UNIVERSITAS
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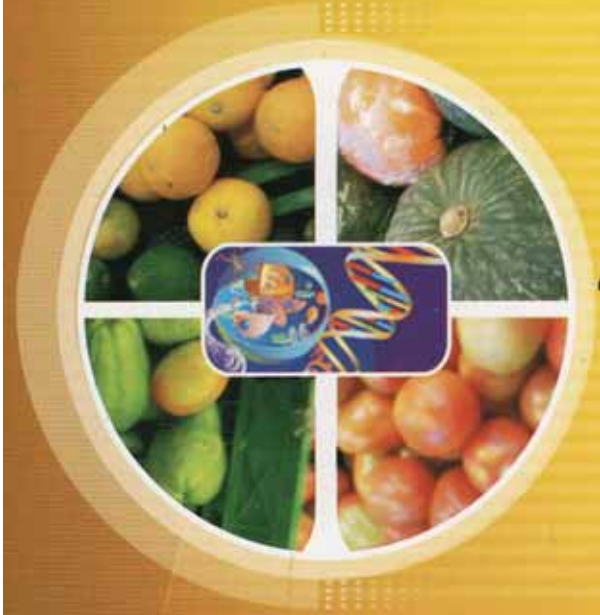
Fakultas Teknobiologi

PROCEEDING



1st International Seminar on
"Natural Resources Biotechnology: From Local to Global"

September 8th – 9th 2015
Faculty of Biotechnology
Universitas Atma Jaya Yogyakarta

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Welcome Speech
Chair of the Seminar Committee

Distinguished Guests,
Honorable Speakers,
Ladies and Gentlemen,

It is a great pleasure to welcome all of you to the International Seminar "Natural Resources: From Local to Global". The Faculty of Biotechnology of Universitas Atma Jaya Yogyakarta runs this seminar to commemorate the 50th Anniversary of the Universitas Atma Jaya Anniversary and the 25th Anniversary of the Faculty of Biotechnology. Your presence is your present for the anniversary of our university and faculty as well.

The Anniversary is not the only reason to run this seminar. A greater reason is behind the seminar. Indonesia is rich in biodiversity. It is a challenge for us, as scientist, to maintain the biodiversity and to develop the potential of the biodiversity for the common good. Through this seminar, the scientific research on Indonesian biodiversity can be shared and probably the finding of the new research can inspire us for further exploration. Therefore, the seminars goal is to facilitate the spread of the research on local potential of biodiversity to the global level. Hopefully, it can attract more researchers to explore the wealth of local biodiversity.

The committee invites speakers who are expertise in the research concerning biodiversity. Our invited speakers are Assoc. Prof. Dr. Michael Murkovic from Graz University of Technology Austria (food scientist), Assoc.Prof. Worawidh Wajjwalku from Kasetsart University Bangkok Thailand (Veterinary disease biotechnology), Dr. Kathryn McMahon from Edith Cowan University Australia (Seagrass biotechnology), Prof. Marco Nemesio E. Montano, PhD from University of the Philippines (Seaweed biotechnology), Prof. Jun Kawabata from Hokkaido University Japan (food biochemist), Endang Semiarti, PhD from Universitas Gadjah Mada, Indonesia (Plant biotechnology), Ign. Pramana Yudha, PhD from Universitas Atma Jaya Yogyakarta (Conservation genetics), Dr Machmud Thohari from Technical Team for Environmental Biosafety, Ministry of Enviroment & Forestry Indonesia (Environmental Biosafety), Dr Harvey Glick from Asia Regulatory Policy & Scientific Affairs Monsanto Company (Regulatory Policy & Scientific Affairs Monsanto). It is a good opportunity to learn from the speakers to enhance and to update our knowledge. I hope this seminar is of benefit to all of us.

In conclusion, I wish you a successful seminar and a pleasant stay in Yogyakarta.

With kind regard
Coordinator of conference program

Dr. rer. nat. Yuliana Reni Swasti, S.TP., MP.

WELCOME SPEECH
DEAN
FACULTY OF BIOTECHNOLOGY
UNIVERSITAS ATMA JAYA YOGYAKARTA

Distinguished Guests,
Honorable Speakers,
Ladies and Gentlemen,

On behalf of the Faculty of Biotechnology, Universitas Atma Jaya Yogyakarta and the Committee of the International Seminar, I would like to first of all to extend our heart-felt thanks for your presence at this Seminar. This seminar is so significant in a sense that it focuses on natural resources with local content but by utilizing biotechnology they will become global and worldwide products and services as well.

Biotechnology has been developed very rapidly and it is believed to be "a new wave in the economic world". Biotechnology has contributed in all aspects of humans' life, such as food production, health, industry, environment, etc. The role of biotechnology for the betterment of human beings, however, is still need to be improved. Indonesia, with its huge biodiversity, has a potency to develop and applied biotechnology nationwide.

The role of biotechnology has increased rapidly. Many are believed that biotechnology has become an integral part of modern industries with high economic values. On the other hand, it needs to be closely managed in order to avoid its negative impacts. The are some example of negative impacts with relate to biotechnology application, such as intellectual property rights, genetically modified organisms (GMOs), environmental degradations, biodiversity issues, indigenous people knowledge, biosafety, etc.

The Seminar covers topics such as: Functional Foods, Food Biotechnology, Biopharmacy, Health/Medical Biotechnology, Environmental Biotechnology, Legal Aspect of Biotechnology, Bioinformatics, and Social-Economic Aspects of Biotechnology. This Seminar will be presented nine (9) invited speakers with different topics and expertise. There will be some papers and posters to be presented also in this Seminar from some participants from the Philippines and Indonesia.

Henceforth, in commemorating its 50th anniversary Universitas Atma Jaya Yogyakarta (UAJY) and 25th anniversary of Faculty of Biotechnology, Universitas Atma Jaya Yogyakarta (UAJY) on September 2015, it is worthy and appropriate to explore the newest innovations in the field of research and development of biotechnology to be applied in many aspects for the betterment of human beings. The Seminar takes this opportunity to discuss and hopefully find ways to solve problems faced by human beings in the world.

I would like to take this opportunity to express my sincere thanks and gratitude to the Committee and in particular to the honorable speakers. Before closing this remarks, allow me to ask the Rector of Universitas Atma Jaya Yogyakarta to open this Seminar officially.

Finally, this is an opportune time for me to wish you all in the two (2) fruitful days of interesting and beneficial programs and hope you have a pleasant stay in Yogyakarta.

Thank you very much and may God bless us all. Amen.

Yogyakarta, 8 September 2015

Dean

Drs. B. Boy Rahardjo Sidharta, M.Sc

Brief Contents

Age Structure of *Babylonia spirata* L 1758 From Gesing Beach, Yogyakarta, Indonesia

Felicia Zahida
Faculty of Biotechnology, Universitas Atma Jaya Yogyakarta
Jl. Babarsari 44, Yogyakarta 55281
Email: feliciazda@mail.uajy.ac.id

Abstract

Babylonia spirata L 1758 from Gesing Beach Yogyakarta has been harvested more than a decade but it seems that the community does not use their potency well. There is a need to understand the real potency and the possibility to be harvested more. Nowadays there is a need to see the specific characteristic of *Babylonia spirata* through its age structure to understand further its population dynamic. The aim of this research was to elucidate and estimate the age structure of *Babylonia spirata* from Gesing Beach Yogyakarta. Method use was sampling 5% of the harvest snails and measure its shell length (mm). Data were then builded in length frequency of the shells. Analysis used was Elefan I from FiSAT package to elucidate growth parameter K and L_∞. Second phase of the analysis were using Bhattacharya and Monte Carlo methods to build the age structure of *Babylonia spirata*. Result shows that growth parameter value were K 0,27 and $L\infty$ was 55.65 mm. K was low means that this species need quite a long time to reach its maximum size. Age structure composed of five (5) age classes using Bhattacharya method, and four (4) classes using Monte Carlo method. Age structure shows tendency of increase and no indication of overfishing.

Keywords: *Babylonia spirata*, Pantai Gesing, Elefan I, metode Bhattacharya dan Monte Carlo

1. INTRODUCTION

Babylonia spirata is well known as Keong Macan in Indonesia. This species is common in Indo-Pacific region. Altena dan Gittenberger (1981) classify it in genus *Babylonia* Schülter 1838, and familia Buccinidae. Distribution in Indonesia is from South Sumatera, Java and Madura. Although Indonesia exportsthis snail to other countries, only fewpeople know about this species as an edible food. Only a few restaurants serve this snail fresh and as one of their delicacy food.

Recently, some peoples posted to the internet that they eager to buy Keong Macan in all size, in an unlimited amount. But again, this is because of the export demand. They offered that opportunity with a good price and this increase the enthusiasm to harvest more.

Since there were some claims of the overfishing status of this species, there is a need to prove the exact status of the species, especially in Yogyakarta. In the other hand fisherman in Yogyakarta can be categorized as new fisherman, since their parents was not a fishermen yet. Other evidence is that local fishermen could not

work in rough big waves. Thatfact supports the possibility condition that the statusof the *B. spirata* is not overfishing yet.This is why basic knowledge on population dynamics needs to be revealed.

One of the part needs to be elucidated is the age structure of the *Babylonia spirata* L, 1758. This is a starting point to understand the population dynamics of the species *B. Spirata*in its habitat in Indonesia. Some researches that were done earlier are:

Yulianda (2009) on the development of *Babylonia. spirata*(Linnaeus 1758) larvae; Andamari (2009) reseached on variation of fishing gear and handicrafts of Keong Macan (*Babylonia spirata* L. 1758) in Cilacap water. Diniah (2009) Utilization of Keong Macan as natural resources using bubu as trap tool.This research emphasized on optimation of folding-bubu's utilization in substrat; Yulianda (2007) on the feed utilization for somatic growth and reproduction Keong Macan (*Babylonia spirata*., L. 1758); Apritia (2006) worked on tendency to eat natural baits of Keong Macan (*Babylonia spirata* L.). This was a laboratory research.Previous reserch done was on catching method, bait, and factors that affect arrest of Keong Macan (Zahida 2014).

This research aim to reveal the age structureof Keong Macan, *Babylonia spirata*, and the age grups number in population. Secondly,this aims to estimate the future tren of the population. Analysis comparation were usingBhattacharya and Monte Carlo methods.

Limitations of this study are, first, there is a dependency to the situation and condition of the local fishermen, specifically in Gesing Beach, Yogyakarta. Rent a boat is to expensive to do. Secondly, there is limitation of the small samplespecimen, because of the net's size/mesh of the trap. This will result in not ideal age structure.

Figure 1. *Babylonia spirata* L fromGesing Beach, Daerah Istimewa Yogyakarta (Photograph by Felicia Zahida)

2. METHODS

Research location was at Gesing Beach, Yogyakarta, Indonesia. The research was done from October 2014 to February 2015.Tools used in this study were Vernier caliper with precision level of 0.05mm and digital weight balance (Sartorius 224D). Sampling was once a month and 5 % of the population catched were measured for its shell length and weight. Data was then tabulated in length frequency. Two methods were used to compare results were using Bhattacharyaand Monte Carlo

methods (Pauly 1984; Sparre and Venema, 1998). FiSAT II package was downloaded from website FAO.Results were analyzed descriptively.

3. RESULTS AND DISCUSSIONS

3.1 Harvest Activity

Data collections of Keong Macan from Gesing beach show irregularity in activity, this indicate that fishermen were depend more on fishes harvest compare to snail harvest. When high price of fishes presence they do not harvest snail. Figure 2 shows the irregularity of the harvest activities recently.

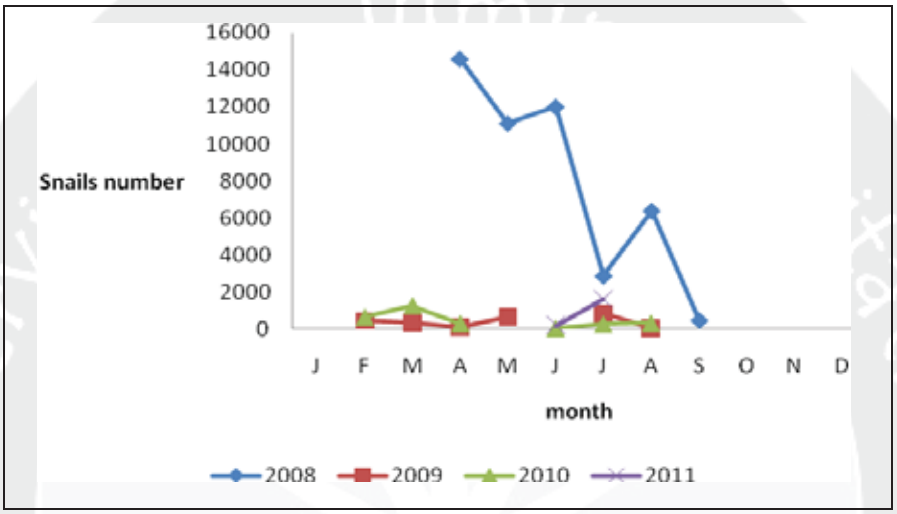

Figure 2. Harvest activities of *B. spirata* from Gesing beach year 2008-2011

Figure 2. shows that snails harvest is an alternative activity when fishes harvest is not satisfactory. Year 2008 was a *booming*time of *B. spirata*, but several years later were not. This may affects the research.

Figure 3. Gesing Beach, Girikarto, Gunung Kidul, Yogyakarta GPS position 8°06'30,30"S and 110°28'05,63"E; 08°06'28,53"S and 110°28'07,56"E (Google Earth.2015)

Gesing Beach was hidden at the western Gunung Kidul municipality. This location is easily accessible from Imogiri rather than Wonosari, as we can see at the map Figure 3. The community is small, so as the harvest results. Peaks were in April-September (2008), February-August (2009, 2010), June-July (2011). The difference of the harvest results were because of the priority of catch fishes.

3.2 Growth Parameter

B.spirata's growth curve can be showed in Figure 4. Prediction number of cohort from population present in that area was formedusing Elefan I analyzes.

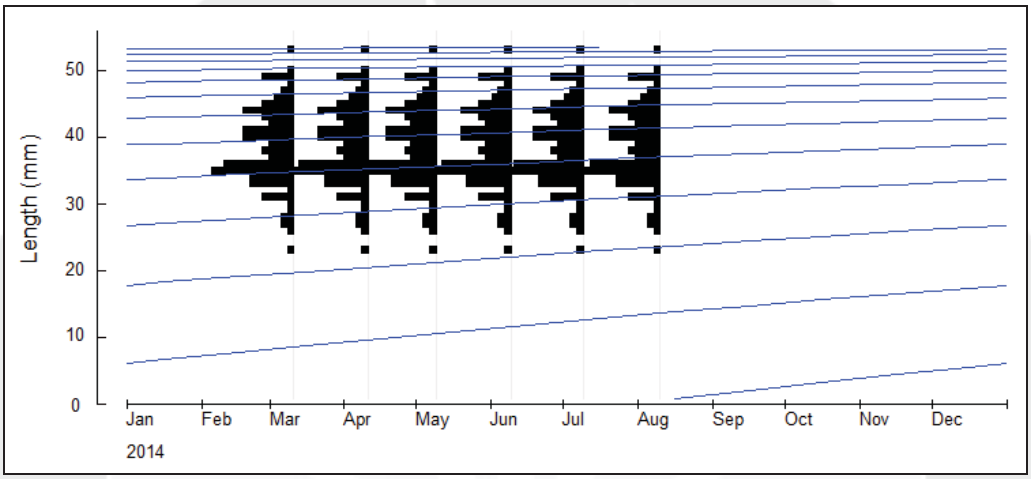

Figure 4. Growth curve of *B. spirata* from Gesing beach

The number of cohort is many, because in tropical country, the reproduction activity was year round. In order to develop age structure, length frequency should be developed into age group. In Figure 4, above, starting of curve, mid August, represent the birth time. Numbers of curves represent number of cohorts.

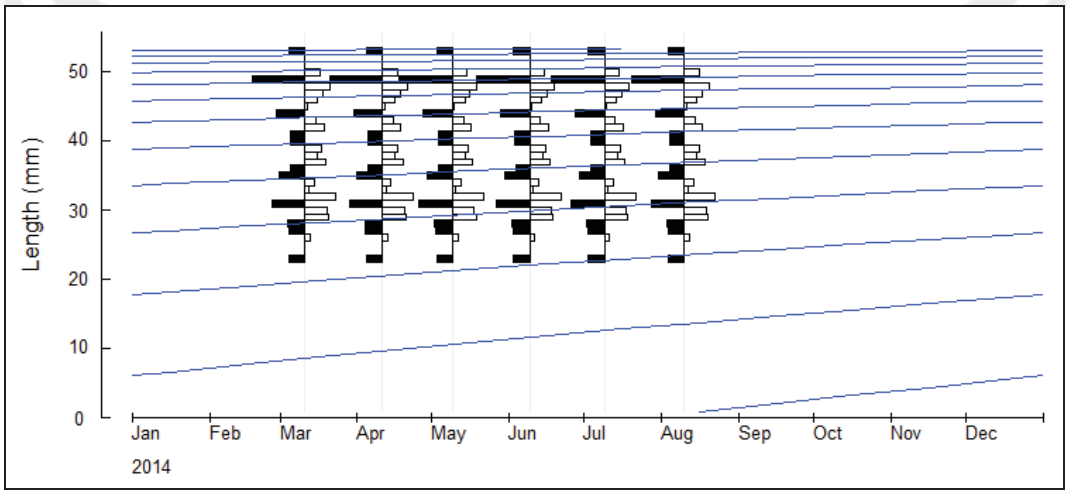

Figure 5. Growth curve with restructured data frequencyof shell length

Figure 5, shows the restructured frequency of shell length and has the same tren as Figure 4. The tren show 10 curves. Note that in tropical area, yearly peak's growth

curve usually developed as bimodal as representative of dry and wet season. If we consider the season, as we have 2 seasons in a year, we understand that the age of *B. spirata* most probably be five years.

Figure 4 and 5 shows the growth of each cohort. Detail of the growth rate can be seen in Table 1.Length at age was develop using von Bertallanffy Growth Formula. Input data was K value from Elefan analysis i.e. 0.27 and L_{∞}55.65.$L_{(t)}$ was developed using t input from 1 to 10 and growth rate could be counted and got.

Tabel 1. Growth rate of *B. spirata* from Gesing Beach

Age t	Shell length L(t), mm	Growth rate $\Delta L/\Delta t$	Length $\bar{L}$ (t) mm
1	13.17	-	-
2	23.22	10.05	18.195
3	30.89	7.67	27.05
4	36.75	5.86	33.82
5	41.22	4.47	38.99
6	44.64	3.42	42.93
7	47.24	2.6	45.94
8	49.23	1.99	48.24
9	50.75	1.52	49.99
10	51.91	1.16	51.33

Table 1 shows fast growth at early stage of life, it reaches13.17mm with in a season. Then, the growth was slowed down, and reaches stationer at size about 51.91 mm.Growth rate shows at collum three. At first season growth rate reaches 10.05 mm, then further decrease every season to 1.99 mm at the eighth season, and 1.16 mm at the tenth season. In the last three year the growth rate only 1-2 mm and continuously decrease and reach its minimum or no more growth. Within about five years, shell length of B. spirata reach its infinity, L_{∞} was 55.65 mm (Elefan analysis). Elefan method's beneficial to get growth parameter and trace yearly growth of cohort, at the time t using shell length.

3.4 Age composition estimation of *B. spirata*

Bhattacharya method beneficial to get age group presence at the population, as shows at Figure 6.

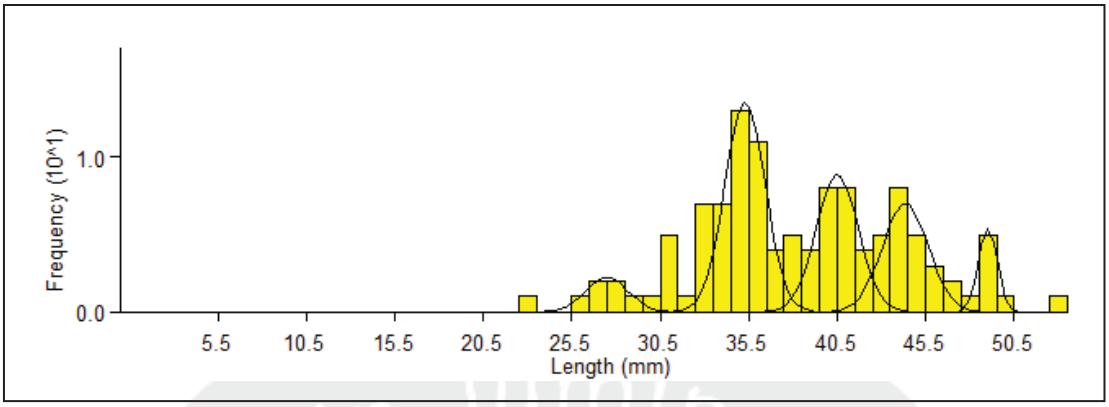

Figure 6. Number of age composition from Bhattacharya analysis

Analysis shows several curves and its peaks of age group of *B. spirata*. Population has been grouped into five (5) groups. Every peak represents one age group. Basically Bhattacharya method separates a number of normal distribution. As a first normal distribution has been achieved, it was removed from the group of total distribution. This procedures had been repeated several times until all population distributed (Sparre and Venema, 1998).

Tabel 2. Decomposition composite distribution using Bhattacharya Methods

Age group (mm)	S.D	Populasi	S.I
27.5	1.2	6.67	n.a
35.29	1.13	38.59	2.38
40.51	1.19	26.52	2.16
44.35	1.29	22.68	2.07
49.02	0.54	7.23	2.12

Annotation S.D.is standard deviasi, and S.I. is separation index.

Tabel 2. Shows five age-group using Bhattacharya. First cohort age group has an average of shell length of 27.5 mm. Then one after the other is, 35.29; 40.51; 44.35; respectively and on the fifth year became 49.02 mm. Every age-group with population percentage of 6.67; 38.59; 26.52; 22.68 respectively and finally 7.23 %. The youngest age group was so few, due to the size net.

Table 3. Monte Carlo simulation from shell length data of *B. spirata*

Age	Frequency	Mean L	S.D of L
0	30	7.12	7.53
1	37	15.40	12.7
2	33	16.87	13.51
3	0	0	0

Tabel 3. Shows Monte Carlo simulation of shell length frequency of *B. spirata*. This technique samples of shell length frequency simulated using computer, with random variability using *roulette* principle. This analysis decrease limitation that presence at length frequency analysis where usually several age group seems mixed. It shows that there are only four cohorts or age groups. This resultis the simples.

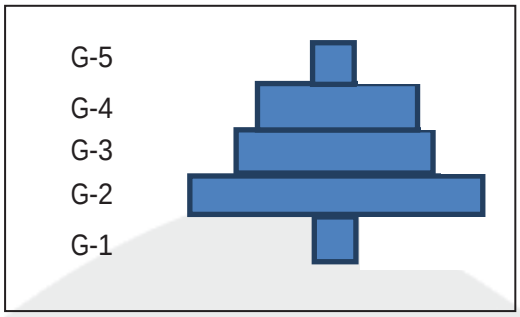

Figure 7. Age structure of *B. spirata* from Gesing Beach. G: age group

This age structure was disregard sex of organisms. The age structureform was also not ideal because the smallest age group was limited by the size of net of the trap. This has a positive effect for the population because it reserves young generation for the next future. This condition also categorized as normal situation, especially for invertebrate which its young age/larvae is in the form of plankton.

Figure 8 and 9 below show local trap for *B. spirata*. called bintur. Bait used was "runcah" or defective fishes. As showed here, it is impossible to catch small snails using this trap. Again, this condition gave an opportunity for small snail to escape and gave them opportunity to get bigger and reproduce.

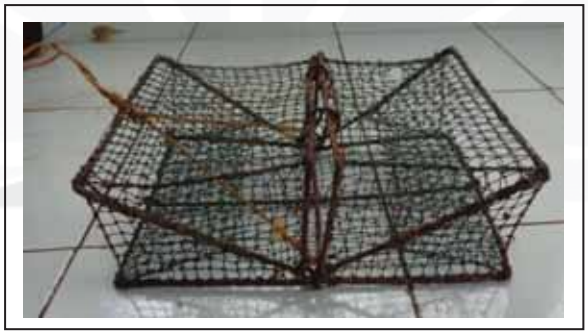

Figure 8. Trap (bintur) for *B. spirata*, square-form with opening in the middle, from Gesing Beach, Yogyakarta

Figure 9. Trap (bintur) for *B. spirata*, shoes-form from Gesing Beach, Yogyakarta

4. CONCLUSION

Elefan analysis shows growth parameter K= 0.27, means that the growth was slow. *B.spirata* growth from birth to adult needs 10 seasons or five years.L_{∞}size was 55.65 mm. Bhattacharya analysis confirm that the number of age group were five. As a comparison Monte Carloanalysisshows that the number of age group was four.

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The Effect of Nitrogen Excess in Medium on Carotenoid and Chlorophyll Content of *Chlorella Zofingiensis* Donz Culture

Eko Agus Suyono[1,2], Umi Muavatun[1], Faridatul Husna[1], Husnul Khotimah[1], Ika Pratiwi[1], Rahmah Husna[1], Fitri Cahyani[1], Yuni Purwanti[1], Thoriq Teja Samudra[1]
[1]Faculty of Biology, Gadjah Mada University
Jl. Teknika Selatan, Sekip Utara, Yogyakarta, Indonesia 55281
[2]Centre for Energy Studies, Gadjah Mada University
Sekip K1A, Kampus UGM, Yogyakarta, Indonesia 55281
Email address: eko_suyono@ugm.ac.id

Abstract

Chlorella zofingiensis Donzis the prospective carotenoid producer. Under unfavorable condition, such as Nitrogen limitation, it has been reported that *C.Zofingiensis*has the ability to synthesize high amounts of carotenoids. However, the effect of nitrogen excess on the carotenoid production of the microalgae is not widely studied yet. Agriculture waste that is rich of nitrogen can be potentially used to enhance the carotenoid as well as the chlorophyll ofnthe microalgae. Therefore, this research aims to study the effect of nitrogen excess on the production of its carotenoids and chlorophylls. This research used medium consisted of commercial micronutrient fertilizer, urea and ZA with the ratio of 0.25: 0,5: 1 (low excessnitrogen medium)and0.5: 1: 2 (high excess nitrogen medium). The parameters measured were dry weight, chlorophyll a and b, and carotenoid. The dry weight was calculated by measuring the difference weight of the wet and dry samples. Both chlorophyll and carotenoid were measured using spectroscopy method. The highest carotenoids, chlorophyll a and b and dry weight were produced in the high nitrogen excess medium. They accounted for 0.5 mgL^{-1}, 2 mgL^{-1} 1.5 $mgL^{-1}$and 80 mgL^{-1}, respectively. Furthermore, the ratio of carotenoids and chlorophyll a and b to the dry weight on high excess nitrogen medium tend to increase.Therefore, the high nitrogen excess treatment was able to enhance carotenoid, chlorophyll a and b and dry weight of the microalgae.

1. INTRODUCTION

C.zofingiensis Dönz is freshwater green algae classified into classes Chlorophyceae, orders Chlorococcales and family Chlorellaceae (Pickett-Heaps, 1975). This algae is non-motile algae and unicellular. Its cell has spherical shape with a diameter from 2µm to 15µm. Chlorophyll a in *C. zofingiensis*is dominant so that the cell is green. This microalgae grows optimally at temperatures between 25 ° C-28 ° C and the salinity with a maximum of 5 ppt (Bold and Wyne, 1985). This microalgae is able to photosynthesize in order to produce organic carbon compounds (Richmond, 2004).

Carotenoids are pigments that most commonly occur in nature and synthesized by all photosynthetic organisms and fungi (Vilchez *et al.*, 2011). The algae is one of the

largest carotenoidproducers. Algae carotenoids show the diversity of structures and about 100 different carotenoids been found in algae (Britton *et al.*, 1995).

In algae, carotenoids play an important role in the process of photosynthesis with chlorophyll. Besides having the photosynthetic pigment chlorophyll, the green algae also have carotenoids as additional pigments. The main carotenoid own green algae including β-carotene, lutein, violaxantin, anteraxantin, zeaxantin, and neoxantin (Burtin, 2003). Currently, microalgae have been used as substrates for biodiesel, pharmaceutical, dietary supplements, and natural feed in aquaculture (Aslull and Omar, 2012).

Corsini and Karidys (1990) states that nitrogen is an important part of the protein, protoplasm, chlorophyll and nucleic acids. Nitrogen is absorbed in the form of ammonium ($NH4^+$) or nitrate (NO_3). N deficiency will also limit growth because there will be no formation of new protoplasm. Meanwhile, excessive nitrogen fertilization will result vegetative growth. Therefore. it is interesting to evaluate the effects of excess nitrogen on the chlorophyll and carotenoid of *C. zofingiensis*.

2. METHODS

2.1 Chemicals

This research used medium agricultural fertilizers: urea: ZA with a ratio of 0.25: 0,5: 1 (low excessnitrogen medium) and 0.5: 1: 2 (high excess nitrogen medium).

2.2 Procedures

The study was conducted in Wukir Sari, Cangkringan, Pakem, Sleman, Daerah Istimewa Yogyakarta (DIY). *C. zofingiensis* was cultured on a mass scale in the 3600 liter pool. Environmental parameters measured were temperature, pH, and density.The mediums were local agricultural fertilizer (farmpion), urea and ZA with ratio of 0.25:0,5:1 (low excess nitrogen medium) and 0,5:1:2 (high excess nitrogen medium). As a control, *C. Zofingiensis* was cultivated in medium with local agricultural fertilizer (farmpion) without the addition of urea and ZA. Nitrogen contentin fertilizers and ZA were 21%, while thenitrogen contentin urea was 46%. Samples were taken every day for 7 days. The parameters measured werechlorophyll, dry weight, and carotenoid.

2.2.1 Chlorophyll Measurement

Samples were taken 10 mL inserted into the tube, then centrifuge at a speed of 3300 rpm for 15 minutes. Samples were spared the supernatant, and then added to 2 mL of acetone, then centrifuged again at a rate of 1800 rpm for 10 minutes. The sample was transferred into a glass cuvette spectrophotometer then inserted and calculated it absorbance at a wavelength of 470, 645 and 662 nm. Based on the absorbance of the spectrophotometer, the concentration of chlorophyll and carotenoids were determined by using the following equations.

$$Ca\left(\frac{mg}{L}\right) = (11{,}75\ xA\ 662\ nm) - (2{,}35\ xA\ 645\ nm)$$

$$Cb\left(\frac{mg}{L}\right) = (18{,}61\ xA\ 470\ nm) - (3{,}96\ xA\ 662\ nm)$$

$$\text{Chlorophyl } a, b \ (ml/m^3) = \frac{(C)(Va)}{(Vc)}$$

2.2.2 Dry weight measurement

Calculation of dry weight by taking a 40 mL sample then adding 2 ml of 5% SDS-MSDO, centrifuging at a speed of 3300 rpm for 10 minutes. After the supernatant was taken and dried in an oven at a temperature of 30 ° C to constant weighed, it was measured its weighusing an analytical balance.

2.2.3 Carotenoid measurement

Samples were taken 10 mL inserted into the tube, then centrifuged at a speed of 3300 rpm for 15 minutes. Samples were spared the supernatant, and then added to 2 mL of acetone, then centrifuged again with a rate of1800 rpm for 10 minutes. The sample was transferred into a glass cuvette spectrophotometer then inserted and calculated absorbance at a wavelength of 470, 645 and 662 nm. Based on the absorbance of the spectrophotometer concentration of carotenoids was determined by using the following equation.

$$Carotenoid\left(\frac{g}{L}\right) = \frac{(A470nm - A662nm)x\ 25\ x\ 1000}{200\ xsamplevolume\ (mL)}$$

3. RESULT AND DISCUSSION

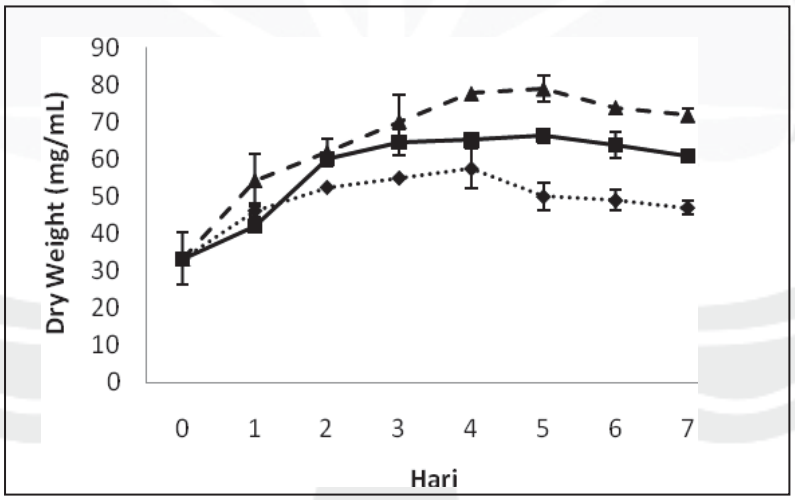


Figure 1. Dry Weightof*C. Zofingiensis* in 7 Days Cultivation
Control Low Excess Nitrogen High Excess Nitrogen

Figure 1. showed that the highest dry weight was obtained in the treatment of high excess nitrogen. This was due to the cells cultured in high nitrogen content, the photosynthesis productwas more stored in the form of carbohydrates rather than used for asexual reproduction through fission (Jiménez *et al*., 2003)

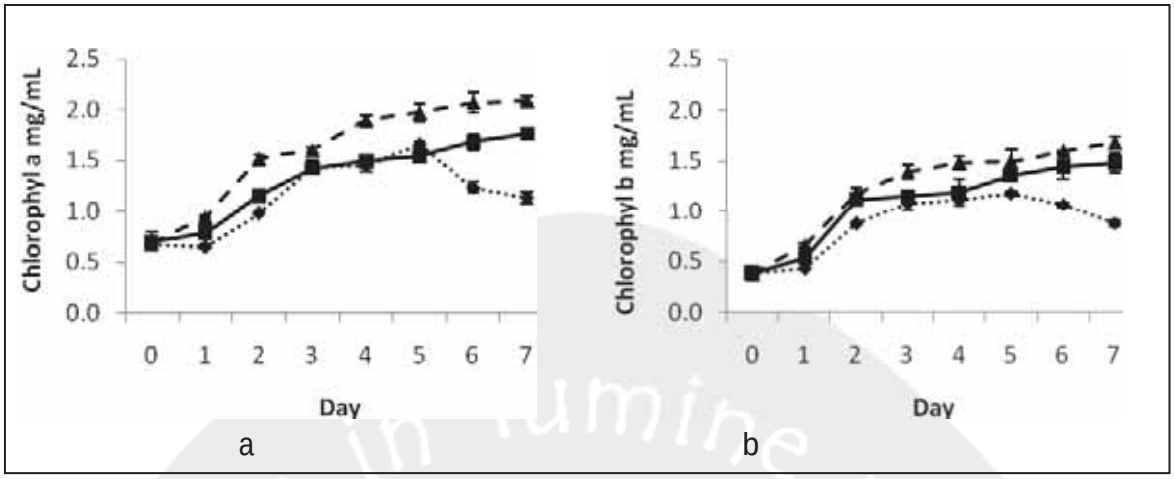


Figure 2. (a) Chlorophyll aConcetration of *C. zofingiensis* (b) Chlorophyll b Concentration of*C. Zofingiensis* in 7 Days Cultivation
Control Low Excess Nitrogen High Excess Nitrogen

According toMulders *et al*., (2014), if microalgae was cultured in medium with high nitrogen content, the production of chlorophyll a and b would tend to increase.Similar to Guedes and Malcata (2012), a high nitrogen content that was 2- 3 times higher than normal concentration in the medium increased the synthesis of pigments in cells, especially chlorophyll. Zhu *et al*., (2014) stated that the increase in chlorophyll a and b were caused by the increased concentration of nitrogen in the medium. Similarly, indicated in figure 2, that the highest chlorophyll a and b were obtained at high treatment of excess nitrogen. Meanwhile, the content of chlorophyll a and b in all treatments tend to be the same (figure 3). Thus, the increase in chlorophyll a and b was followed by an increase in dry weight.

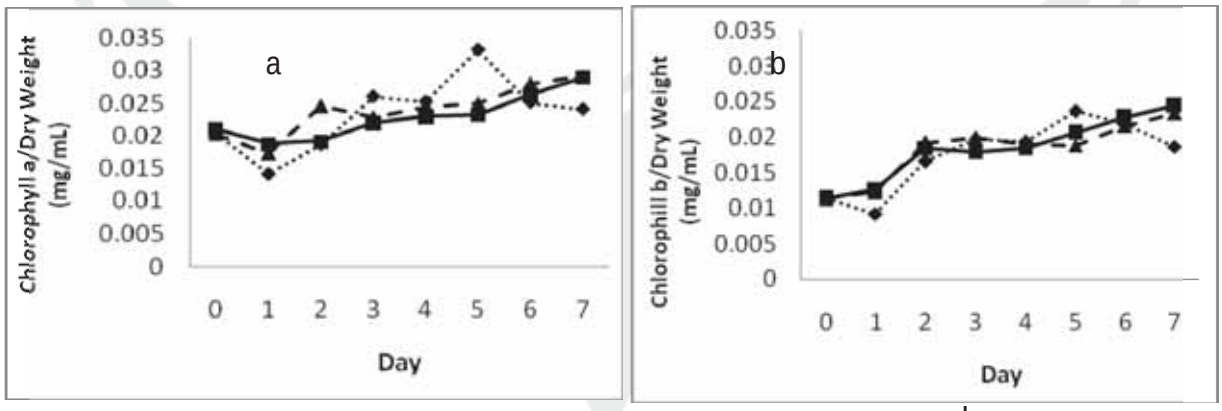
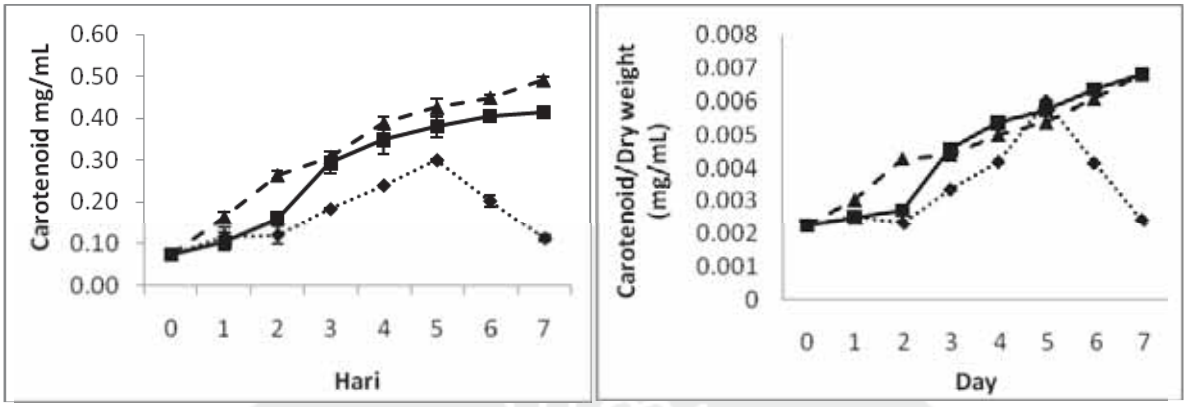


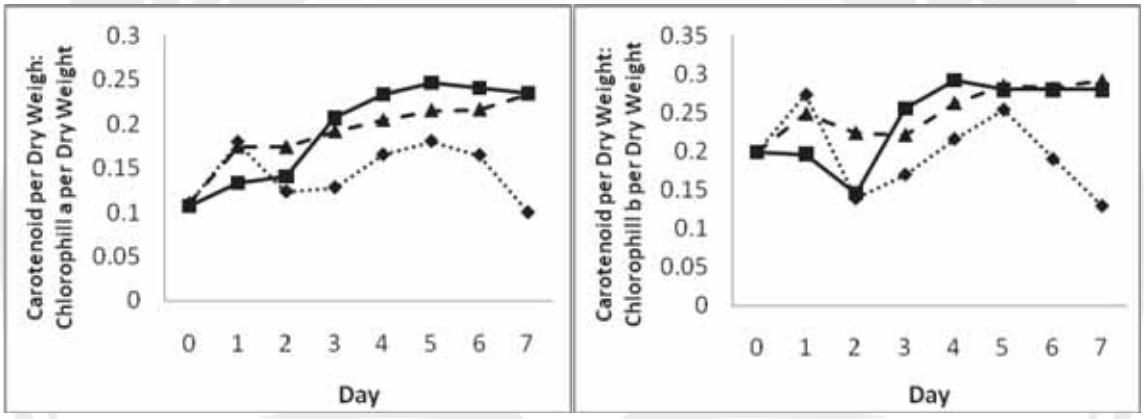
Figure 3. (a) Chlorophyll a Contentper Dry Weigth Content of*C. zofingiensis*in 7 Days of Cultivation (b) Chlorophyll bContent per Dry Weigth of*C. zofingiensis*in 7 Days of Cultivation
Control Low Excess Nitrogen High Excess Nitrogen



a b

Figure 4. (a) CarotenoidContent of*C. zofingiensis* (b) Carotenoid Content per Dry Weigth of*C. zofingiensis*

Control Low Excess Nitrogen High Excess Nitrogen



a b

Figure 5. (a) Ratio of Carotenoidper Dry weigth content with Chlorophyll a per Dry weigth in *C. zofingiensis* (b) Ratio of Carotenoidper Dry weigth content with Chlorophyll b per Dry weigth in *C. zofingiensis*

Control Low Excess Nitrogen High Excess Nitrogen

In figure 4 and 5, showed that the carotenoid content tend to increase in conditions of low and high excess nitrogen. Similarly, the ratio of carotenoid content per dryweight to chlorophyll a and b per dry weight. According Mulders *et al*., (2014), a high nitrogen concentration increased the growth of cells as well as caused stress on the culture. Furthermore, increased cell growth culture under stress would also increase the synthesis of secondary metabolites for cell protection. Carotenoids are synthesized when the culture experiencing environmental stress including nitrogen stress (Wang *et al*., 2003).

4. CONCLUSIONS

In conclusion, The highest carotenoids, chlorophyll a and b and dry weight were found in the high nitrogen excess medium. They accounted for 0.5 mgL-1, 2 mgL-1 1.5 mgL-1 and 80 mgL-1, respectively. Furthermore, the ratio of carotenoids and chlorophyll a and b to the dry weight on high excess nitrogen medium tend to

increase. Therefore, the high nitrogen excess treatment was able to enhance carotenoid, chlorophyll a and b and dry weight of the microalgae.

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Enzymatic Modification of Chicken Feathers Waste As Livestock Feed Rich in Nutrients

Ditya Lasarati[1], *Maharani Pertiwi Koentjoro[2], and Endry Nugroho Prasetyo[1]
[1]Biotechnology Laboratory, Department of Biology, Institut Teknologi Sepuluh Nopember, Gedung H 1st floor, Kampus ITS Keputih Sukolilo Surabaya, 60111 Indonesia
[2]Laboratory of Environmental Microbiology, Department of Biological and Environmental Science, Faculty of Agriculture - Shizuoka University & Structural Biology Research Center, Inter-University Research Institute Corporation - High Energy Accelerator Research Organization (KEK), Tsukuba-Ibaraki, Japan
*Corresponding author email address: maharanipertiwi@yahoo.com

Abstract

Feathers is organic waste consists of 90% keratin protein structure that link by disulfide and hydrogen bonds. Structures and linkages of keratin make feathers waste unsoluble in the water and very difficult to degrade. The alternative and innovative solution to overcome abundant of feathers waste is by the utilization of keratinolytic microorganism capable of producing keratinase and degrade keratin become amino acids and peptides. Chicken feathers waste containing high keratin protein has the potential to be used as an alternative sources of protein and can be applied in the manufacture of animal feed. This research aimed at utilization of chicken feather waste that are modified enzymatically by keratinase to produce water soluble protein and converted into alternative protein source in livestock feed that are cheap and rich in nutrients. Keratinase produced by *Bacillus* sp. SLII-I through fermentation using FM media. Keratinase isolated by centrifugation method then activity and protein content of keratinase is measured. This researched reported that *Bacillus* sp. SLII-I capable of producing crude keratinase with 2.08 (mg/second)/ ml enzyme activity that can increase water soluble protein level of feathers waste until 22.06%. Broiler chicken (*Gallus domesticus*) that consumed feed containing 5% feather meal indicated production performance of 1194.8 gram/head of feed consumption, 567 gram/head of addition of weight, and 2.1 of feed conversion ratio. An enzymatic engineered chicken feathers waste showed the performance of broiler chicken that is better than soybean meal as conventional sources of protein but could not yet substitute the use of conventional protein sources of fishmeal.

Keywords: *Bacillus* sp. SLII-I, Keratinase, Keratin, Feed, Broiler chicken.

1. INTRODUCTION

Chicken feathers is organic waste that generated in bulk quantities as a by-product in poultry industry. In general, each bird has up to 125 gram of feather (Lakshmi *et al*.,, 2013) that represent 5-7% of the total weight of mature chickens (Matikevičienė *et al*., 2009). Meanwhile, more than 400 million chicken being processed every week worldwide (Lakshmi *et al*., 2013) so that the accumulation of feather waste reaches five million tons (Han *et al*., 2012). Most feather waste is land filled or burned that cause global environmental issue such as pollution of both air and underground

water resources (Cai *et al*., 2008; Matikevičienė *et al*., 2009) and feather protein wastage (Cai *et al*., 2008).

Chicken feathers are high protein resource consist of 90% keratin (Matikevičienė *et al*., 2009; Cai *et al*., 2008). keratin protein that have α-helix (α-keratin) or β-sheet (β-keratin) structure link by disulfide and hydrogen bonds (Riffel and Brandelli, 2006; Mazotto *et al*., 2011). The structures fold and form complex structures (Kreplak *et al*., 2004). Structures and linkages of keratin make keratin have high mechanical stability (Mazotto *et al*., 2011) and resistance to degradation by common proteolytic enzymes such as trypsin, papain, and pepsin (Mousavi *et al*., 2013).

Feathers waste is poorly recycled in nature and has limited utility due to the chemically unreactive nature of keratin (Lakshmi *et al*., 2013). Despite the rigid structure of keratin, it can be degraded by mechanical, chemical, and biological methods (Mousavi *et al*., 2013). The major drawback of mechanical and chemical degradation methods is requires great input energy, give rise to environmental problems, and are destructive to certain amino acids such as methionine, lysine and tryptophan and also in the formation of non-nutritive amino acids such as lysinoalanine and lanthionine (Marcondes *et al*., 2008) that leads to low protein quality and digestibility (Zerdani *et al*., 2004) so the feathers waste that converted into feed supplement conventionally resulting in feed of poor quality which is nonviable economically (Acda, 2010).

The alternative and innovative solution to overcome abundant of feathers waste is by the utilization of keratinolytic microorganism capable of producing keratinase. Keratinase belongs to hydrolase group that capable of hydrolyze keratin more efficient compared to other protease (Vigneshwaran *et al*., 2010; Kanmani *et al*., 2011). Keratinase attack disulfide bonds to degrade keratin (Agrahari, 2013). Biodegradation of keratin using keratinase produce peptide and rare amino acids such as serine, cysteine and proline (Mousavi *et al*., 2013) and essential amino acids such as threonine, valine, methionine, isoleucine, leucine, lysine, histidine and tyrosine (Ali *et al*., 2011). Chicken feathers waste containing high keratin protein has the potential to be used as an alternative sources of protein and can be applied in the manufacture of animal feed (Sastry *et al*., 1986) that are cheap and rich in nutrients (Balaji *et al*., 2008; Khardenavis *et al*., 2009). Hence, this research conduct to utilize chicken feather waste that are modified enzymatically by keratinase that are produced by *Bacillus* sp. SLII-I and then converted into alternative protein source in broiler chicken (*G. domesticus*) livestock feed.

2. METHODS

2.1 Keratinase Production and Isolation

Bacillus sp. SLII-I (10%) inoculated in feather meal broth and feather meal media to make it adjust to feathers as carbon source. The flasks were incubated at room temperature for 24 hours at 110 rpm. Keratinase produced by *Bacillus* sp. SLII-I through fermentation using feather meal media (FM) containing 0.5 g/L NaCl, 0.3 g/L K_2HPO_4, 0.4 g/L KH_2PO_4 and 10 g/L of feather meal. Then, keratinase isolated when culture in early stationer stage by centrifugation method at 3500 rpm for 30 minutes. The supernatant was collected for keratinase activity and protein determination.

2.2 Keratinase Activity

The keratinase activity was assayed as follows: 1.0 gram keratin properly diluted in 160 ml phosphate buffet (50mM, pH 7.0-7.2) was incubated with 0.16 ml keratinase enzyme at 50 °C in waterbath for 2 hours. The reaction is stopped by cooling the solution. Then, solution was filtered through Whatman No.1. Obtained filtrate containing water soluble protein that was determined based on Bradford method (1967). Keratinase activity in this research was defined as the ability of keratinase hydrolyze keratin into 1 mg water soluble protein every second compared to the control and calculated by the following equation:

$$\text{Keratinase Activity ((mg/second)/ml)} = (\Delta DP/T)/V \times DF$$

Where:

ΔDP = Total water soluble protein compared to the control (mg)
T = Incubation time (second)
V = Keratinase volume (ml)
DF = Dilution factor

2.3 Protein Determination

Protein content was analyzed using Bradford method with bovine serum albumin as standard protein (Bradford, 1967). Readings were carried out in a spectrophotometer at 595 nm.

2.4 Enzymatic Modification of Chicken Feathers Waste

Enzymatic modification of chicken feather was done by means of reaction between feathers and keratinase directly. One gram feather meal properly diluted in 160 ml phosphate buffet (50mM, pH 7.0-7.2) was incubated with keratinase enzyme (0.04 ml, 0.08 ml, 0.12 ml, 0.16 ml, and 0.20 ml) at 50 °C in waterbath for 2 hours. The reaction is stopped by cooling the solution. Then, solution was filtered through Whatman No.1. Obtained filtrate containing water soluble protein that was determined based on Bradford method (1967). An enzymatic engineered feathers waste which have the highest increase level of water soluble protein converted into alternative source of protein.

Conversion of feathers into feed was done using Poovendran *et al*., (2011). The flask containing solution of feather meal, phosphate buffer, and enzyme after incubated, were taken out and boiled. Simmering continued until all of the liquid was vaporized and a dry powder was left is feather meal that used as an alternative sources of protein in livestock feed.

2.3 Experimental Diets and Management of Animals

A total of 30 broiler chickens (DOC/ Day Old Chick) were provided by the local broiler hatchery and were used in this research. Broiler chickens were brooded for 6 days on crumbled standard commercial starter that were provided ad libitum with water and food supplement. On the 7th day, chicken were weighed and randomly allocate pens. The chickens were placed in $70 \times 70 \times 60$ cm pens with 5 chickens in each pen. The chickens were fed ad libitum experimental diets for 4 weeks during which feed intake and body weight gain were assessed weekly and the feed conversion ratio are calculated. Food supplement such as vitamin and mineral were given via water. The composition of experiment diets were shown in Table 1.

Table 1. The Composition of Experiment Diets for Broiler Chicken

Ingredients	Diet 1 (1st Control)	Diet 2 (2nd Control)	Diet 3
Yellow Corn Meal (%)[1]	55	55	55
Rice Bran (%)[2]	3	3	3
Soybean (%)[3]	42	37	37
Fish Meal (%)[3]	0	5	0
Feather Meal (%)[3]	0	0	5
Protein (%)	27.95	28.15	29.35
Fat (%)	2.77	2.82	2.79
Energy (Kkal/Kg)	3336.76	3297.91	3342.69

Source: 1. Murtidjo (1987)
2. Tamalludin (2014)
3. Laboratory Analysis

3. RESULTS AND DISCUSSION

3.1 Keratinase Production and Isolation

Keratinase produced by *Bacillus* sp. SLII-I through fermentation using feather meal media (FM) containing feather as carbon source in the form of keratin. Fermentation by *Bacillus* sp. SLII-I on FM media show the change visually (Figure 1) on texture of feathers becomes soft as a result of the hydrolysis of protein (Deliani, 2008) and color of white feathers become yellowish after 24 hours incubation as a consequence of the browning reaction caused by activity of bacteria producing oxidize enzyme (Winarno, 2002). Fermentation of chicken feather is also causing unique smell as a result of protein degradation that can produce peptone, amino acids, and components may inflict foul odor like NH_3 and H_2S (Deliani, 2008).

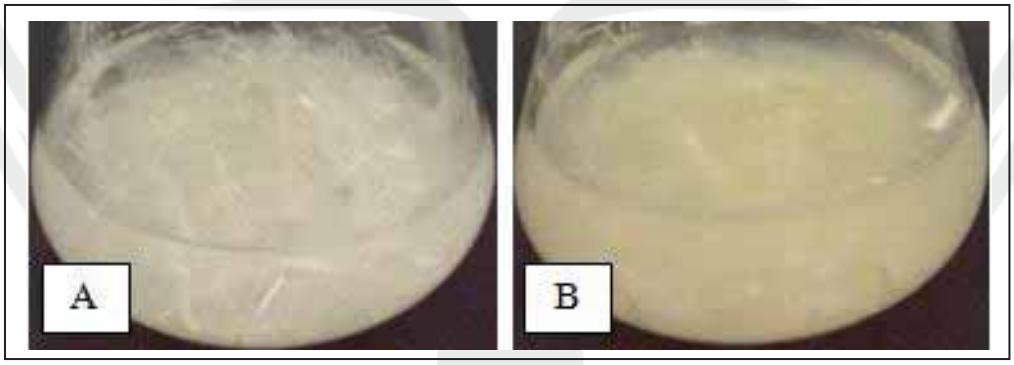

Figure 1. Fermentation of Chicken Feathers by *Bacillus* sp. SLII-I in FM media A: Chicken Feather Without Fermentation; B: Chicken Feathers After 24 h Fermentation

Keratinase isolation was done after fermentation. Growth curve of *Bacillus* sp. SLII-I was necessary to know the right time for isolating keratinase. Figure 2 show the highest keratinase production happened on 14th hours when culture has entered stationary phase and keratinase accumulating maximally in media (Anitha and Eswari, 2012). The growth of *Bacillus* sp. SLII-I in stationary phase due to activating *Ker A* gene encoding keratinase that will be expressed when there are keratin substrates. Keratinase hydrolyze keratin in order to meet the needs of carbon source

of *Bacillus* sp. SLII-I. Keratinase isolated when culture in early stationer stage by centrifugation method at 3500 rpm for 30 minutes. The supernatant was collected for keratinase activity and protein determination.

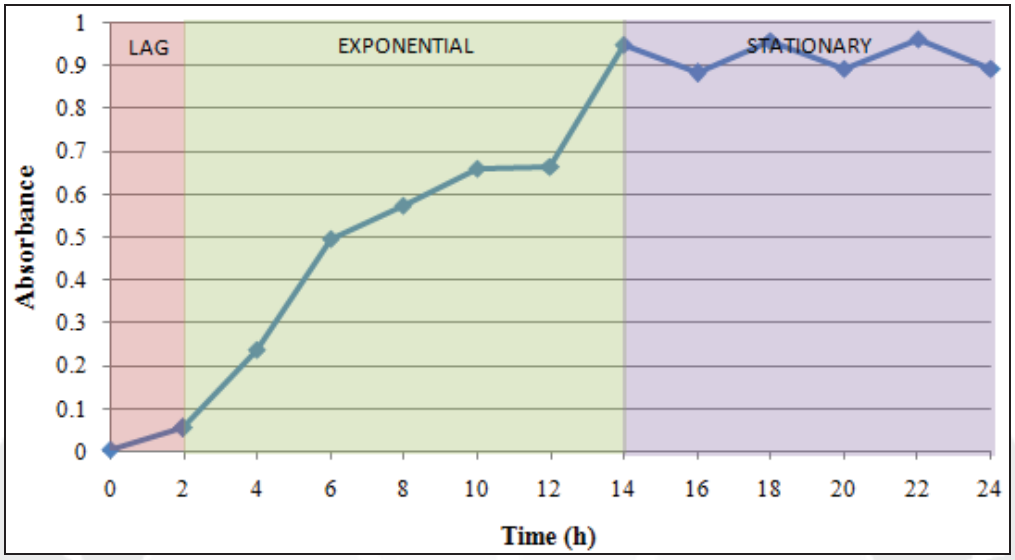

Figure 2. Growth Curve of *Bacillus* sp. SLII-I in FM Media for Keratinase Production

3.2 Keratinase Activity and Protein Determination

Keratinase activity and protein determination is necessary to confirm that enzyme produced in the process of fermentation and isolation is keratinase. *Bacillus* sp. SLII-I is capable of producing enzyme with 2.08 (mg/second)/ml keratinase activity and 6.6 mg/ml protein content. This means that enzyme produced by the *Bacillus* sp. SLII-I is keratinase. Keratinase belongs to hydrolyses group that capable of hydrolyze keratin more efficient compared to other protease (Vigneshwaran *et al*., 2010; Kanmani *et al*., 2011). The hydrolysis of keratin by keratinase produced amino acids and peptide (Mousavi *et al*., 2013) that soluble in water.

3.3 Water Soluble Protein of Enzymatic Engineered Chicken Feathers Waste

Keratin protein in chicken feathers are insoluble in water and have low digestibility (Joshie *et al*., 2007). Keratinase had a role in hydrolyze keratin via termination of hydrogen and disulfide bonds to produce amino acids and peptides (Mousavi *et al*., 2013) that are indirectly increasing digestibility of chicken feathers waste (Lee *et al*., 1991). Figure 3 show the highest increase level of water soluble protein of 1.0 gram chicken feather waste that are modified enzymatically by 0.16 ml keratinase. This is the optimum incubation condition where keratinase hydrolyze keratin efficiently. Gaman and Sherrington (1992) said increase level termination of hydrogen and disulfide bond during hydrolysis also increasing the level of protein that can be absorbed by the body and result in growth. The condition that resulting in enzymatic engineered feathers waste which have the highest increase level of water soluble protein is applied in the conversion feather into alternative protein source in livestock feed.

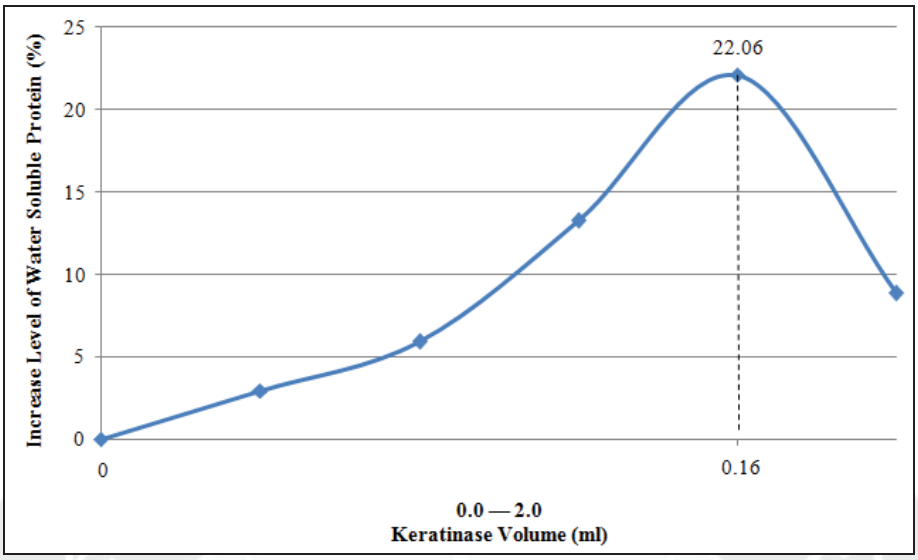

Figure 3. Increase Level of Water Soluble Protein of Enzymatic Engineered Chicken Feathers Waste

3.4 Performance of Broiler Chicken

Table 2 show that difference protein source in diets had a considerable influence ($P<0.05$) to the performance of Broiler chicken (*G. domesticus*). Broiler chicken (*G. domesticus*) that consumed feed contain fishmeal protein source give the highest production performance than broiler chicken that consumed feed contain only soybean meal and feather meal. It was because of feed contain fishmeal have higher energy-protein level than feed contain only soybean meal. Pesti (2009) states that feed utilization efficiency depend on dietary protein and the energy level. Feed with high energy and protein content is more efficient to produce growth than feed with low energy and protein content (Tamalludin, 2014). Feed contain hydrolysate feather meal show higher value of feed consumption and addition of weight than feed contain soybean meal protein but not as good as fishmeal. It was because of low digestibility of feather meal than fish meal. Although, feed contain feather meal have the highest energy and protein value (Table 1).

Table 2. Performance of Broiler Chicken (*G. domesticus*)

Diet	Feed Consumption (gram /head)	Addition of Weight (gram/head)	Feed Conversion Ratio
Diet 1 1st Control (Soybean Meal)	983.6^{A}	232.6^{A}	4.27^{B}
Diet 2 2nd Control (Fish Meal)	2244.3^{C}	1203.0^{C}	1.86^{A}
Diet 3 (Feather Meal)	1194.8^{B}	567.0^{B}	2.11^{A}

Information: Values with the different notation $^{A, B, C}$ are significantly different.

The difference of feed intake of broiler chicken (*G. domesticus*) is affected by the balance of nutrient content in feed. Fish meal contain high essential amino acids of lysine and methionine (Rahman, 2002) while soybean meal have low methionine amino acid (Ketaren, 2008). Feather meal is rich in leucine, isoleucine, and valin amino acids but low in methionine and tryptophan (Ketaren, 2008). Fish meal have better quality of protein and balance amino acids than soybean meal and feather meal so it gives high feed intake followed by high body gain weight resulting in feed conversion ratio that meet standard. The declining of protein content and deficiency in essential amino acids (Burman dan Burgess, 1986; Trisiwi *et al*., 2004; Ketaren, 2008) had harmful consequences for growth and lowered feed intake (Grazziotin, 2008; Waldroup *et al*., 2012)

However, feather protein is considered an excellent source of metabolizable protein (Wang and Parsons, 1997). Despite the limited nutritional value of keratin, both the digestibility and amino acids of feather protein might be improved by enzymatic modification of keratin by keratinase. This research indicated that protein hydrolysate of feather meal could substitute about 5% of soybean meal protein and showed the performance of broiler chicken that is better than soybean meal conventional sources of protein. But, feather meal could not yet substitute fish meal although showed comparable feed conversion ratio value (P>0.05) with fish meal. This problem can be resolved with the utilization of soybean meal, fish meal, and feather meal resulting to good quality of protein and balanced amino acids in livestock feed.

4. CONCLUSIONS

Bacillus sp. SLII-I is capable of producing keratinase with 2.08 (mg/second)/ml keratinase activity and 6.6 mg/ml protein content that can increase water soluble protein level of feathers waste until 22.06%. An enzymatic engineered chicken feathers waste could substitute about 5% of soybean meal protein and showed the performance of broiler chicken that is better than soybean meal conventional sources of protein but could not yet substitute the use of conventional protein sources of fishmeal.

5. ACKNOWLEDGEMENT

The authors are grateful for the academic grant provided by Biomaterial and Enzyme Technology Research Team (2014/2015) supervised by Dr. techn. Endry Nugroho Prasetyo, MT. in which the study was conducted. Special thanks are also given to father Tjahjo Harsojo and mother Theresia Puspita for the motivation, support and prayers; to family of Scylla serrata 2011 and Biomaterial and Enzyme Research Team (2014/2015) for the motivation, support and assistance during the study.

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Detection of Bovine Viral Diarrhea Virus for Identification of Persistently Infected Animal in Dairy Cattle Herds

*P. Anika[1], R. Wasito[2] and H. Wuryastuti[2]
[1] Departement of Inter University Center, Faculty of Biotechnology, University of Gadjah Mada, Jogjakarta, Indonesia
[2] Departement of Pathology, Faculty of Veterinary Medicine, University of Gadjah Mada, Jogjakarta, Indonesia
[3] Departement of Internal Medicine, Faculty of Veterinary Medicine, University of Gadjah Mada, Jogjakarta, Indonesia
neeka.theprayer@gmail.com

Abstract

Bovine viral diarrhea disease (BVD) is an infectious diseases caused by BVD virus that affects economic loss worldwide due to abortion and infertility. Most of the BVD eradication programs are focus on finding persistently infected animal because it can shed large amount of virus in secretions and excretions throughout its lifetime and are primary route of virus transmission. This research objectives were:1) To detect the presence of BVD virus in dairy cattle herds in East Java, Jogjakarta and Central Java and 2) To identify the occurance of persistently BVDV infection among the herds. For the first screening, 87 serum samples from unvaccinated cows in East Java, Yogyakarta and Central Java that had reproductive problems historically were tested by ELISA antibody-BVDV. Results showed that of the 87 samples, 65 positive, 20 negative, and 2 suspected. Seronegative samples were further tested by RT-PCR to find the BVDV antigens followed by ACE method to detect protein E^{rns} as spesific protein abundantly produced by PI animal. Based on RT-PCR test results, from 22 seronegative samples, 11 were negative and 11 were positive BVDV. However, only 10 out of the 11 samples were coming from PI animals. Based on the results, it can be detected and identified persistent BVDV infection in a group of dairy cows in East Java, Yogyakarta and Central Java.

1. INTRODUCTION

Bovine viral diarrhea virus (BVDV) has spread throughout the world and resulted in economic losses. The disease is endemic in most of the cattle population in the world (Radostits *et al*., 2000). Clinical symptoms caused by BVDV disease varies greatly ranging from mild clinical symptoms such as mild fever and leukopenia to clinically fatal such as abortion, stillbirth, congenital defects, weak calves, stunted growth, and mummification (Baker, 1987).

According to Sandvik (2005), observation of clinical symptoms is not enough for identification the occurance of BVD in cattle herd since the disease often goes without any symptoms (subclinical). Therefore, appropriate laboratory tests must be conducted in order to find the source of infection. According Linberg and Alenius (1999), BVD disease control programs are generally intended to identify the presence of persistently infected animals (PI animals) in a group because it is the main source of infection from BVD virus. PI calf was born from a cow infected with

BVDV during the first trimester of pregnancy. This calf will act as a virus factory which will produce and spread the virus continuously throughout his life (Meyling *et al*.,, 1990). The presence of PI animals in a population characterized by a high prevalence of seropositivity (> 90 %) (Houe and Meyling, 1991).

Accurate and effective diagnosis are required for the detection of PI animals. Several diagnostic methods that have been used for detecting the BVD are virus titration, serum neutralization test, ELISA, immunohistochemistry (IHC), virus isolation, RT-PCR and quantitative RT-PCR. The type of the method selected were based on the type of sample collected such as blood, milk, saliva, follicular fluid, tissues, ear notches, nasal swabs and serum (Lanyon *et al*., 2014). According to Saliki *et al.,*. (2000), specific and sensitive diagnostic methods for detecting the PI animals were virus isolation, IHC, RT-PCR and ELISA Ag. In this study, the methods used for detecting the PI animals were total antibody ELISA, RT-PCR and, antigen capture ELISA (ACE). This research objectives were:1) To detect the presence of BVD virus in dairy cattle herds in East Java, Yogyakarta and Central Java and 2) To identify the occurance of persistently BVDV infection among the herds.

2. METHODS

2.1 Chemicals

Eighty-seven blood samples were collected from cattle that historically have never been vaccinated against BVDV and have experienced of having reproductive disorders were used in this study. The cows were coming from several locations such as Central Java, Yogyakarta and East Java. Other chemical used include ammoniumchloride 0.85% (w/v), Tris 0.2% (w/v) pH 8.0, a solution of phosphate buffered saline (PBS), lysis buffer solution, ELISA kits (for BVDV antibodies and BVDV antigen), RNA isolation kit, One-Step RT-PCR (Invitrogen) kit, chloroform (1:1), and a DNA marker.

2.2 Procedures

Blood is collected aseptically in lavender tube, kept into a cooler and sent to the lab ± 1 day later. Blood samples were then centrifuged at 2000 rpm for 20 min for serum collection. Subsequently, the samples were tested using the antibody ELISA kits commercially available. Seronegative samples were then tested by RT-PCR. Isolated RNA were synthesized to cDNA using One-Step RT-PCR (Invitrogen) kit with a total volume of reaction was 25µL each tube. The composition of the reaction were as follows : RT - Mix 12.5 mL, RT - Taq enzyme 0.5 mL, $MgSO_4$ 1µL, template RNA of 2.5 mL (5 □ 10 ng), 1µL forward primer (10 pmol), reverse primer 1µL (10 pmol). The program used is one cycle at 42°C for 60 min, followed by denaturation at 94°C for 30 seconds, annealing 54°C for 1 min, elongation 68°C, 1 min for the total of 30 cycles, extra elongation at the end of the amplification was at 68°C for 10 minutes. Amplification products were then visualized by 1.5 % agarose gel electrophoresis. For the identification of persistent infection, the sample were further tested using antigen capture ELISA in accordance with the test procedures commercially available kits.

3. RESULTS AND DISCUSSION

In this study, first step screening for detecting the BVDV infection in a dairy cows herds were done serologically using ELISA antibody techniques against BVDV. According to Tan *et al*., (2006), in the group of cows that were not vaccinated, serologic testing is a convenient method to determine the prevalence of the disease. Furthermore, the serological cases were considered to have been the result of a natural infection. Results showed that BVDV infection were occured in 65 out of 87 total samples tested (Swasthikawati, 2015). Twenty-two seronegative samples were further tested by RT-PCR using specific primers that amplify the gene 5'-UTR BVDV. Of the total 22 samples tested, 11 samples showed a positive result (Figure 1).

Table 1. Test result of ELISA antibody anti-BVDV, RT-PCR and ACE

Test	Negative	Positive	Suspected
ELISA antibodyanti-BVDV*	20 of 87	65 of 87	2 of 87
RT-PCR	11 of 22	11 of 22	0 of 22
Antigen Capture ELISA (ACE)	1 of 11	10 of 11	0 of 11

* Swasthikawati (2015)

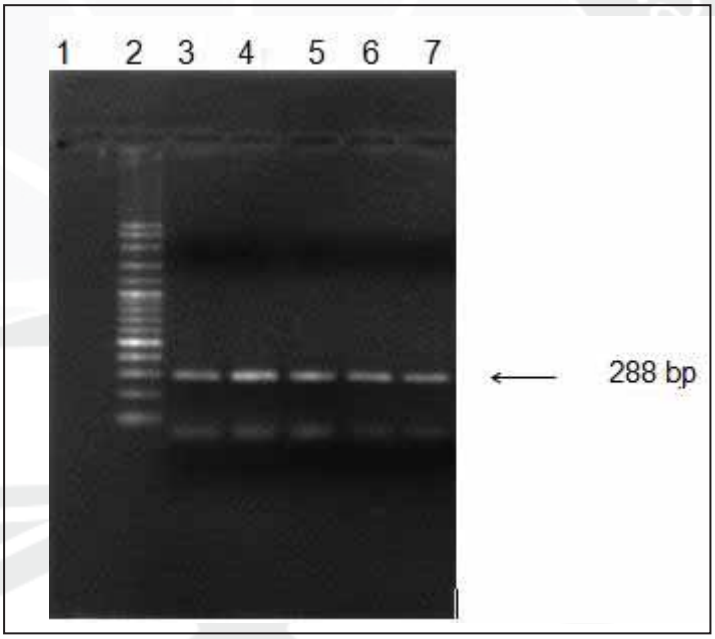

Figure 1. The results of RT - PCR (288 bp) after running on a 1.5 % agarose gel. 1 : negative control BVDV (dH2O); 2 : 100 bp DNA marker; 3 : BVDV positive control; 4 - 7 : BVDV positive field samples

Initial screening showed that the rate of BVDV prevalence in dairy cows was 74.71 % with OD values > 0.7. According to Lanyon *et al*., (2014), Brownlie *et al*., (2000) and Houe *et al*., (1995) high titer antibodies to BVDV and high prevalence rates against BVDV is one indicator the presence of IP animals within a group. Based on those facts, the blood samples from BVDV positive animals with the method of RT-PCR were then furtherly tested by using the antigen capture ELISA (ACE) to confirm the presence of protein Erns, a structural proteins that are expressed virus during replication and were produced by persistently infected animal (Table 1). Erns protein

has RNase activity that is able to inhibit the synthesis of IFN type- I. Since IFN is a defense mechanism against viruses in the early stages of intrauterine development, disturbance in this natural immune response leads to persistent infection by BVDV (Peterhans *et al*., 2010).

ACE test results showed that 10 out of the 11 positive samples were coming from BVDV persistently infected animals and 1 sample was from acutely infected animal. Acute infection is a common form of BVDV infection. In acutely infected animals, their immune system will respond approximately two weeks after virus infection (Meyling *et al*., 1990). In cows, acute infection will cause infertility due to changes in ovarian function and production of gonadotropin and progesterone hormones. While the bulls are likely to excrete the virus in their semen for a short period of time during and immediately after infection and can temporarily decreased their fertility (Fray et al., 2002). In this study, 10 out of 87 (11.49 %) samples tested came from persistently infected animals. Sandvik (2005) explained that the key to persistent infection is the virus' ability to penetrate the placental barrier in non-immune animals and infect the fetus. Persistenly infected animals got infection before their immune system has been well developed so that the PI animal will look healthy and normal until reaching adulthood, but the nature of the immunotolerant make the virus able to replicate throughout his life. As a consequence, PI animal will act as a source of infection for other animals that are sensitive and not vaccinated. Find a persistently infected animal in a herd is very necessary for the implementation of BVDV control program. According to Radostits (2000), regardless of the level of the prevalence, PI animals are the main source of infection in the group.

4. CONCLUSIONS

Based on the results of this study it can be concluded that ± 75 % of dairy cattle originating from the group of cattle in East Java, Yogyakarta and Central Java has been infected with BVDV and ± 11.5 % are considered persistently infected. Follow-up to the PI animals needs to be done for BVDV control program in Indonesia.

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The Study of Bioactive Compound Lesser Yam (*Dioscorea esculenta*), Wild Yam (*Dioscorea hispida*), and Arrowroot (*Maranta arundinacea*) Tubers as Source of Antioxidants

Ari Yuniastuti[1], Retno Sri Iswari[1], Nanik Wijayati[2]
[1]Department of Biology, Mathematics and Natural Sciences Faculty, State University of Semarang
[2]Department of Chemistry, Mathematic and Natural Scianece Faculty, State University of Semarang
ari_yuniastuti@yahoo.co.id

Abstract

Indonesia is currently facing the problem of increasing the prevalence of degenerative diseases. It caused a decline in the health status of society. One of the causes of degenerative diseases is a free radical, which is a compound having unpaired electrons that are highly reactive. It snatches electrons from other molecules around it to stabilize them. An increase in free radicals causing oxidative stress and lead to dysregulation of adipose tissue as an early pathophysiology of degenerative diseases such as hypertension, atherosclerosis, coronary heart disease, stroke, diabetes and other vascular diseases. Free radicals can be resisted by antioxidants. Identification of potential bioactive compounds obtained from local tubers such as lesser yam, gadung, and arrowroot that have the physiological effect as an antioxidant, and will be developed as a functional food through nutrigenomics approach.This study was an exploratory study to identify the content of the antioxidant compound on local tubers such as lesser yam, gadung, and arrowroot obtained in the area of Gunungpati, Semarang. The study was conducted in the laboratory of Biochemistry, Department of Biology,Unnes in April-July 2015. The materials needed were gembili, gadung and garut tubers as well as chemicals and equipment necessary for the analysis of total phenol and antioxidant activity. The antioxidants analysis was done descriptively. The results of phenol total on lesser yam was 0,8865%, whereas arrowroot was 1,8959% and wild yam was 2,7132%. While antioxidant activity test by DPPH methods were 21.2422%, 20.2845% and 19.8476%, respectively. Lesser yam, arrowroot and wild yam tubers are potential as a functional food to be applied as an antioxidant

Keywords: tubers, antioxidants, functional food

1. INTRODUCTION

Recently, the rapid development of science impactsthe increasing of industrialization, urbanization, development, free markets, and economic and social welfare. It is also impact on the lifestyle changes i.e. low frequency of exercising, a diet that high in calorie, and low-fiber food consumption. This condition is significantly influence the health and nutritional status of people, particularly in developing countries, including Indonesia. Diet is a major cause of obesity. Modern humans tend to be busy with a variety of life activities until could no longer eat healthy and nutritious foods. Instant

and junk food are getting popular for most people who are exposed to modern life. Obesity is happening followed by increasing of fat metabolism would lead to the production of Reactive Oxygen Species (ROS) and free radicals, both in circulation and in adipose cells. Free radicals (ROS) are defined as the atom/ molecule/ compound containing one or more unpaired electrons (Fang *et al*., 2002). In chemistry, the unpaired molecules of free radicals tend to react with a molecule of body cells. Then cause abnormal compounds (free radicals new, more reactive) and start a chain reaction that cause cell damage (Winarsi, 2007).

Increased of ROS in adipose cells could cause the balance of oxidation-reduction reactions (redox) is disturbed, resulting in decreased of the antioxidant enzyme in the circulation. This situation called oxidative stress (Fang et al., 2002). The increasing of oxidative stress is causing the dis-regulation of adipose tissue as well as an early pathophysiology of degenerative diseases such as hypertension and atherosclerosis, diabetes mellitus, cancer, hyper-lipid with sickness "derivatives" such as coronary heart disease (CHD), stroke, failed kidneys, arthritis, Alzheimer and Parkinson (Furukawa *et al.*, 2004; Hernani, 2005; Yunanto *et al.*, 2009).

Indonesia is currently facing more nutritional problems such as obesity, vascular disease (coronary heart disease and atherosclerosis), diabetes mellitus and cancer, or commonly known as degenerative diseases. Degenerative diseases triggered by unhealthy eating patterns, causing obesity and the increase of free radicals (ROS) and oxidative stress (Caves and Munos, 2003).

In fact, human need to get antioxidants from diet. It produces by the body to experience a decline in circulation as a result of competition with free radicals. Antioxidants from the outside can be obtained from fruits and vegetables, or other foods that contain antioxidants, known as exogenous antioxidants. Various studies and studies of antioxidants have been widely performed as the content of the antioxidant compounds in seaweed (Olsen *et al*., 2013; Namvar *et al*., 2013; Fiedor and Burda 2014), honey (Bohdanov *et al*., 2008), antioxidants red fruit (Tjahjani and Khiong, 2010).

The study in antioxidants is still needed to be done in the view of great benefits to health. The study on the local sources of antioxidants such as local tubers i.e.lesser yam, wild yam, arrowroot is potential. Those tubers are containing with bioactive compounds such as antioxidant, anthocyanin, dioscorin, diosgenin, and phenol (Mar'atirosyidah and Teti, 2015). As one of the efforts to optimize the use of natural sources of Indonesia as well as improving public health, it is necessary to study to explore bioactive compounds that have physiological effects as antioxidants in the local tubers.

Tubers group can be regarded as a functional food because it contains one or more compounds that have a specific physiological function and health benefits. When the bioactive compounds in the tubers are either directly or indirectly affect the human genome, which in its action can change the expression of the gene structure which called asnutria-genomics (Muller and Kersten, 2003).

2. MATERIALS AND METHODS

2.1 Materials

The raw materials used in this study includedlesser yam, wild yam,and arrowroot

obtained from the Gunungpati, Semarang, Central Java with a shelf life of no more than seven days after harvesting. The chemicals were water, NaCl, sodium metabisulfite, methanol, alcohol, acetone, distilled, concentrated HCl, solution Buffer KCl, Na-acetate buffer solution ($CH_3CO_2Na.3H_2O$), petroleum ether, ethanol 98% of Bratacho chemical, Folin Ciocalteu phenol from Merck, gallic acid from Sigma, sodium carbonate from Merck, aluminum foil (Klin pack), radical DPPH (2,2-diphenil-1-picryldihydrazil radical) from Sigma.

2.2. Methods

2.2.1 Preparation and Treatment of Materials

Lesser yam, wild yam and arrowroot flour was prepared in the following manner: the tubers were sorted, peeled, washed, sliced with a thickness of ± 0.2 cm and soaked in water with the addition of sodium metabisulphite for ± 5 minutes. Slices of each tuber then drained and dried in the sun for ± 2 days to dry, milled and sieved using a sieve of 80 meshes. The next flour packed in plastic and stored at room temperature.

2.2.2 Estimation of total phenolic content

Total phenolic content was estimated using the Folin-Ciocalteu method (Lachman *et al*., 2000). Samples (100µL) were mixed thoroughly with 2 ml of 2% Na2CO3. After 2 min. 100 mL of Folin-Ciocalteu reagent was added to the mixture. The resulting mixture was allowed to stand at room temperature for 30 min and the absorbance was measured at 750 nm against a blank. Total phenolic content was expressed as grams of gallic equivalents per 100 grams of dry weight (100g g-1DW) of the plant samples (Ruba and Mohan, 2013; Therasin and Baker, 2009).

2.2.3 Determination of antioxidant activity with DPPH (1,1-Diphenyl-2 Picrylhidrazyl)

Sample extract of tubers with a concentration of 20,000 ppm was taken as 2 ml, and thenit poured into a test tube. DPPH solution was made by 7.5765 x 10-5 mol/l in ethanol, and thenit was taken for 1 ml and was added by 3 ml of distilled water. The absorbancewas measured at 516 nm wavelength and must obtained absorbance at 0.8. In order to measure the sample absorbance, as much as 1 ml sample of the antioxidant was added by 3 ml of DPPH solution. The mixture was measured at a wavelength of 516 nm for 20 minutes. Determination of DPPH radical capturing capability was measured against standard curve of Gallic acid (0, 50, 100, 250, 300 ppm).

3. RESULTS AND DISCUSSION

The results of the percentage of total phenol content was highest in the wild yam (*Dioscorea hsipida*) (2.7132%), followed by arrowroot (*Maranta arundinacea*) (1.8959%), and lesser yam (*Dioscorea esculenta*) (0.8865%). In determining the levels of phenolic compounds, total gallic acid used as a standard solution. Gallic acid obtained maximum absorption at a wavelength of 750 nm. In the beginning, the standard curve of Gallic acid was made as a standard level to determine the levels of total phenolic compound. Making the standard curve was useful to help determine the levels of phenol in the sample through a regression equation of the standard

curve. Based on the standard curve, the regression equation Y = 0,0021x + 0.733 was obtained. The correlation coefficient (R^2) = 0.9926. The value of R^2 value proved that the regression equation was linear. Tables and Gallic acid standard curve can be seen in Table 1 and Figure 1.

The concentration of the sample solution can be determined by using a calibration curve by measuring the absorbance of samples, and then the total phenolic content in the tubers was calculated using linear regression equation. Total phenolic content of the ethanol extract of the tuber lesser yam (*Dioscorea esculenta*), arrowroot (*Maranta arundinacea*) and Wild Yam (*Dioscorea hispida*) are presented in Table 2.

Table 1. Results of measuring the absorbance of standard solution of Gallic acid at a wavelength of 750 nm using a spectrophotometer

No	Concentration (mg/L)	Absorbance
1	0	0,7328
2	100	0,8953
3	150	1,1021
4	250	1,2961
5	300	1,3819

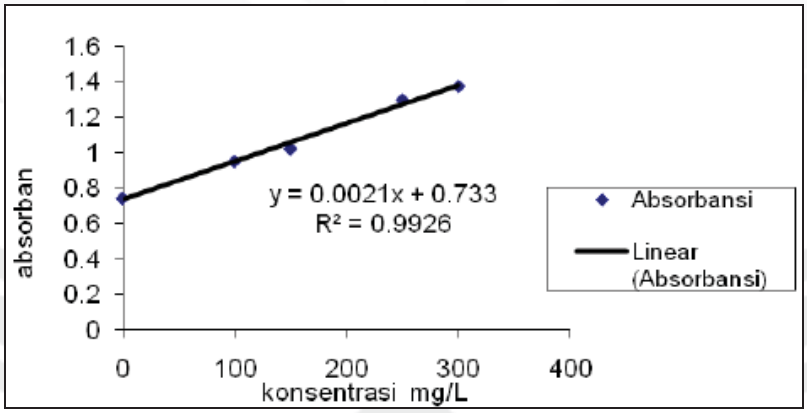

Figure 1. Calibration curve of Gallic acid in a solution of phenol at a wavelength of 750 nm

Table 2. Percentage Content of phenols several types of tubers

No	Tubers	Mean of Absorbance	Fenol concentration (%)
1	*Lesser yam (Dioscorea esculenta)*	0,8952	0,8865
2	*Arrowroot (Maranta arundinacea)*	0,9123	1,8959
3	*Wild yam (Dioscorea hispida)*	1,2865	2,7132

3.1 Radical capturing activity with DPPH (1,1-Diphenyl-2-Picrylhidrazyl)

The ability to capture free radicals is another term for the activity of aantiradical compound. This activity is measured by the value of DPPH. The ability to capture the highest percentage of radicals contained in lesser yam (*Dioscorea esculenta*) was 21.2422%, arrowroot (*Maranta arundinacea*) was 20.2845% and the lowest was wild yam (*Dioscorea hispida*) 19.8476%. This condition indicates that the activity was not affected by the antiradical polyphenol compounds contained. However, high amount of polyphenol compoundsdid not affect the activity.

The method used in part of testing of the antioxidant activity was DPPH radical absorbance method. This method is simple, easy, and requires only samples in small amounts, a short time (Hanani *et al*., 2005). In the determination of the ability to capture radicals,Gallic acid was used as a standard solution. Gallic acid obtained maximum absorption at a wavelength of 516 nm. Before the determination of ability to capture radicals, the standard curve of Gallic acid was made.The standard curve was useful to help to determine the ability to capture radicals in the sample through linear regression equation of the calibration curve. From the determination of the standard solution of Gallic acid calibration curve obtained by regression equation Y = 0,0014x + 2.4078 and correlation coefficient (R^2) = 0.9819. Table and figure of Gallic acid standard curve can be seen in Table 3 and Figure 2. The concentration of the sample solution can be determined by using a calibration curve by measuring the absorbance of samples, and then the percentage of radical capture capabilities was calculated using linear regression equation. Percentage ability to capture radicals in the ethanol extract of tubers lesser yam (*Dioscorea esculenta*), arrowroot (*Maranta arundinacea*) and wild yam (*Dioscorea hispida*) are presented in Table 4.

Table 3. The results of the absorbance measurement of a standard solution of DPPH at 516nm using a spectrophotometer

No	Concentration (mg/L)	Absorbance
1	50	2,1642
2	100	2,2604
3	250	2,1437
4	300	2,0271

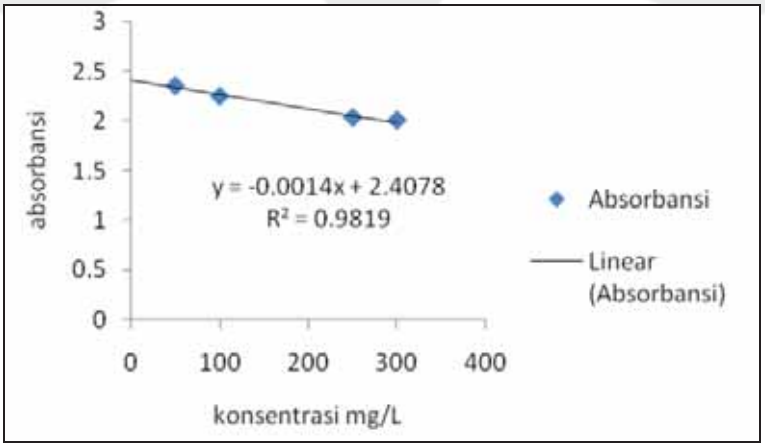

Figure 2. Calibration curves DPPH gallic acid in solution at a wavelength of 516 nm

Table 4. Percentage of antioxidant activity of several types of tubers

No	Tubers	Mean of absorbance	Anti free radicals activity (%)
1	*Lesser yam (Dioscorea esculenta)*	0,8952	21,2422
2	*Arrowroot (Maranta arundinacea)*	0,9123	20,2845
3	*Wild yam (Dioscorea hispida)*	1,2865	19,8476

On a sample of tubers after DPPH solution added, it was resulting in a color change in the solution of DPPH in ethanol, which was originally colored dark violet to yellow. This was in accordance with Andayani *et al*., (2008), the measurement of the antioxidant activity of the sample performed at a wavelength of 516 nm which is the wavelength of maximum DPPH. The presence of the antioxidant activity of the sample was proved by a color change. DPPH is a free radical that is stable at room temperature and is often used to evaluate the antioxidant activity of several compounds or extracts of natural ingredients. DPPH accept electrons or hydrogen radicals will form a stable diamagnetic molecule. DPPH antioxidant interaction with either the transfer of electrons or hydrogen radicals on DPPH will neutralize free radicals of DPPH character. If all the electrons in the free radical DPPH are into pairs, then the color of the solution changed from dark purple to yellow light and the absorbance at 517 nm wavelength will be lost. These changes can be measured in accordance with the stoichiometric amount of electrons or hydrogen atoms captured by DPPH molecules due to antioxidants (Gurav *et al*., 2007).

4. CONCLUSIONS

Phenol content and antioxidant activity of tubers in this study can be concluded as follows:

1. The percentage of the highest content of phenolic compounds found in wild yam (2.7132%), arrowroot (1.8959%), and lowest was found in the lesser yam (0.8865%).

2. Antiradical activity was highest in lesser yam (21.2422%), followed by arrowroot (20.2845%), and was lowest in wild yam (19.8476%).

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Phenolic Compound and Antioxidant Activity of Arganically and Conventionally Grown Vegetables as Potential Functional Food Ingredients

Ignasius Radix A.P. Jati
Department of Food Technology, Faculty of Agricultural Technology, Widya Mandala Catholic University Surabaya, Surabaya, Indonesia
radix@ukwms.ac.id

Abstract

Vegetables are rich sources of bioactive compound that could act as antioxidant. Nowadays, there are many campaigns of organically grown vegetables which claimed to be healthier than the conventional one. The objectives of this research are to determine the phenolic compound and to examine the antioxidant activity of organically and conventionally grown garlic, red bell pepper, and rucola leaves. In this research, phenolic compound was measured by Folin Ciocalteau method. Meanwhile, the antioxidant activities were examined by DPPH radical scavenging capacity, FRAP, and superoxide radical scavenging activity. The result shows that organically grown garlic have the highest phenolic content (24,35 mg GAE/g dry sample), followed by organic red bell pepper (14,76 mg GAE/g dry sample) and conventional rucola leaves (12,42 mg GAE/g dry sample). The DPPH method reveal that organically grown garlic have highest DPPH scavenging capacity followed by organic red bell pepper and conventional rucola leaves with 72%, 62%, and 47%, respectively. Similar trends were found in FRAP with 931,65 µmol $FeSO_4$/l; 692,48 µmol $FeSO_4$/l; and 376,43 µmol $FeSO_4$/l, respectively, and also superoxide radical scavenging activity with 67%, 52%, and 43% respectively.

1. INTRODUCTION

The number of research in the field of functional food is increase rapidly. Functional food could help to fight against numerous incidences of degenerative diseases such as coronary heart disease, diabetes, cancer, stroke, and premature aging by providing bioactive compound which have functional properties that can be beneficial to reduce the onset of such diseases. Degenerative diseases are believed to be caused by unhealthy lifestyles which among them are poor dietary habit, lack of physical activity, excessive consumption of alcohol and cigarettes, environmental pollutions, and life stress (Gutteridge *et al*., 1993). These factors induce the unbalance metabolism of the body resulted in the over production of pro oxidant, which responsible for the oxidation of components in the body such as DNA, fat, and protein leads to diseases. As the number of pro oxidant in the body increased, human body react by producing indigenous antioxidant. However, the excessive numbers of pro oxidant will outcast the indigenous antioxidant if stress condition occurred. Therefore, intakes of exogenous antioxidants are needed to meet the balance condition of the metabolism process.

Vegetables are rich sources of antioxidant compounds. The most abundant

compound found in vegetables is phenolic. Phenolic compound is a secondary metabolites of plants which responsible for fighting against pathogens and ultraviolet radiation (Shahidi and Naczk, 2003). Vegetables that rich in phenolic compound are garlic, green leafy vegetables, and red to black colored vegetables. The phenolic compound in vegetables are reported to have high antioxidant activity (Huang *et al*., 2009) which could inhibit the growth of cancer cell (Kris-Etherton *et al*., 2002), decrease the formation of atherosclerotic plaque (Morton *et al*., 2000), and give the positive response in maintaining blood glucose level (You *et al*., 2012). The high phenolic content and antioxidant activity are however reduce significantly when the vegetables are subjected to cooking or heat processing. The heat treatments are believed transform the antioxidant compound into other compounds which have low antioxidant activity. In response to these findings, researchers suggested that minimally processed vegetables are suitable to be consumed in term of maintaining the antioxidant activity. As evidence were shown from widely investigated Mediterranean diet which consists of minimally processed vegetables and reported to have health effect and increase the life quality of people in the Mediterranean region (Sofi *et al*., 2010). Salad is the most popular minimally processed food served in the diet, especially in Europe. Among the ingredients, red bell pepper and rucola leaves are the most common vegetables found. Meanwhile garlic is spices that widely used in Asia and the numerous number of research demonstrated the health properties of garlic (Rahman, 2007).

Other concerns in consuming minimally processed vegetables are the methods of growing. Recent findings reported that conventionally grown vegetables were found to have chemical substances which postulated to be from the fertilizer and insecticide used (Kipopolou *et al*., 1999). These chemicals could not be metabolized and if accumulated in the body will lead to development of diseases (Khan *et al*., 2008). Organically grown vegetables are the response to the concern of chemical residue. Organic planting is one of methods for growing plants without using any chemical substances starting from the preparation of the soil to the harvest of the yields. Manures and other biological substance such as plant extract were used for organic planting. Organic planting produces vegetables without any chemical residues. On the other hand the quality of vegetables in terms of productivity and size are lower and the susceptibility to insect and pest are higher compared to the conventional one.

Although report on the methods of organic farming, quality and quantity of yields are available (Lampkin *et al*., 2000), research on the effect of organic and conventional planting methods on phenolic compound and antioxidant activity are limited. Therefore, the objectives of this research are to determine the phenolic compound and to examine the antioxidant activity of organically and conventionally grown garlic, red bell pepper, and rucola leaves.

2. METHODS

2.1 Chemicals

All chemicals such as Folin ciocalteu, Gallic acid, Sodium carbonate, 2,2-diphenyl-1-picryl hydrazyl radical (DPPH), riboflavin, methionine, Nitroblue tetrazolium (NBT), $FeSO_4$, Butylated Hydroxytoluene (BHT), methanol, and HCl used were purchased from VWR International GmbH and Merck KGaA (Darmstadt, Germany) unless

stated otherwise. Millipore Milli-Q, Q-Gard 2 (Eschborn, Germany) was used to produce ultrapure water used for extraction.

Vegetables sample which are organic and conventional grown garlic, rucola, and red bell pepper were purchased from local market in Plieningen, Stuttgart, Germany

2.2 Procedures

2.2.1Sample treatment

The vegetables were immediately chopped into small pieces. Then samples were placed into round-bottomed flasks and stored in a freezer (-20°C) overnight prior to freeze drying (Virtis, Freeze mobile 25 EL, Gardiner, New York). Each freeze-dried sample was subsequently milled using a food processor (Philips) and passed through 30 mm mesh size sieve. The samples were stored in dark brown bottles and kept in a refrigerator (4°C) until used for analysis.

2.2.2 Methanolic extract of sample

Briefly, 500 mg of sample was placed in 15 ml falcon tubes and 5 ml of methanol-HCl (1%) solution was added. The tubes were vortexed and subsequently placed in a roller extractor for 15 min. After that, the tubes were centrifuged at 2790 x g for 20 min at 4°C. The mixture was then filtered using Whatman No.1 filter paper and the supernatant were collected. The extraction was repeated three times and the supernatants were combined, dried using rotary evaporator, and stored until further used.

2.2.3 Phenolic analysis

Folin Ciocalteau method by Singleton and Rossi (1965) was applied to determine total phenolic content. Briefly, 0.1 ml extract was placed in a tube, and 0.5 ml Folin Ciocalteu reagent mixed with ultrapure water (1:1) was added, mixed, and allowed to stand for 8 min. Then, 4.5 ml of 2% sodium carbonate (Na_2CO_3) solution were added, mixed and stored in a dark room for 1 h at room temperature. Absorbance of the resulting blue complex was then measured at 765 nm using a spectrophotometer. Methanol was used as the blank and gallic acid was used as standard. The results were expressed as mg Gallic acid equivalents/100 g dry weight of sample. Data were reported as means ± SD for three replications.

2.2.4 DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity

The DPPH radical scavenging activity was measured according to method described by Astadi *et al*., [18]. In brief, 0.5 ml of freshly prepared 0.5 mM DPPH solution was vigorously mixed with 0.1 ml of extract. Then, 4 ml of methanol was added to the mixture and vortexed thoroughly before allowing to stands for 60 min in a dark at room temperature. The absorbance was then measured using spectrophotometry at 516 nm against a blank. DPPH radical scavenging activity was calculated using the following formula, % DPPH radical scavenging = [(control absorbance-sample absorbance)/control absorbance] x 100%. BHT was used for reference. Data were reported as means ± SD for three replications.

2.2.5 Ferric Reducing Antioxidant Power (FRAP)

The FRAP was determined following a method reported by Vadivel and Biesalski (2012). Briefly, 1.8 ml freshly prepared FRAP reagent was mixed with 180 µl distilled water and 60 µl extract. Then the mixture was incubated at 37°C for 30 min. The absorbance readings were taken immediately at 593 nm using a spectrophotometer. The reducing power was calculated from the calibration curve prepared with different concentrations of Fe [II] ($FeSO_{4.7}H_2O$, 100–2000 mM). Methanol and BHT were used for the reagent blank and positive control, respectively.

2.2.6 Superoxide radical scavenging capacity

The superoxide radical scavenging activity of extracts was measured based on method reported by Zhishen *et al*., (1999). Briefly, 4.9 ml of reagent (riboflavin, methionine and NBT in 0.05 M phosphate buffer pH 7.8 with final concentration of 3 X 10^{-6}, 1 X 10^{-2} and 1 X 10^{-4} mol/l, respectively) was mixed with 100 µl of extract. The mixture was then illuminated at 25°C for 25 min using a 20 W fluorescent lamp. The un-illuminated reaction mixture was used as a blank and the absorbance was measured at 560 nm.

2.2.7 Statistical Analysis

All of the experiments were performed in three replications. SPPS version 13 was used for statistical analysis, and significant test was performed with Least Significant Difference (LSD) test. Data were reported as means ± SD for three replications.

3. RESULTS AND DISCUSSION

The number of research in the field of functional food is increase rapidly. Functional food could help to fight against numerous incidences of degenerative diseases such as coronary heart disease, diabetes, cancer, stroke, and premature aging by providing bioactive compound which have functional properties that can be beneficial to reduce the risk of such diseases. In recent years, researchers widely explore various foods and investigated their bioactive properties and the effect on human health, thus could be claimed as functional food. Generally, the research on functional food began from the local or indigenous knowledge of community on certain foods that claimed to have beneficial effects on health. This belief has been existed for decades or even centuries. From the local knowledge, scientist is trying to find the scientific answers behind such indigenous wisdom. Some research found the scientific evidence thus brought the traditional food to a broader scope (Salminen *et al*., 1998).

This research focused on the phenolic compound and antioxidant activity of garlic, red bell pepper, and rucola leaves. The phenolic content of samples can be seen in Table 1.

Table 1. Phenolic content of samples (mg GAE/g dry sample)

Vegetables	Organic	Conventional
Rucola	5,12[a]	12,42[b]
Red bell pepper	14,76[c]	10,24[d]
Garlic	24,35[e]	15,88[f]

From Table 1, it can be seen that garlic has the highest phenolic content in both organic and conventional groups followed by red bell pepper and rucola. The phenolic compound in this research was determined by Folin Ciocalteu method. This research in line with previous report by Lu *et al*., (2011) stated that garlic has a high content of phenolic compound as well as other bioactive substances. Even though rucola and red bell pepper contents of phenolic were lower than garlic, however both vegetables are having considerable amount of phenolic compound and could supply such bioactive compound for the diet if consumed regularly. Meanwhile, organically grown garlic and red bell pepper are having higher phenolic compound compared to the conventional one. This could be due to the fact that plant which grown using organic method will have less nutrients available for growth and moreover, the nutrient from soil and manure will not easily soluble with water and therefore resulted in the difficulties of roots to pick the nutrients up and utilized. This condition leads to the deficiency of nutrients and increase the susceptibility of plants to insect and any other disease. To protect itself, plants then needs to alter their metabolism. The products of plant metabolism are compound better known as bioactive compound which among them is phenolic compound (Shahidi and Naczk, 2003). This result in line with previous report by Asami *et al*., (2003) suggested that organically grown plants were having higher phenolic content compared to the conventional one. On the other hand, the conventionally grown rucola has the higher phenolic content compared to the organic one. This result is also supported by previous finding (You *et al*., 2011) which postulated that not all organic treatment will result in higher bioactive compound. It depend on the plant variety and secondary metabolites produced by the plant

In this research, antioxidant activities of vegetables extract were examined using three different methods. The first method is DPPH. This method determines the ability of extract to scavenge DPPH radical. The purple color of DPPH solution will turn to pale yellow if it successfully scavenged by the vegetable extract. The result is shown in Figure 1.

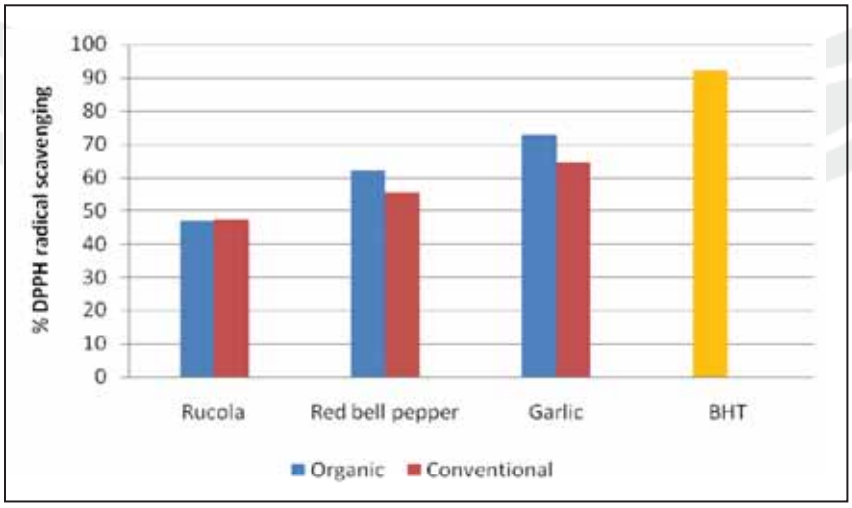

Figure 1. DPPH radical scavenging activity of extract

Figure 1 revealed that samples having higher phenolic content were also having higher antioxidant activity. The DPPH method reported that organically grown garlic

have highest DPPH scavenging capacity followed by organic red bell pepper and conventional rucola leaves with 72%, 62%, and 47%, respectively. The finding on the positive correlation between phenolic content and antioxidant activity using DPPH method are in line with previous result which stated that sa *et al*., 2011).. Even though all of the extract exhibit strong ability to scavenge DPPH radical, however compared to BHT as positive control, the activity of extract are lower. BHT was used because it is usually added to foods as synthetic antioxidant as preservative agent. DPPH are simple and rapid method to examine antioxidant activity. DPPH will react with substances that act as hydrogen donor. Some drawbacks however exist for extract having strong color such as red, black, or purple which will interfere with yellow color as the end product of reaction.

Other method used for antioxidant activity is FRAP. The principle of FRAP method is the ability of bioactive compound of the extract to reduce ferric to ferrous ion. The result of FRAP are shown in Figure 2.

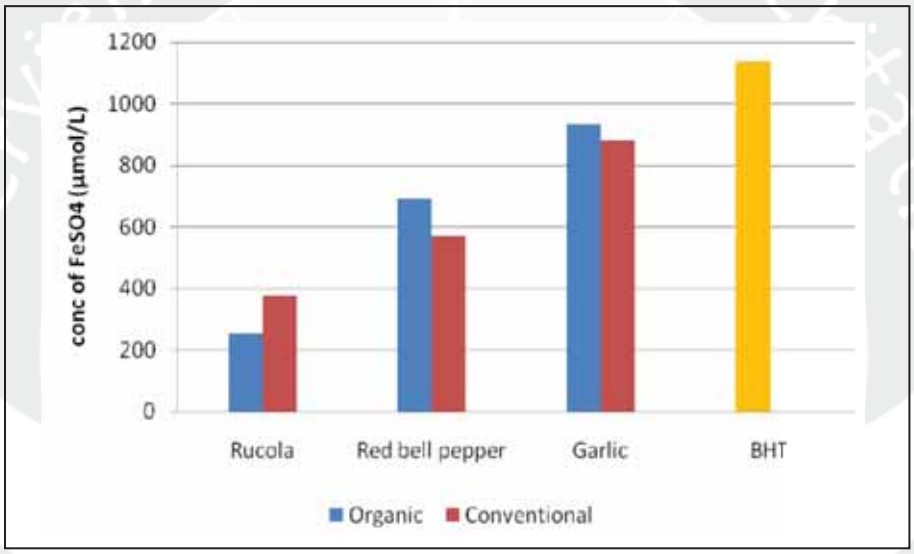

Figure 2. Ferric Reducing Antioxidant Potential (FRAP) of extract

Similar trend as DPPH were found in FRAP method (Figure 2) that organic garlic has the highest antioxidant activity (931,65 µmol $FeSO_4$/l), followed by organic red bell pepper (692,48 µmol $FeSO_4$/l), and conventionally grown rucola leaves (376,43 µmol $FeSO_4$/l). Similar reason with DPPH could explain this condition which is due to the higher phenolic compound found in organic garlic and red bell pepper. Meanwhile conventional rucola leaves also have higher phenolic compound compared to the organic one. The suitability of phenolic compound to reduce ferric ion were also reported in previous research (Dudonne *et al*., 2009). From the result, it can be suggested that bioactive compound in the extract had the capacity of reducing ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) in FRAP method. The ability of phenolic compound to inhibit the superoxide radical formation was due to its chemical structure and substitution pattern of the hydroxyl bond (Bors *et al*., 1990).

Superoxide radical scavenging capacity was other method used to examine antioxidant activity of extract. This method based on the mixture of methionine, riboflavin, and nitroblue tetrazolium (NBT). The mixture was then illuminated at 25^{O}C

for 25 min, so that the photochemically reduced riboflavins generated superoxide radicals, which reduced NBT to form a blue formazan. The extract which was added to the reaction mixture was going to scavenge superoxide radicals, thereby inhibiting the NBT reduction. The un-illuminated reaction mixture was used as a blank and the absorbance was measured at 560 nm. The result of ability of extract to scavenge superoxide radical are presented in Figure 3.

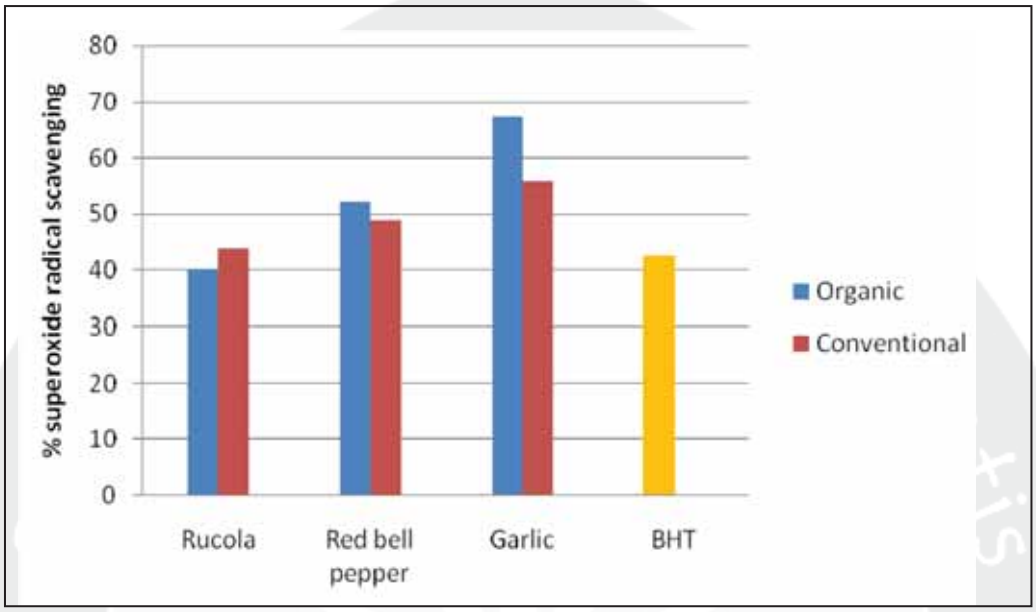

Figure 3. Superoxide radical scavenging capacity of extract

The result shows that organic garlic has the highest antioxidant activity compared to other sample and BHT as positive control, followed by conventional garlic, organic red bell pepper, conventional red bell pepper, conventional rucola, and organic rucola. All of the results are having positive correlation with phenolic content of the extract. Superoxide radical scavenging capacity is also simple and rapid method. However, the sensitivity of the riboflavin, methionine, and NBT mixture to lights made it difficult to predict the time needed for oxidation process. The distance of the mixture containing tubes to the light source was also give significant effect on the oxidation rate. Moreover, the chamber used for placing the tubes and light source was a closed chamber therefore it is difficult to detect whether there are changes in the mixture.

4. CONCLUSIONS

Garlic, red bell pepper, and rucola leaves are having high content of phenolic compound. All of samples also exhibit high antioxidant activity examined using DPPH, FRAP, and TBARS. Organically grown garlic and red bell pepper are having higher phenolic content and antioxidant activity compared to the conventional one. On the other hand, conventionally grown rucola are having higher content of phenolic and antioxidant activity compared to the organic one.

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Hypoglycemic In Vivo Bioassay of Protein Isolate from Cowpeas (*Vigna unguiculata*) Sprout

Bayu Kanetro
Food Technology Department, University of Mercu Buana Yogyakarta Indoensia
Jl Wates km 10 Yogyakarta
bayu_kanetro@yahoo.co.id

Abstract

This research was aimed to determine the potency of hypoglycemic activity of protein isolate from cowpeas sprout through in vivo bioassay by using Sprague Dawley male rats. The treatments of the research were rat conditions (normal and diabetic rats) and feed treatments (standard and protein isolate feed). Blood glucose of rats were analysed on 3^{th}, 6^{th}, 9^{th}, 12^{th}, 15^{th} days for the treatment and before treatment as control. The result of this research showed that the potency of hypoglycemic activity were shown by decreasing of blood glucose level in diabetic rats with protein isolate treatment. While the blood glucose of diabetic rats with protein isolate feed reduced to normal level on 12^{th} and 15^{th} days, that was indicated that protein isolate from cowpeas sprout could normalize blood glucose.

Keywords: cowpeas, sprout, hypoglycemic, protein isolate

1. INTRODUCTION

The development of protein isolate consumption or vegetable-based meat alternatives in the future will increase in the future along with increasing in vegetarian group, due to their potency prevent the onset of many degenerative diseases. Soybean seed has popularly known as functional food for preventing degenerative diseases, esspecially diabetes. it is due to its ability to reduce blood glucose. Soybean protein had hypoglicemic effect due to its potency for stimulation of insulin secretion, and its ability to reduce blood glucose. The potency of soybean sprout protein stimulated insulin secretion was higher than soybean protein (Kanetro *etal*, 2008). The other reseacher had shown that the germination of soybean (Pathak, 2005), rice (Usuki *et al.*, 2007) could increase the potency for decreasing blood glucose. Soybean sprout protein also showed the role as *insulin-like protein* (Pathak dan Martirosyan, 2011).

Hypoglicemic property of soybean related to the composition of amino acids, in particular arginine (Kanetro *etal,* 2008). Amino acids may influence insulin secretion via a number of possible mechanisms, including generation of metabolic coupling factor, depolarization of the plasma membrane, or enhancement of mitochondrial function (Newsholme *et al*., 2006). The specifics amino acids that are known as insulin stimulation can activate mitochondrial metabolism in pancreatic β-cell via the tricarboxylic acid (TCA) cycle, resulting in the formation of ATP. The rise in ATP levels leads to closure of ATP-dependent K^+ channels, which in turn depolarizes the cell membrane, thus opening of voltage-dependent Ca^{2+} channels and increasing

intracellular Ca^{2+} concentration, which triggers insulin exocytosis and hence facilitating insulin secretion from pancreatic β-cell (Argmann and Auwerx, 2006; Newsholme *et al*., 2007).

The increasingly high price of soybean in Indonesia encourage research to replace soybean as a functional food. Based on previous research it was known that the protein isolate from cowpea sprout contained arginine (Kanetro dan Dewi, 2013). But the hypoglycemic ability of protein isolate from cowpea sprouts has not been known. The purpose of this research was to study the hypoglycemic ability of protein isolate from cowpea sprouts through in vivo bioassay

2. METHODS

2.1 Chemicals

The main materials of this research were cowpea seed (*Vigna unguiculata*) from Beringharjo market in Yogyakarta, and Sprague-Dawley rats obtained from Animal Experiment Development Unit UGM, Yogyakarta for in vivo biological testing. The other materials were chemicals to the feed and bioassay, including alloxan (Sigma), corn starch, casein, vitamin mix, mineral mix, sucrose, choline bitartat, soy oil, cholesterol kit (DiaSys Diagnostic System GmBH & Co.), and chemicals for protein isolation ie HCl (Merck), and NaOH (Merck). Chemical agents, such as aloxan, glukosa kit (*DiaSys Diagnostic System GmBH & Co*), dan kholesterol kit (*DiaSys Diagnostic System GmBH & Co*) were purchased from Sigma Chemical Co.

2.2. Procedures

2.2.1 Isolation of Protein from Cowpea Sprout

Cowpea seeds were soaked for 8h, and then germinated for 36h. Proteins of cowpea sprout were isolated along with Yusniardi *et al.,* (2010). The protein were extracted at pH 9 and then precipitated at pH 4. The precipitates of protein were dried by oven at 50^{o}C before stored and analyzed.

2.2.2 In Vivo Bioassay

The *in vivo* bioassay was done to determine the potency of hypocholesterolemic of protein isolate from cowpeas sprout by using 20 *Sprague Dawley* male rats. The experiment sequences of the step were adaptation of rats for 3 days, diveded rats into 4 groups, treated rats for 15 days with the condition of rat and feed treatments, and analysed the blood glucose for the treatment of rats on 3th, 6 th, 9 th, 12 th, 15 th days and before treatment as control (0th). The experimental design of this research was randomized complete design with 2 factors. The first factors were rat condition treatments, that were normal rats and diabetic rats which was induced by aloxan injection. The second factors were feed treatments, that were standart feed according to AIN–93 (Reeves *etal,* 1993) and protein isolate feed which was prepared by subtitution of casein protein in standart feed with the protein isolate from cowpeas sprout. The data of this experiments was statistical analysed by Anova (analysis of varian) and DMRT (Duncan Multiple Range Test).

3. RESULTS AND DISCUSSION

The body weight of rats during the experiment shown in Table 1 The weight of

normal rats increased, while the body weight of diabetic rats decreased despite the decline in the treatment of diabetic rats with fed protein isolates occur at the beginning of the experiment or until 12th day and subsequent the weight increased. The percentage change in body weight of rats on day 15 (end of treatment) compared to day 0 (before treatment) shown in Table 1 showed that the treatment of diabetic rats fed protein isolate was relatively stable or declining as the standard feed treatment. This indicated that feeding protein isolate could be expected to improve the condition of diabetic rat to normal, because of the potential for protein isolates of cowpeas sprout as functional food described in further discussion.

Tabel 1. Body weight of rats during the experiments (g)*

Rats conditions	Feed treatments	0th day	3th days	6th days	9th days	12th days	15th days	% icrease (+)/decrease (-) in weight
Normal	Standar	201.4b	204.4b	207,b	209.6b	214.4c	220.8b	+9,6
	Protein isolate	202.0b	205.4b	208.4b	212.6b	218.2c	224.2b	+11,0
Diabetic	Standar	195.2a	189.2a	187.8a	185.4a	183.0a	179.8a	-7,9
	Protein isolate	199.9b	193.2a	194.6ab	197.2ab	199.2b	203.2b	+1,7

*The same notation of statistic in the table showed not significantly differences at the same column

Table 2 showed that blood glucose level of all rats before treatment were 72.2 – 73.1 mg/dL. This indicated that all rats were normal or not diabetic. The glucose level of human diabetic condition was higher than 180mg/dL (Burtis *et al*., 1988), while The glucose level of rat diabetic condition was higher than 109mg/dL (Garrison, 2013). The glucose level after aloxan injection at 3th days increased significantly and these rats were diabetics. The potency of hypoglycemic were shown by decreasing of blood glucose level in diabetic rats with protein isolate oyek. On 15th days treatment, The blood glucose of the diabetic rats with standard feed increased and they were still diabetec. While the blood glucose of diabetic rats with protein isolate feed treatment reduced 52.78% on 15th days after the treatment. This indicated that protein isolate of cowpeas sprout was potential to normalize blood glucose.

Table 2. The effect feed treatment of protein isolate of germinated cowpeas on glucose level of normal and diabetic rats (mg/dL)

Rat conditions	Feed treatment	0th day	3thdays	6th days	9th days	12th days	15th days
Normal	Standar	72.2a	72.5a	72.4a	73.6a	73.3a	74.6a
	Protein isolate	73.1a	73.1a	72.8a	72.6a	71.3a	71.8a
Diabetic	Standar	72.4a	223.2b	223.6b	226.2b	225.2b	228.3b
	Protein isolate	72.5a	221.9b	221.4b	189.7b	160.3b	104.8ab

* The same notation of statistic in the table showed not significantly differences at the same column

4. CONCLUSIONS

The potency of hypoglycemic were shown by decreasing of blood glucose level in diabetic rats with the treatment of protein isolate of germinated cowpeas. This result indicated that protein isolate might be used to prevent and cure diabetic. Protein isolate of cowpeas sprout could be potential to be added to the product as functional food.

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Effect of Combination Between Carrying material and Different Store Duration on Production of Biofungisides *Trichoderma harzianum* pellet

Juni Safitri Muljowati, Purnomowati and Aris Mumpuni
Faculty of Biology, University of General Soedirman, Purwokerto
junisafitri@gmail.com

Abstract

This research was aimed to know the effect combination between carrying material and different store duration on viability of *T. Harzianum* biofungiside pellet in relation to the highest yielding conidia. This experimental research was done using a Completely Randomized Design (CRD) with factorial pattern. The first factor was type of carrying material which is sticky rice meal, mixed of 75% white sticky rice meal and 25% mungbean meal, mixed of 75% white sticky rice meal and 25% soybean meal, and mixed of 75% white sticky rice meal and 25% skim milk. Each of those carrying material was subjected to be inoculated by *T. harzianum* at 10^8 conidia/ml. The second factor was store duration with three different levels they were 0, 3, 6, and 9 weeks. Obtained data were analyzed by analysis of variance (F test) at significant level of 95% and 99%, then followed by an Honestly Significant Difference (HSD) test. Current results showed that there was interaction between types of carrying material with store period that showed significant effect on viability of *T. harzianum*. Carrying material type of white sticky rice meal with store period of 9 weeks resulting the highest percentage (78,19%) in yielding the conidia of *T. harzianum*.

Keywords: Viability, pellet of *Trichoderma harzianum*, carrying material, storage duration

1. INTRODUCTION

Trichoderma one among those antagomnistic fungi with specific ability on reducing the patogen density in a particular inoculum as well as pressing germination of pathogenic conidia through competition, antibiosis, microparasitisms soil pathogen. This character makes this fungus becomes commonly used as biocontrol agent. Papavizas (1985) stated *Trichoderma* can be used as biocontrol agent for soil fungi like *Rhizoctonia solani*, *Fusarium oxysporum*, and *Schlerotium rolfsii* which commonly attack horticulture plants. The *Trichoderma* produces cellulose which contain a complete enzyme namely C_1 (selobiohidrolase) that able to change natural sellulose, β-glukanase to change liquidified cellulose (CMC-*Carboxyl Methyl Cellulose*) and β-glukosidase. Those three components are synergistically breaking a particular substrate (Salma and Gunarto, 1996).

Among those species within the genus of *Trichoderma*, *T. Harzianum* is the most commonly used as biocontrol agent. This type of fungus has been being developed commercially in several form to control the spread of soil fungi (Roco and Perez,

2001). Alexopoulos *et al.,* (1996), grouped *T. Harzianum*, a soil fungus, in the class of Deuteromycetes, Hypocreales Order and Hypocreaceae Family. Furthermore, Gandjar, (1999) stated that this fungus presencecosmopolitie, easy to be isolated, fast growing, able to produce millions spores and so has a high competitive character (Chang and Baker, 1986). It was also reported as able to live in a poor environment, microparasites antagonistics, and produces kitinase enzyme, β-1-3 glucanaseand β-glucosidase (Wahyudi, 2001).

Apart from its antagonistics character in depressing growth of soil ptahogen, utilization of *T. harzianum* as biocontrol agent could also be used to speed up the growth of the plant (Djatmiko and Rohadi, 1997; Wahyudi, 1999). *Trichoderma* has been being appllied in various substrates like combination between rice bran and saw dusts, sand and rice husks, sand and corn meal fortified with rice husks (Dharmaputra and Suwandi, 1998 *dalam* Salamiah *et al.*, 2003). In a large scale application, the use of those above substrates are concluded as not an effective way. Knowing type of carrying material as well as the formulation are then become prerequisites. The carrying material must contain nutrition needed by the fungus (*T. harzianum*). Stamets and Chilton (1983) stated nutritions are needed by the fungus for its various cell's metabolism processes for energy sources during its live. According to Papavizas (1986), the *Trichoderma* needs nutritions like carbon (), and nitrogen (N) for its live. Wahyudi (1999) stated there are three different types of *T. harzianum* biofungiside apllied so far, namely: pellet, granules, and liquid. Each of them has different carrying material and so its application (1) pellet, a product in a tablet-like form made of mixed material betrween rice husks, rice bran or rice powder and the *Trichoderma* conidia, the pellet is 1 cm X 1 cm in size; (2) granules. A product in a granule form made of a mixture between matrix and conidia of *Trichoderma*; and (3) liquid. This product can either be formulated as a suspension or liquid. Of those three formulas, because of its small size, Salamiah *et al.,* (2003) reported pellet was the most practicable one to be applied in a large scale.

2. METHODS

2.1. Materials

Materials used : sticky rice powder, mung bean powder, soybean powder, skim milk, garlic powder, Isolate of *T. harzianum* (culture collection of Laboratory of Mycology and Plant Pathology Fac. of Biology Unsoed, PDA media, plastic wrapper, distilled water, alcohol 70%, and methanol.

2.2. Procedures

Preparation of T. Harzianum biofungiside pellet. Four different matterial namely: pure white sticky rice meal, mixed of white sticly rice with either mung bean meal, soy bean meal or skim milk (treatmen) was weight for 100 gram each. Each type of meal was then added with 5 g grlic powder (as an antibiotic component) and covered with envelope, and sterilized at 80°C for 24 hours. When the powder got warm then poured into a 14 cm diameter petridshes, and added by 60 ml sterile aquadest to make it as a dough. A 20 ml conidia suspension of *T. harzianum* at the concentration of 10^7 konidia/ml was then poured into the dough and homogenized and pressed to 1 cm thickness. The dough was then drilled to make pelletes with 1 cm diamter and dried 40°C for 24 hours. Dried *T harzianum* biofungiside pelletes (Figure 3) then

covered with an alumna foil or plastic container and incubated as the duration requestedi.e.: 0, 3, 6, and 9 weeks. All steps were done in an aseptical ways (Salamiah *et al.*, 2003).

Research design. Current experimental study was design as a Completely Randomised Design (CRD) with a factorial. Carrying material was treated as the main factor with 4 levels. Firstly is 100% white sticky rice meal (T1); (T2) a mixture between 75% white sticky rice meal and 25% mung bean meal; (T3) a mixture between 75% white sticky rice and 25% soy bean meal; and (T4) a mixture bertween 75% white sticky rice and 25% skim milk. Second factor was store durations with 4 levels also i.e.: 0 week (W1), 3weeks (W2), 6 weeks (W3), and 9 weeks (W4). Each treatment was applied in triplicates so the total were 48 units.

Observed variable was the *T. harzianum* viability which was represented by total number of germinated conidia. Intial total number of conidia and final were subjected as the main parameter, the duration of colonies appear on the media, pH, temperature, humidity of the incubation room, C/N ratio were applied as supporting parameters. Formula as stated by Hadioetomo (1994) was applied in calculating the *T. harzianum* conidia viability. Data were then analysed using an F-test and follwed by the Honest significant different (HSD) test at the significant levels of 95% and 99% (Steel and Torrie, 1991).

3. RESULTS AND DISCUSSION

Current data showed the highest viability of *T. harzianum* conidia, as represneted by total number of germinated conidia (%) in different carying material and store duration was T1W4. It came from carrying matrial of 100% white sticky rice and the longest store duration (9 weeks) and percentage of viability was 78,19%. On the other hand, the lowest viability rate was acchieved by the treatment of T3 W1 i.e.: a mixrture between 75% white stickly rice and 25% soy bean meal and store duration of 0 week i.e. 51,08%. Complete data are performed in Figure 1.

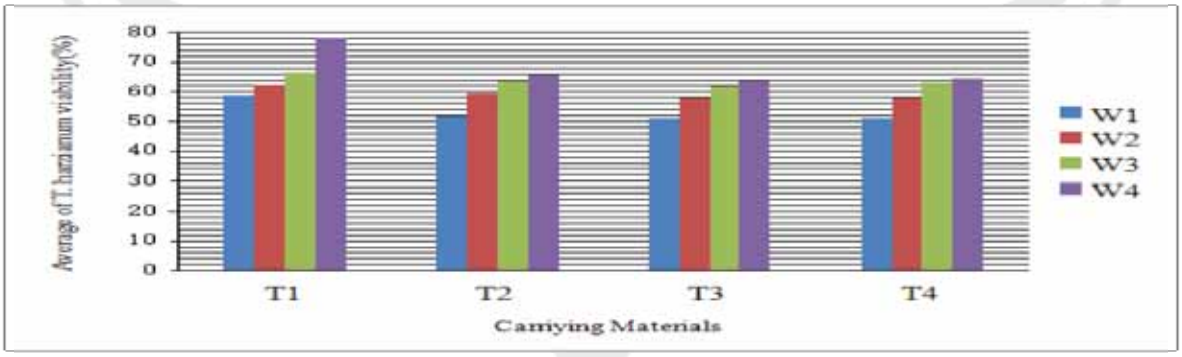

Figure 1. Histogram of correlation between carrying material and store duration on the *T. harzianum* (%) conidia viability

For their optimal growth fungi require sufficient nutrition as energy sources from their substrate. Utrilization of some carrying material has been aimed to provide nutrition sources for the fungi. Nutrition contents of the carrying material are then important factors for the growth and viability of *T. harzianum*. Papavizas (1986), atated

Trichoderma requires nutrition as energy sources in its live. The main nutritions are: carbon (C) and nitrogen (N). The presence of vitamins and minerals in its growing medium could then be ignored. Bilgrami and Verma (1978) stated Carbon and Nitrogen are strongly needed by the fungi in order to increase its conidida viability. Moreover, Carbon is required for synthesis of cell's components, energy source, and replacing the broken cells. Carbon can be supplied in form of Carbohydrate like monosaccharides, disaccharadies and polysaccharaides. Meanwhile, Nitrogen is required to support the process of vegetative growth as well as formating the cell's organeles. The Nitrogen element can be in form nitrate (NO_3^-), nitrit (NO_2^-), ammonium (NH_4^+), N organik, amino acids and protein (Garraway and Evans, 1984). Tjokrokusumo *et al.,* (2004), most of the carbon element is required by fungi as energy source in their growth and cells formation, but nitrogen is required for its growth through protein synthesis.

Long store duration of the pellet might also affect the viability. Salamiah *et al.,* (2003), type of carrying material as well as store duration of *T. harzianum* pellets affected its viability.

Table 1. Anova of *T. harzianum* viabillity (%) on different carrying material and store duration

Variation Source	Degree of freedom	Total Quadartic	Quadrat Mean	F-calculated	F-table	
					5%	1%
Treatment	15	2055,624	137,042	20,390 **	2,01	2,70
T	3	480,859	160,286	23,849 **	2,92	4,51
W	3	1418,810	472,937	70,367**	2,92	4,51
T x W	9	155,955	17,328	2,578*	2,21	3,07
Residual	32	215,054	6,721			
Total	47	2270,680				

An F-test using an Anova at the significant level of 95% and 99% (Table 1) showed interaction between crrying material and store duration gave a significant effect on increasing the viability of *T. harzianum* conidia. A further analsysis of a Honest Significant Different of significant level of 95%, was also done in order to know the differences between treatments of carrying material and store duration. The results were as follows.

An HSD test at 95% significant different of the T1W4 (100% white sticky rice meal, and 9 weeks store duration) showed the highest result on the viability of *T. harzianum* conidia (78,19%). The lowest viability of *T. harzianum* conidia was obtained when the treament of T3W1 (a mixture between 75% white sticky rice meal and 25% soy bean meal, and 0 week store duration) was given (51,08%). It shows if the viability of *T. harzianum* conidia here was affected by interaction between carrying material and store duration. The best interaction was obtained when 100% white sticky rice meal and store duration of 9 weeks was applied to measure the viablity of *T. harzianum* conidia. According to Papavizas (1986), the *Trichoderma*

requires nutrition as its energy source in its growth. The main components supposed to available in its growth media are carbon (C) and nitrogen (N). The presence of vitamins and minerals in its growth media are then can be ignored. Bilgrami & Verma (1978) stated if carbon and nitrogen are strongly required by fungi to increase the viability of its conidia.

Table 2. The HSD test of *T. Harzianum* conidia viability (%) on different carryying material and store duration

Treatments	Average germinated conidia of *T. harzianum* (%)
T1W1	58,89 ef
T1W2	62,28 bcde
T1W3	66,55 b
T1W4	78,19 a
T2W1	51,69 g
T2W2	59,54 def
T2W3	63,75 bc
T2W4	65,48 bc
T3W1	51,08 g
T3W2	57,88 f
T3W3	61,94 cde
T3W4	63,39 bc
T4W1	51,16 g
T4W2	57,99 f
T4W3	63,16 bcd
T4W4	64,33 bc

Carbon is required as energy siurce and synthesis of cell's components along the growing processes as well as replacing broken cells. This element can be either in monosaccharides, disaccharides or polysaccharaides. The nitrogen however, is required to support the processes of vegetative growth as well as formation of cell's organels in form of nitrate (NO_3^-), nitrit (NO_2^-), ammonium (NH_4^+), or ganic N, amino acids and protein (Garraway and Evans, 1984). According to Tjokrokusumo *et al.,* (2004), most of the available corbon is used as energy source for its growth and cells formation, but the nitrogen is used for growth through protein synthesis. Analysis of C, N, and C/N content of those four different carrying material are shown in the Table.

Table 3. Carbon, nitrogen,and C/N content of four different carrying material

No	Carrying material	C (%)	N (%)	Rasio C/N
1	100% white sticky rice meal	26,3	1,82	14,29
2	A mixture of 75% white sticky rice meal and 25% mung bean meal	52,0	5,44	9,56
3	A mixture of 75% white sticky rice meal and 25% soy bean meal	38,4	6,21	6,12
4	A mixture of 75% white sticky rice meal and 25% skim milk	48,1	6,78	7,08

Moerdiati *et al.,* (1999) reported one among those important factor in the quality of fungal growth media was the C/N ratio. Current study noted if the highest C/N ration was 14,29 (100% white sticky rice meal), in the contrast, the lowest was 6,12 (a mixture between 75% white sticky rice meal and 25% soy bean meal). According to Musnamar (2004), the over C/N ratio in such a substrate might depress fungal growth. Lopez (2002), stated a substrate with an over carbon but low in nitrogen could depress growth of fungal's mycelium. Aiman (1999), suggested if the C/N ratio in the fungal growth media similar to that in the soil (10-20) was optimum for growth, at this ratio fungal will increase production of conidia, whereas the over high C/N ratio amy depress myceliums growth and production of conidia.

Apart from nutrition content of the carrier, storing duration was also found to affect viability of the *T. harzianum* conidia. Current study noted that the longer storing duration from 0 week to 9 weeks increased the viability of *T. harzianum* conidia in each carrier. As supported by the data of increasing total number of conidia in the pellet at pre stored as well as total number of germinated conidia (post storing duration), leads to the increase of viability of the *T. harzianum* conidia in each carrier. The longest storing duration of 9 weeks, showed the highest result on increasing viability of the *T. harzianum* conidia in each carrier. This might happen due to the available nutritions in each type of carrier were sufficient to support the growth of *T. harzianum* condida up to 9 weeks.

Salamiah *et al.,* (2003) reported the viabilty of *T. harzianum* conidia which were formulated as pellets in the white sticky rice meal, IR 66 rice, sweet corn and storing duration of 8 weeks showed increase of viability. However, if the storing duration given is longer than 8 weeks it might give different effects, since nutrition available contained in the carrying materials have gradually decrease.

Nutritions availbality contained in the carrier could increase vianility of the *T. harzianum* conidia. Pelczar and Chan (1986), defined the situation where total number of cells are doubling is then called as exponential or logaritmic phase, leads to a balance in growth. Based on the data, current study predicted if the storing duration given is longer than 9 weeks, it might reduce the viability of the *T. harzianum*, that might due to the fungal has reached stationer phase and followed by lag phase and death. Pelczar and Chan (1986), claimed the stationer phase will appear when nutritions available in the growth media started to finish and some cells are death, the rest cells are then divide themselves to make the total living cells are stable. Whereas, lag phase is a particular phase where many cells are dead due to minimum nutrition availability in its growth medium. Total dead cells are even larger than those exist during the logarithmic phase. Though it still depend on the organism types, but in most of the cases such a microorganism dead within several days, weeks, or months.

T. harzianum is an antogonistic fungal that might be utilized as biocontrol agent, and it might also be formulated in various types of applications. Current study, formulated the conidia of *T. harzianum* as pellets, applying some different carrying material namely: 100% white sticky rice meal, a mixture of 75% white sticky rice meal and 25% mung bean meal, a mixture of 75% white sticky rice meal and 25% soy bean meal,, and a mixture of 75% white sticky rice meal and 25% skim milk. Nutrients availability and storing duration of the pellets are the two main factors affected

viability of *T. Harzianum* conidia. In compared with other combinations, current study noted combindation betwen the carrier of white sticky rice meal and 9 weeks storing duration performed the best result on total number of germinated conidia of *T. Harzianum*. It happened because, the white sticky rice meal conatin suficient nutrients to support the growth of *T. Harzianum*. Moreover, the storing period of 9 weeks is the logarithmic phase on *T. Harzianum* life cycle, in this phase the *T. harzianum* continuosly grow. Apart from those factors, nutrition availablity and storing duration, environment factors like: medium pH, temperature, humidity and light intensity are also important factors in affecting the fungal 's conidia viability, sporulation, and antagonistics charaters. Moerdiati *et al.,* (1999), stated that C/N ration of the media is one among the most important factors to support fungal growth. Musnamar (2004), the over high C/N ratio in the growth medium of a particular microorganism might become barrier on the fungal growth. Lopez (2002) stated when a substrate contain over amount of the Carbon but the number of N is poor might become barrier in growth of fungal mycelium. Aiman (1999), stated the C/N ratio of 10-20 which is similar to the situation in the soil, is the best condition for fungal growth as well as production of conidia, however, the over limit of C/N ratio might affectoppositedly.

4. CONCLUSIONS

Based on current data obtained, it might then be concluded as follows: 1) interaction between type of the carrying material and pellets storing duration affected viability of the *Trichoderma harzianum* conidia, and 2) the best interaction was 100% white sticky rice meal and 9 weeks storing duration affected the highest impact on niability of *T. harzianum* conidia.

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Optimization Production and Characterization of Chitin Deacetylase by Thermophilic *Bacillus* Sp. Sk II-5

Qintan Istighfarin Atmaja[1*], Nur Shabrina[1], Maharani Pertiwi Koentjoro[2], Endry Nugroho Prasetyo[1]
1. Biotechnology laboratory Institut Teknologi Sepuluh Nopember, Gedung H Kampus ITS Keputih Sukolilo Surabaya 60111 Indonesia
2. Laboratory of Environmental Microbiology, Department of Biological and Environmental Science, Faculty Agriculture-Shizuoka University.and Structural Biology Research Center, Inter-University Research Institute Corporation-High Energy Accelerator Research Organization (KEK), Tsukuba-Ibaraki, Japan.
*Corresponding author email address: iqintan@gmail.com

Abstract

Chitosan is a product of chitin deacetylation which has many benefits in various fields. Solubilization of chitin into chitosan has been done through chemical process which is harmful to the environment. The current study approaches the problem through green technology in which chitin deacetylase is optimized by *Bacillus* sp. SKII-5 as biological agent of chitin solubilization. The production optimizations of chitin deacetylase conducted in this study include the temperature, pH and enriched medium. The protein content was measured by Bradford method with BSA (Bovine Serum Albumin) as standard. The enzyme was purified by ammonium sulfate precipitation and characterized by measuring the isoelectric point, enzyme activity, protein content and molecular weight. Optimum enzyme activity was achieved through combination of medium shrimp shells as the carbon source with pH 7 at 60ºC. with the highest enzyme activity results of purification of ammonium sulfate at 60-75% fraction of 0.00528 U / ml with a protein content of 0.0024 mg / ml. Chitin deacetylase from *Bacillus* sp SK II-5 has an isoelectric point at pH 5 and molecular weight of 45 kDa.

1. INTRODUCTION

Crab and shrimp shells existing in fishery solid waste are abundant of chitin, a polymer which constitutes the cell walls of Zygomycetes and Crustaceans (Prameela *et al.*, 2010; Arbia *et al.,* 2013; Dong Gao *et al.*, 1995; Galed *et al*., 2005). Chitin is comprised of N-acetylglucosamine monomers, and when the second atom on the acetyl group is modified, it will be deacetylated into chitosan (Sharp, 2013). Chitosan is more favorable to be applied in various industries (pharmaceutical, biochemical, biotechnological, biomedical, food, paper, textile, agricultural and health industries) because it is more soluble in acidic solvents (Sharp, 2013; Raval *et al.*, 2013; Choi *et al.,* 2004; Darmawan, 2007).

Conversion of chitin into chitosan is usually done by deproteinization (NaOH 4%) and demineralization (HCl 4%) (Arbia *et al*., 2013). However, those methods may reduce the amount of chitin in the crab and shrimp shells (Bhaskar *et al.,* 2007). Alternative solution which is more efficient for chitin processing is through fermentations of lactic acid bacteria and *Vibrionacea* group (Prameela *et al.,* 2010; Hunt *et al.,* 2007). In

addition, *Colletotrichum lindemuthianum*, *Serratia* sp. and *Bacillus* sp. are also common as biological agents in the enzymatic technology development of chitin deacetylase (Tsigos *et al.,* 1995; Kaur *et al.,* 2012; Mathur *et al.,* 2011).

Chitin deacetylase (CDA) is a glycoprotein with a molecular mass of 24-150 kDa, optimum enzyme activity at 50ºC, and optimum pH range from 4.5 to 8.5 (Jeraj *et al.,*, 2006). CDA was first extracted from cell walls of *Mucor rouxii*. However, fungi produced low chitin deacetylase because of their slow growth and complicated fermentation process (Zhao *et al.,* 2010).

Bacillus sp. has the potential to produce more efficient CDA than fungi because of easier cultivation and faster growth (Kaur *et al*., 2012). However, production of CDA by *Bacillus* sp. on a large scale has not yet been conducted. Hence, this study aims to optimize the production and characterization of CDA with high enzyme activity by utilizing fisheries waste as the carbon source.

2. METHODS

2.1 Organism and cultivation condition

Bacillus sp. SK II-5 in this study was from the collection of Microbiology Laboratory of Institut Teknologi Sepuluh Nopember (ITS) (Surabaya, Indonesia). The bacteria was grown on Nutrient Agar and Nutrient Broth at 27ºC for 24 hours and maintained at 4ºC until use.

2.2 Optimization of pH, temperature and carbon source medium

The Taguchi method was initially used to determine the approximate optimum enzyme activity by combining various pH, temperature and medium. Effect of pH was determined by measuring the enzyme activity at pH 4, 5, 7, and 8 with acetate and phosphate buffers. Effect of temperature was determined by incubating the bacteria at temperature of 30-60ºC. And effect of production medium was determined by adding chitin powder, crab powder, shrimp powder in the medium as positive control and without carbon source medium as negative control in production medium. Then, the culture was incubated on a rotary shaker at 130 rpm.

2.3 Production and isolation of CDA

Bacillus sp. SK II-5 was cultured in 250 ml Erlenmeyer flasks containing 100 ml of production medium with the composition (g/l): chitin/ crab powder /shrimp powder 0.5 g, yeast extract 0.2 g, ammonium sulphate 0.2 g, KH_2PO_4 0.1 g, tryptone 0.1 g, and $MgSO_4.7H_2O$ 0.01 g. Bacteria was transferred into production medium to be optimized with OD value of 0.6-0.8. The enzymes were harvested by centrifugation at 8000 rpm for 15 min at 4°C (Emmawati *et al.*, 2007). The filtrate obtained was the crude enzyme and was used to determinate crude enzyme activities, followed by purification and protein content determination.

2.4 CDA purification

Precipitation of crude enzyme was done by adding 0-30%, 30%-45%, 45%-60% and 60%-75% ammonium sulfate, stirred for 15 minutes, and incubated overnight at 4°C. Furthermore, the enzyme was centrifuged at 3000 rpm for 15 minutes. The result of precipitation was washed with 10 mL of 0.1 M phosphate buffer pH 7 (Suri *et al.,* 2013).

2.5 CDA assay

CDA activity was measured using chitin as the substrate. Standard enzyme assays were done in glucosamine (10 mM) and the reaction was initiated by the addition of 3 ml enzyme solution, 8 mg chitin and 1 ml buffer (Ischaidar *et al*., 2014). Enzyme was incubated for 30 min at 50□ Cand the reaction was terminated by the addition of 200 µl of 33% (v/v) acetic acid and 200 µl $NaNO_2$ 5%. Upon termination of the reaction, the concentration of glucosamine residues produced by deacetylation reaction was estimated by oxidation using $NaNO_2$. The tubes were then shaken and left standing for 10 minutes, at which time of the deamination was completed. The excess of nitrous acid was then removed by adding 500 µl of a 0,1 mM ascorbic acid and the mixture was shaken for 30 min. About 800 µl of HCL 5% and 80 µl indole in 1% ethanol absolute was added subsequently. The mixture was then immersed for 5 min in a boiling water bath and an intensive orange color was created. After the mixture was cool, 800 µl of ethanol absolute was added and shaken. The absorbance was measured at 492 nm (Tokuyasu *et al*., 1996).

2.6 Protein measurement (Bradford assay)

The protein concentration was determined by the method of Bradford (1976) using bovine serum albumin (BSA) standard. Readings were carried out in a spectrophotometer at 595 nm.

2.7 Isoelectric point

About 1 ml of CDA was added in each of the six test tubes, followed by addition of 1 ml of acetate buffer solution (pH 3- 8) in each tube. Test tube was whipped and the degree of turbidity was recorded after 0, 10, and 30 minutes. The test tubes were observed for the maximum precipitation. Furthermore, all of the tubes were heated over a water bath. The isoelectric point was indicated by the rapid or massive formation of sediment turbidity.

2.8 SDS-PAGE electrophoresis

SDS-PAGE was carried out by using the method of Bollag and Edelstein (1991). The protein was stained by Coomassie Brilliant Blue.

3. RESULTS AND DISCUSSION

3.1. Optimization of CDA production

Optimization of CDA production was done to determine the combination of the medium, pH, and temperature which produce the maximum value of enzyme activity. The yield of CDA activities at varying temperatures are presented in Figure 1.

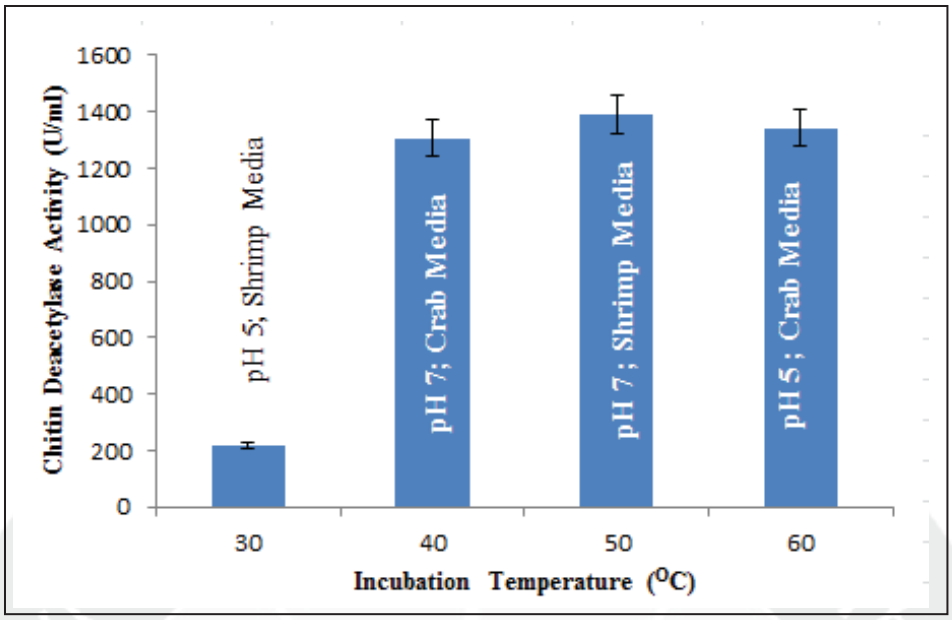

Figure 1. Optimization of CDA production

Figure 1 shows that the highest CDA activity (1390.44 U / ml) achieved by combining shrimp shells as the substrate medium with temperature of 50ºC and pH of 7. CDA activity with the lowest temperature of the varying temperatures used (30°C and pH 5) was 218.14 U / ml. On the other hand, the lowest value of CDA activity was demonstrated by using crab as the substrate medium at 50ºC and pH of 4 (191.48 U / ml). CDA activity of *Bacillus* sp. SK II-5 increases with the rising of temperature. This was because *Bacillus* sp. SK II-5 was originally isolated from Dieng crater and belongs to the thermophilic bacteria, thus affects the production temperature (Tsurayya, 2013). However, suppose the temperature exceeds the optimum temperature, the enzyme activity would decrease as a consequence of molecular structure damage of the enzyme protein (Pelczar, 1972; Setyahadi *et al*., 2006).

High activity value was achieved in the medium pH of 7 (Fig. 1) because pH 6-7 is the optimum pH for the growth of *Bacillus* sp. (Raevuori and Genigeorgis, 1975). CDA production is highly dependent on the pH of the medium. If the pH was appropriate for the growth of microbes, CDA activity will also be optimum (Setyahadi *et al*., 2006).

Utilization of shrimp shells as the carbon source achieved the highest CDA activity. This was due to the differences in polymer structures in which the structure of shrimp shells polymer is more spread than crab shells and powdered chitin, thus shrimp shells were easily hydrolyzed (Arbia *et al.,* 2013).

The optimum combination that generates the highest CDA activity value (Fig. 1) was different from the results of analysis using statistical Taguchi test (*General Linear Model Annova test)* which was achieved by the combination of temperature of 60°C at pH 7 with shrimp shells as the substrate. Nevertheless, the average enzyme activity from the optimum combination of the two values was not significantly different based on the calculated F value between temperature of 50ºC and 60ºC.

3.2. Purification and characterization of CDA

Purification of CDA was done by ammonium sulfate purification (Juan, 1990). Result of CDA purification using varying salt concentrations is presented in Figure 2.

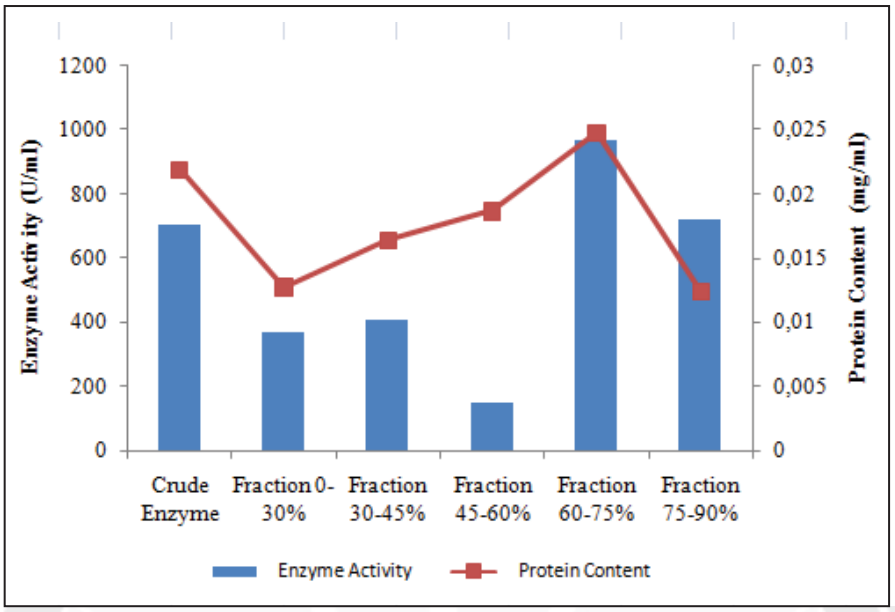

Figure 2. Protein content and enzyme activity in each fraction of ammonium sulphate purification

Ammonium sulphate purification was carried out at 60ºC, pH 7 with shrimp shell as the substrate. It was shown in Fig. 2 that the value of CDA activity reached the highest at 60-75% fraction (969.14 U / ml), indicating that CDA protein was concentrated in this fraction. To confirm the result of CDA purification, characterization was carried out by protein content assay, isoelectric point and SDS-PAGE. The highest activities at 60-75% fraction showed high concentration of total protein (of 0.024 mg/ ml), while the isoelectric point was reached at pH 5 (Fig. 3a) (Tsigos *et al*., 2000). The isoelectric point of the protein molecule is the condition in which the protein has equal positive and negative charges, reaching neutral charge (Tsigos *et al*., 2000).

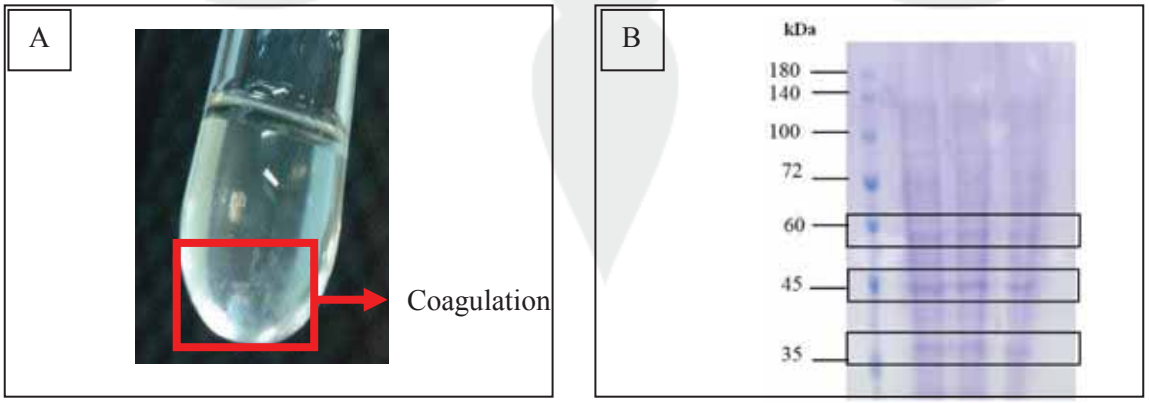

Figure 3. Characterizations of CDA. A) Isoelectric point of CDA, indicated by coagulation; B) Protein bands visualized by Coomassie Brilliant Blue stain

Result from SDS-PAGE electrophoresis showed that there are three bands of protein with molecular weight of 60 kDa, 45 kDa and 35 kDa (Fig. 3b). However, of all the three bands, 45 kDa band was most apparent. Thus, it was most likely that the molecular weight of CDA from *Bacillus* sp. SK II-5 was 45 kDa. This result was comparable with the study by Raval *et al.,* (2013) in which the molecular weight of CDA from *Bacillus* was in the range of 30-45 kDa.

4. CONCLUSIONS

Optimization of CDA production by *Bacillus* sp. SK II-5 with the highest enzyme activity of 1390.44 U / ml was obtained at 50ºC with pH of 7 and using shrimp shells as the substrate medium. Ammonium sulphate purification may increase the enzyme activity by 37% in the 60-75% fraction with protein content of 0.024 mg/ml. Isoelectric point was achieved in the condition of pH 5 with the dominant molecular weight of 45 kDa.

5. ACKNOWLEDGEMENT

The author is grateful for the academic grant provided by Biomaterial and Enzyme Technology Research Team (2014/2015) supervised by Dr. techn. Endry Nugroho Prasetyo, M.Eng. in which the study was conducted. She was also thankful for her father Guntur Tri A. and mother Siti Rochmatun for the support and prayers, also for *Scylla serrata* 2011 family and members of Biomaterial and Enzyme Technology Research Team (2014/2015) for the motivation, support and assistance.

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The Antioxidant Activities of The Extracts of Red Fruit (*Pandanus conoideus* Lam.) Pre-dried by *Détente Instantanée Contrôlée* (DIC)

*Ratih[1], Kohar, Indrajati [1], Anesia Qalbye[1], Hadiyat, M. Arbi [2], and Allaf, Karim[3]
[1]Faculty of Pharmacy, The University of Surabaya, Jl. Raya Kalirungkut, Surabaya, Indonesia.
[2]Departement of Industrial Engineering, The University of Surabaya, Indonesia.
[3]University of La Rochelle, Laboratory of Engineering Sciences for Environment LaSIE UMR - 7356 CNRS, France. Phone: +33 68 58 16 9 12; +33 5 4645 8766. kallaf@univ-lr.fr
ratih1006@gmail.com

Abstract

Red fruit is an indigenous fruit from Irian Jaya, is known contains large amounts of polyphenolic compounds, β-carotene and α-tocopherol wich has antioxidant capacities, and these may prevent oxidative damage of DNA. *Détente Instantanée Contrôlée* (DIC) which is a high-steam pressure treatment, is also categorized as a High Temperature Short Time (HTST) process. It increases the material porosity as well as the specific surface area and reduces the diffusion resistance of moisture during the final dehydration step. This research was directed to appraise the antioxidant activity of the ethanol and the hexane extract treated with DIC as a pre-drying/texturing method and compare it with the untreated one, evaluate antioxidant activities using *in vitro* methods of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and FRAP's radical scavenging. The results were analyzed by one-way ANOVA. From this study, it is indicated that the DIC-assisted extraction had better impact in terms of antioxidant activity compared to extract without DIC pre-drying.

Keywords: *Détente Instantanée Contrôlée*, Red Fruit (*Pandanus conoideus* Lam.), Antioxidant, Pre-drying, DPPH, FRAP's.

1. INTRODUCTION

Red fruit (*Pandanus conoideus* Lam.), is one of the fruit commonly consumed by many local communities in Papua, Indonesia. They believe that Red fruit (*Pandanus conoideus* Lam.) can treat many illnesses such as cancer, arteriosclerosis, rheumatoid arthritis, and stroke [6]. Red fruit contains large amounts of polyphenolic compounds, with antioxidant capacities, and these may prevent oxidative damage of DNA [17]. Red fruit is also rich in flavonoids and other polyphenols, β-carotene and α-tocopherol [17], that have been shown to possess a wide range of biological and pharmaceutical benefits, including anticarcinogenic, antioxidative, and hypolipidemic activities [7, 19].

Red fruit has a high moisture content that can induce enzymatic reaction, hydrolysis and microbiological contamination which can decrease its quality. So, the moisture content must be reduced by drying method before extraction stage of the fruits. Drying method of each *simplicia* must be considered because a different drying method can affect the quality of the *simplicia* itself [18].

The conventional drying method, like sun drying is the method mostly used in Indonesia. Long time exposure and long thermal treatments during the drying process cause significant deterioration. Antioxidants, well-known for their healthy properties related to the prevention of degenerative diseases, are damaged by long thermal treatments.

For that reason, a new process of drying by instantaneous controlled pressure drop, called *Détente Instantanée Contrôlée* (DIC), was used as pre-treatment of hot air drying [1]. DIC treatment is categorized as a High Temperature Short Time (HTST) process. Texturing step permits to modify the material texture, which would then affect the dehydration kinetics, the product physical properties, including water and oil holding capacity, and the microbial decontamination. The DIC step can lead to a porous structure, which considerably increases the mass transfer within the product and accelerating the last drying phase and increasing extraction rate [9].

Natural antioxidants, particularly in fruits and vegetables have gained increasing interest among consumers and the scientific community because epidemiological studies have indicated that frequent consumption of natural antioxidants is associated with a lower risk of cardiovascular disease and cancer. Antioxidants are vital substances, which possess the ability to protect the body from damage caused by free radicals inducing oxidative stress [14].

According to Rohman *et al.,* (2010), the antioxidant activity of the ethyl acetate extract of red fruit can be used as natural antioxidant source to prevent diseases associated with free radicals. Thus, the results from the previous study proved that DIC-assisted extraction is better than those not implying such texturing stage. The responses used for analyzing this impact and comparing DIC and non-DIC samples were the contents of three substances (flavonoids, total phenol, and α-tocopherol). Furthermore, for the optimum conditions of DIC pretreatment and the solvent extraction confirmed that DIC-assisted extract gave higher content of the three substances than the extract without DIC pre-treatment [10, 11].

According from the statement above, there are five samples that are tested in this research. The first sample is red fruit of optimum DIC pre-treatment (0.25 MPa, 4 cycles,15 s, each) followed by optimum extraction condition (60% ethanol, 30°C, 1 h). The second is red fruit dried by conventional drying followed by optimum extraction condition (60% ethanol, 30°C, 1 h). The third sample is red fruit of optimum DIC pre-treatment (0.15 MPa, 2 cycles, 15 s, each) followed by optimum extraction condition (hexane, 45°C, 1.5 h). The fourth sample is red fruit dried by conventional drying followed by optimum extraction condition (hexane, 45 °C, 1.5 h). And the last sample is red fruit oil. The antioxidant activity of red fruit oil is also analyzed because red fruit oil is considered as the representative of the juice (the red part of the red fruit) which the antioxidant activity of the red fruit juice will also be observed.

The determination of the antioxidant activity needs using 2,2-diphenyl-1-picrylhydrazyl (DPPH) [5,8]. DPPH assay is said to be the most profitable, the simplest, and the cheapest way. It only needs the reagent, some cuvettes, and a UV–Vis spectrophotometer. The latter is found even in the most rudimentary laboratories. Both approaches report extent of reaction and ignore reaction rates [2].

The aim of this study is to evaluate the antioxidant activity of the ethanol and the hexane extracts from DIC-assisted solvent process compared with the untextured samples of red fruit oil, using the DPPH method.

2. METHODS

2.1 Chemicals

Ethanol 96%, Cab-O-Sil, Whatmann filter paper #41, Demineralized water (Laboratory of Faculty of Pharmacy, University of Surabaya), DPPH p.a (Sigma), Sodium acetate 3 H_2O (Riedel de Haen, Germany), Acetic acid (BDH Laboratory Supplies, England), 2,4,6-tripyridil-s-triazine (TPTZ) p.a, Fluka Chemicals, Switzerland, HCl, $FeCl_3.6H_2O$ p.a. (BDH).

2.2 Instrumentations

Ultrasonic Bath (Branson 1200, Connecticut, USA), Spectrophotometer UV-Vis (Hitachi U-2000), Gram Balance (NHK), Analytical Balance (Sartorius), Micropipette, Glasswares: cuvette, beaker glass, volumetric flask, measuring flask, stirring rod, funnels, volume pipettes, test tube.

2.3 Procedures

19.7 mg DPPH were weighed, dissolved in 100.0 ml of ethanol 80% in a volumetric flask, then 2 ml of the solution was pipetted into a 10.0 ml volumetric flask, and ethanol was added to the mark in order to get 0.01 mM DPPH. This solution was immediately used, kept at low temperature and protected from light. A part of this solution was poured into the cuvette. The maximum wave length with the highest absorbance from the DPPH solution was determined. 1.0 mL of sample solution and 3.0 mL of 0.01 mM 2,2-diphenyl-1-pycrylhydrazyl solution were pipetted into a test tube. 1.0 mL of ethanol 80% was introduced to the tube and the absorbance was written and the minutes, which were giving the stable absorbance from two different concentrations were observed.

Each red fruit extract (bulk) was accurately weighed (500 mg) and dissolved in ethanol 80% to a 100.0 ml volume flask to obtain a solution with a concentration of 5000 ppm. Then it was diluted to make various concentrations of 20 ppm, 40 ppm, 60 ppm, 80 ppm and 100 ppm.

We added 3.0 ml of 0.01 mM DPPH solution into the test tube containing 1.0 mL of the test solution of red fruit extract. Then 1.0 mL of ethanol 80% was introduced and stand according to the extract reaction time. The extract absorbance was determined and the radical scavenging percentage was calculated following Eq. 1:

$$\left(1 - \frac{As}{Ac}\right) * 100 \qquad \textit{Eq. 1}$$

Where; A_C =absorbance of control and A_S =absorbance of sample solution. The data were analyzed by one way ANOVA statistical method using MINITAB (version 16) program.

3. RESULTS AND DISCUSSION

3.1 Anti Oxidant Activity Test

The antioxidant activity of various foods can be determined accurately, conveniently, and rapidly using DPPH testing. The trend in antioxidant activity obtained by using the DPPH method is comparable to trends found using other methods reported in the literature. This method can be used successfully for solid samples without prior extraction and concentration, which saves time [16].

3.1.1 Quantitative Analysis of Antioxidant Activity using DPPH (2,2-diphenyl-1-picrylhydrazyl)

The quantitative measurement of the scavenging of DPPH radical allows one to determine exclusively the intrinsic ability of substance to donate hydrogen atom or electrons to this reactive species in a homogenous system. The method is based on the reduction of methanol-DPPH solution with the presence of antioxidant substances having hydrogen donating groups (RH) such as phenolics and flavonoids compounds due to the formation of non radical DPPH-H form [15]. The primary reaction, which takes place, is the formation of free radical R**.** and the reduced form of DPPH (Figure 1).

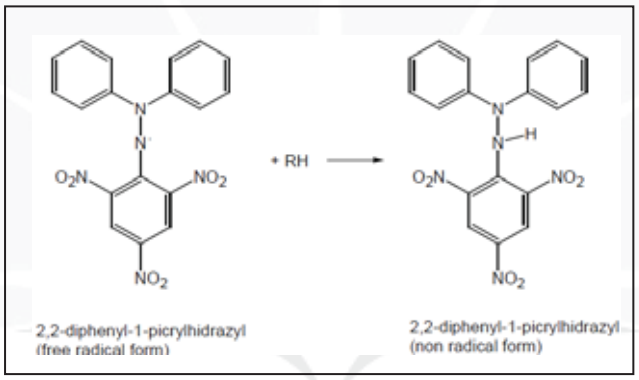

Figure 1. Structure of DPPH and its reduction form by the antioxidant RH (Rohman *et al*., 2010)

The parameter used to measure/evaluate the radical scavenging activity of extracts and fractions was IC_{50}, defined as the concentration of antioxidant required for 50% scavenging of DPPH radicals in a specified time period. The smaller the IC_{50} value, the higher the antioxidant activity [12]. On the wavelength scan it was found that the maximum wavelength for these experiments was 521 nm, while the time scan was 30 min and 34 min for the conventionally dried and for the DIC fruits, respectively.

Table 1. Antioxidant activity (IC50 value) of Red Fruit Extracts and Red Fruit Oil by DPPH method

No		DIC-assisted Ethanol Ext	Conv. Drying Ethanol Ext	DIC-assisted Hexane extr	Conv. Drying Hexane extr	Oil
1	y=	0.1506x+1.6771	0.0729x+2.1058	0.0011x+0.5221	0.001x-2.4677	0.0099x+1.9138
	IC50(ppm)	320.87	656.99	44979.91	52467.7	4857.19
2	y=	0.1326x+4.1219	0.0724x+3.485	0.0011x+0.4311	0.001x-2.7523	0.0098x+2.4401
	IC50(ppm)	345.99	642.47	45062.64	52752.3	4853.05
3	y=	0.1412x+2.5194	0.0743x+3.2137	0.0011x-0.1239	0.001x-2.4225	0.0101x+1.5917
	IC50(ppm)	336.26	629.69	45567.18	52422.5	4792.09
Average (ppm)		334±13	643±14	45203±318	52548±179	4834±36

Table 2. IC50 value of the extracts, oil, and positive control equal to the concentration in extracts

Extracts	IC_{50} Value ± SD (mg/L)
Ethanol Extract of DIC red fruit powder	334.37 ± 12.66
Ethanol Extract of conventionally dried red fruit powder	643.05 ± 13.65
Hexane Extract of DIC red fruit powder	45203.24 ± 317.88
Hexane Extract of conventionally dried red fruit powder	52547.50 ± 178.80
Red Fruit Oil	4834.38 ± 35.98
Positive Control (α-Tocopherol)	38.73 ± 0.66
Positive Control (Catechin monohydrate)	19.70 ± 0.18
Positive Control (Gallic Acid)	9.90 ± 0.03

The ethanol and the hexane extracts obtained from the DIC red fruit powder had antioxidant activities approximately better than the un-treated ones. From the data above, all of the red fruit extracts and red fruit oil were not as potent as the positive controls of antioxidant. The positive controls which were used were α-tocopherol, catechin monohydrate and Gallic acid, and the concentrations were taken equal to their concentrations in the extracts. The intensity of antioxidant activity of active substances using the DPPH method can be classified according to the values of IC_{50} [3].

Table 3. The intensity of antioxidant activity with the DPPH method

Intensity	IC_{50} Value
Very strong	< 50 µg/mL
Strong	50-100 µg/mL
Moderate	101-150 µg/mL
Weak	> 150 µg/mL

As seen from IC_{50} value, red fruit extracts and red fruit oil were classified as weak antioxidants. All of the ethanol and hexane extracts and the red fruit oil cannot be considered as potent antioxidants. Besides, according to Molyneux *et al.,* (2004) if the IC_{50} value of active substances at the concentrations of 200-1000 ppm, the substances is less active but still have an antioxidant activity. But, when observed from the active substances content of the red fruit extract, antioxidant activity from the substances in its extract can be classified as a potent antioxidant.

Table 4. IC_{50} value of red fruit extracts and red fruit oil based on its flavonoid content

No.	Sample	Flavonoid	Extract in bulk	Flavonoid in extract	IC_{50} Value from flavonoid content (ppm)
1.	Ethanol Extract of red fruit powder dried conventionally	0.53 %	23.89 %	2.22 %	13.81 ± 0.88
2.	Ethanol Extract of red fruit powder pre-dried by DIC	0.71 %	29.62 %	2.39 %	7.99 ± 0.30
3.	Red Fruit Oil	0.79 %	-	-	38.19 ± 0.28

Table 5. IC_{50} value of red fruit extracts and red fruit oil based on its total phenol content

No.	Sample	Total phenol	Extract in bulk	Total phenol in extract	IC_{50} Value from total phenol content (ppm)
1.	Ethanol Extract of conventionally dried red fruit powder	0.92 %	23.89 %	3.87 %	24.88 ± 0.49
2.	Ethanol Extract of DIC red fruit powder	1.27 %	29.62 %	4.28 %	14.31 ± 0.54
3.	Red Fruit Oil	0.10 %	-	-	4.83 ± 0.13

Table 6. IC50 value of red fruit extracts and red fruit oil based on its α-tocopherol content

No.	Sample	α-tocopherol	extract in bulk	α-tocopherol in extract	IC_{50} Value from α-tocopherol content (ppm)
1.	Hexane Extract of conventionally dried red fruit powder	0.17 %	46.85 %	0.36 %	339.02 ± 2.39
2.	Hexane Extract of DIC red fruit powder	0.34 %	45.60 %	0.75 %	189.17 ± 0.64
3.	Red Fruit Oil	3.54 %	-	-	171.13 ± 0.13

All of the extracts cannot be considered as a potent antioxidant because the percentage of the active substances on the whole extracts are low. This condition may be due to the high percentage of Cab-O-Sil in the bulk, causing the active substances that act as antioxidant in the extracts cannot dissolve completely because the extracts is bound to a high percentage of Cab-O-Sil.

The solvents that are used to pull the active substance out were ethanol and hexane. Ethanol is a polar solvent due to its hydroxyl (OH) group, with the high electronegativity of oxygen allowing hydrogen bonding to take place with other molecules. While hexane is a non-polar solvent due to the bonds between carbon and hydrogen in hexane are uniform. Since in this research ethanol was used only as a polar solvent and hexane as a non polar solvent, a further study is needed to better study the impact of other solvents for extraction. Probably, a semi polar solvent can be used to dissolve not only polar, but also non polar active substances.

Another reason is may be due to the dynamic maceration as extraction method, because it should not allow optimally extracting all the active substances. Further studies need to be achieved using the same dynamic maceration as extraction method but with extraction of the red fruit powder repeatedly.

3.1.2 Data Analysis

The results of antioxidant activity in the red fruit extracts and the red fruit oil were analyzed statistically by one-way ANOVA using MINITAB program. This aimed at establishing the effect of DIC treatments on the antioxidant activity of the red fruit extracts. In this experiment, the extract concentrations act as a factor (independent variable), while IC_{50} Value as a response (dependent variable).

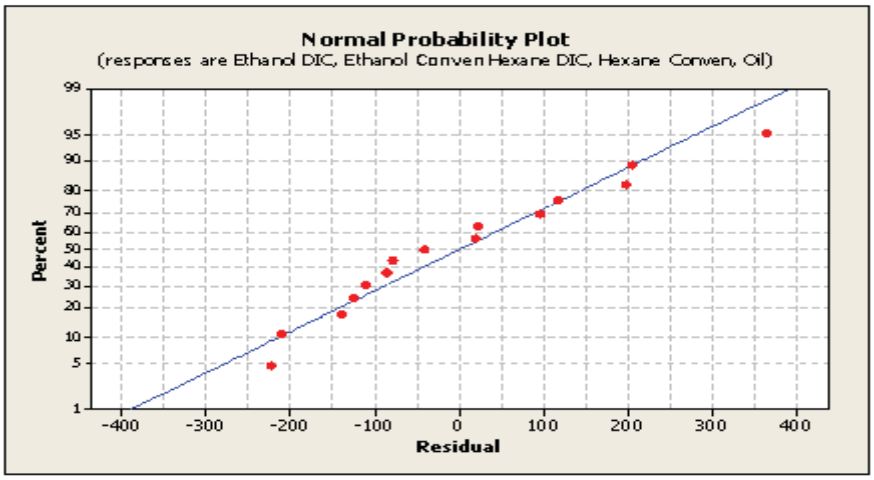

Figure 2. Normal Probability Plot of Red Fruit Extracts and Red Fruit Oil

Minitab Output 1. One-way ANOVA *DIC, Ethanol Conven, Hexane DIC, Hexane Conven, Oil*

```
Source  DF          SS          MS         F      P
Factor   4  8050054747  2012513687  74661.83  0.000
Error   10      269551       26955
Total   14  8050324297

S = 164.2   R-Sq = 100.00%   R-Sq(adj) = 100.00%


                              Individual 95% CIs For Mean Based on
                              Pooled StDev
Level           N   Mean  StDev  +---------+---------+---------+---------
Ethanol DIC     3    334     13  *
Ethanol Conven  3    645     17  *)
Hexane DIC      3  45203    318                                  *
Hexane Conven   3  52548    179                                       *
Oil             3   4834     36     *
                              +---------+---------+---------+---------
                              0     15000     30000     45000
```

```
Grouping Information Using Tukey Method
                N   Mean  Grouping
Hexane Conven   3  52548  A
Hexane DIC      3  45203    B
Oil             3   4834      C
Ethanol Conven  3    645        D
Ethanol DIC     3    334        D
Means that do not share a letter are significantly different.
```

The output from this analysis showed there were 4 groups of results (A to D), where the extracts in a same group do not have significant difference in antioxidant activity measured with IC_{50} value.

Group A consists of Hexane extract of Conventional dried fruits. Group B consists of Hexane extract of DIC fruits. Group C consists of Red Fruit Oil, and for the last group (group D) consists of Ethanol extract of DIC fruits and Ethanol extract of conventionally dried fruits.

Although the means or averages of IC_{50} values from the first group (group A-C) are high, but the antioxidant activity is lower than that of the last group (group D), because the higher the IC_{50}value, the lower the ability in scavenging the activity of free radicals the IC_{50} value, the lower the antioxidant activity.

From the data above using one-way ANOVA, ethanol extract of DIC fruits and ethanol extract conventional dried fruits are at the same group. Even so, the ethanol extract of DIC fruits had the highest antioxidant activity because the value of the IC_{50} was the smallest among the others.

Based on the experiment, the IC_{50} values of the ethanol extract of the DIC fruits, the ethanol extract of the conventionally dried fruits, the red fruit oil, the hexane extract of the DIC fruits, and the hexane extract of the conventionally dried fruits were 334.37 ppm; 643.05 ppm; 4834.38 ppm; 45203.24 ppm; and 52547.50 ppm, respectively. The antioxidant activity of the red fruit extracts was much higher for DIC fruits than conventional dried fruits. From this study, the red fruit extracts and the red fruit oil are categorized as weak antioxidants. Also the hexane extract from the red fruit powder pre-dried by DIC has antioxidant activity better than that of dried conventionally.

In addition studies of antioxidant activity of the Red Fruit extracts were also carried out by Frap Method [4].

3.2 Quantitative Test of the Antioxidant Activity (Frap Method)

3.2.2 Reagent preparation:

1. 300 mmol/L acetate buffer, pH 3.6 (3.1 g Sodium acetate 3 H_2O + 16 ml acetic acid per Liter of buffer solution)
2. 10 mmol /L TPTZ in 40 mmol/L HCL = 10 x 312.34 mmol = 3123.4 ppm.
3. 20 mmol/L $FeCl_3.6H_2O$ = 20 x 270.5 mg/mmol = 5410 mg/L

FRAP reagent was prepared by mixing 25 ml acetate buffer + 2.5 ml TPTZ solution and 2.5 ml Ferric chloride solution. TPTZ trihydrate in aqueous ethanol, reacts with ferrous ion to yield intense violet color over pH range 3.4- 5.8 with maximum absorption $(Fe(TPTZ)_2^{2+}$ (water) at 593 nm (ε 22,600). At low pH the reduction of iron (III) tripyridyltriazine (FeIII TPTZ) is carried out to become iron (II) (Fe IITPTZ), which can be observed from color changes to blue intensive color. This color is measured at λ of 593 nm. The change of absorbances is in accordance with the antioxidant activities.

The sample was dissolved in methanol in a concentration of 50 µg/mL then FRAP solution (50 µg/mL was added (volume of sample: volume of FRAP solution = 1:1).

The mixture was incubated for 20 min and the absorbances were measured at 593 nm.

The antioxidant activity was measured as the percent capacity of the sample and is calculated using the equation:

$$\%\,Capacity = (1 - T_s)x100 \qquad \textit{Eq.2}$$

Note :
T_S = Transmittance of FRAP solution after the adding of the test sample: $T_S = 10^{-As}$
A_S = Absorbance of FRAP solution after the adding of the sample.

3.2.3 Quantitative Analyses of the Sample by FRAP's Method

50.0 mg of each extract was weighed and dissolved in about 5 ml MeOH in a sonicator. Then it was filtered to 10.0 ml volume flask and MeOH was added to make 10.0 ml (= 5000 ppm)
From this solution 5 more dilutions were made:

2.5 ml solution was pipetted into a volume flask and MeOH was added to make 5.0 ml solution (2500 ppm).
1.0 ml solution was pipetted into a volume flask and MeOH was added to make 5.0 ml solution (1000 ppm).
1.0 ml solution was pipetted into a volume flask and MeOH was added to make 10.0 ml solution (500 ppm).
0.25 ml solution was pipetted into a volume flask and MeOH was added to make 5.0 ml solution (250 ppm).
0.25 ml solution was pipetted into a volume flask and MeOH was added to make 10.0 ml solution (125 ppm).
Then 2.0 ml of Frap solution was added to 2.0 ml sample solution, the absorbance was measured, and then the T was calculated.

The absorbance was measured at 593 nm with MeOH:distilled water: HCl 0.04 M as the blank. Incubation time was 20 minutes in the dark.

Table 3. Antioxidant capacity of red fruit products by FRAP Method

No		DIC-assisted Ethanol Ext	Conv. Drying Ethanol Ext	DIC-assisted Hexane extr	Conv. Drying Hexane extr	Oil
1	Y =	0.104 x+12.32	0.082x+7.103	0.080x+5.670	0.034x+8.186	Cannot be analyzed by FRAP method.
	A 50 (ppm)	362.31	523.13	554.13	1229.82	
2	Y =	0.105 x + 12.01	0.076 x + 8.452	0.085 x + 4.503	0.034 x + 5.680	
	A 50 (ppm)	361.81	546.68	535.26	1303.53	
Av of A 50 ppm)		362.06±0.35	535±17	545±13	1267±52	

The oil could not be analyzed by FRAP method because the addition of FRAP reagent caused the formation of precipitate, which disable the reading in the Spectrophotometer.

4. CONCLUSIONS

The antioxidant activity (using DPPH method) was identified to follow the following order, from the highest to the lowest level: DIC-assisted ethanol extraction of red fruit, ethanol extraction of conventionally dried red fruit, oil, DIC-assisted hexane extraction of red fruit, and conventionally dried red fruit, respectively. In this study, the red fruit extracts and oil obtained from the red fruit powder pre-treated and untreated by DIC have antioxidant activity, but cannot be considered as potent antioxidant activity.

The FRAP experiment as an additional experiment, although did not give similar results as the DPPH experiments, has confirmed this order of the antioxidant activity.

It proofed that *détente instantanée contrôlée* (French for "Instant controlled pressure drop") DIC-assisted solvent extraction can enhance the extraction of the active compounds from the plant cells and in do so also enhance its antioxidant activity.

5. ACKNOWLEDGEMENT

This study was supported by The Grant from The Research Centre of The University of Surabaya. Additional support and laboratory facilities were made available by The Faculty of Pharmacy, University of Surabaya, Indonesia. The author would like to thank Mrs. Vicenta Blasco, General Manager of Abcar DIC Process, La Rochelle, France for providing the DIC apparatus, and the Department of Chemical Engineering of The University of Surabaya for allowing the usage of the DIC.

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Using species specific primers for detecting DNA in a wildlife feces

Sena Adi Subrata[1]
[1]Department of Forest Resources Conservation, Faculty of Forestry, Gadjah Mada University, Yogyakarta, Indonesia
adisubrata@ugm.ac.id

Abstract

Conservation genetic research frequently involves molecular genotyping. For effective and efficient genotyping, a critical step is ensuring the availability of DNA of targeted species in genetic samples. Recently, availability of DNA of targeted species is detected by sequencing PCR product. It may become prohibitive when involving many samples due to the high cost of sequencing. The challenge is how to detect the availability of DNA of targeted species reliably but at low cost. Here we applied two-steps screening to address the challenge: morphological recognition of genetic sample and amplification part of its mt-DNA. As a case study, we applied this screening to two species of medium-sized mammals: Binturong (*Arctictis binturong*) and Common Palm Civet (*Paradoxurus hermaphroditus*). We collected feces samples from Gembira Loka Zoo, Yogyakarta and identified it morphologically according to the literature. We extracted DNA from feces using QIAamp Stool Mini kit (Qiagen) with a modified protocol. We designed species-specific primers from cytochrome-b sequences of both species. We amplified targeted DNA fragments using the primers and a PCR kit (Kappa 2G Fast Ready mix) and run PCRs according to suggested protocol. We also applied the primers for DNA amplification of closely related species Small Indian Civet (*Viverricula indica*) as a control. We detected the availability of amplicons in 1.2% agarose gel. We successfully detected the availability of DNA in feces of both species. Visualization of the gel shows bands of amplicons of Binturong and Common Palm Civet but no amplicon for Small Indian Civet. This result is consistent with our primer design that expect amplicons in the length of 261 bp for Binturong, 426 bp for Common Palm Civet and no amplicon for other species. It suggests that using morphological recognition and species-specific amplification result in reliable detection of DNA availability in feces using only PCR and agarose gel electrophoresis.

1. INTRODUCTION

Conservation genetic research frequently involves molecular genotyping for identifying individuals. The research uses genetic theory and technique to understand an ecological process that causes population extinction (Frankham *et al*., 2002). Since individual variations within populations may elucidate the mechanism, individual identification is very important. Currently, the identification relies on molecular genotyping. Phenotypic identification, as may be indicated by morphological measurements, frequently results in misidentification.

For effective and efficient genotyping, particularly employing microsatellite loci, a critical step should be done is ensuring the availability of DNA of targeted species in genetic samples. For threatened wildlife species, the samples are most likely collected in the field and found in imperfect condition. Contained DNA may be fragmented, degraded and compounded with other species.Although microsatellite is a species-specificmarker (Selkoe and Toonen, 2006), however polymerase chain reaction (PCR) may fail or result in unspecific products due to absenceof DNA of targeted species. Avoiding this undesired PCR results, ensuring the availability of DNA of targeted species is very important. Recently, availability of targeted DNA in a sample is detected with sequencing the PCR products and matching it into a database (Pereira *et al*., 2008) or roughly indicated by visual recognition of genetic samples.

In a wildlife research involving many samples of feces collected from fields, the critical step may become prohibitive due to ambiguous results of visual recognition or high cost of sequencing. The challenge is how to detect the availability of DNA of targeted species reliably but at low cost. Here we applied two-steps screening to address the challenge: morphological recognition of feces sample and amplification part of its mt-DNA. As a case study, we applied this screening to feces of two species of medium-sized mammals: Binturong (*Arctictis binturong*) and Common Palm Civet (*Paradoxurus hermaphroditus*).

2. METHOD

2.1. Chemicals

We collected feces from Gembira Loka Zoo, Yogyakarta. We preserved the feces in a 50 mL falcon tube containing ethanol absolute until DNA extraction. We used QIAamp Stool Mini kit (Qiagen Inc) to isolate and purify DNA. Extra ASL buffer was needed as we performed additional pre-treatment steps. A 25 uL PCR reaction was conducted involving 12.5 uL PCR mix (Kapa2G Fast ReadyMix), 0.5 uM forward and reverse primers, 2.5 uL BSA (0.1 ug/mL), 3 uL DNA template (containing 3-6 ng DNA/uL), and 4.5 uL PRC-grade water.Primer pairs were designed specifically for the Common Palm Civetand Binturong. PCR products were run for electrophoresis in 1.2% agarose stained with Ethidium bromide.

2.2. Procedures

Feces was collected early in the morning to avoid further contamination and exposure to solar radiation. We collected the feces of the Civet, Binturong and Small Indian Civet *(Viverricula indica).* We measured length and diameter each feces and take a photograph before preserving it in ethanol. We added 1-3 g of feces into 5 mL ASL buffer and incubated for an hour before proceededto DNA extraction according to manufacturer protocol. All extracted DNA was diluted into a concentration of approximately 3-6 ng/uL.

We run PCRs using above-mentioned ingredients with cycling parameters as follow: 1 minute initial denaturation and followed by 15 seconds denaturation step at 95°C, 15 seconds annealing step at 60°C, 15 second extension step at 72°C and 1 minute final extension step at 72°C. We run PCR for 35 cycles. We checked the presence of PCR product in agarose gel. Template for PCR reaction was DNA from Common

Palm Civet, Binturong andSmall Indian Civet. We involved DNA from the later species as a control.

Before we run PCR, a species-specific primers was designed. We selected DNA sequence from cytochrome-b (cyt-b) gene as a molecular marker. We aligned 62DNA sequences of cyt-b of 30 species. The species were closely related with Common Palm Civet and Binturong (Veron and Heard, 2000) or classified as Javan carnivores (Sody, 1989). We obtained the sequences from NCBI database and aligned using MEGA 5 (Tamura *et al*., 2011). We searched for conservative sequences that flank sequence unique to targeted species (e.g. Paradoxurus hermaphroditus or Arctitis binturong). The conservative sequences then were listed as primer candidates that were screened further based on PCR product length and common characteristic of good primers (primers length, melting temperatures, GC content, GC clamp, and minimal secondary structures). We did it using SP-Designer (Villard and Malausa, 2013) and Oligonalizer 3.1 (Owczarzy *et al*., 2008). As we consider that DNA in the feces most likely fragmented and there was a possibility of feces confusion, we expected PCR product length less than 500 bp and the difference between amplicon lengths should be more than 100 bp. We selected a pair of primers for each species that best suits the criteria.

3. RESULTS AND DISCUSSION

We successfully selected DNA sequences of *cyt-b* gene as species-specific marker of Common Palm Civet and Binturong (table 1). The sequences were the best suites according to good primers criteria, although primer dimers were still possibly produced. The possibility cannot be avoided as we consider many criteria to design primers that amplify degraded DNA fragment of targeted species. Moreover, we also consider that the PCR product of both species-specific primer should be easily separated in agarose gel to avoid confusion. Therefore, we considered the dimer was a disadvantage that should be minimized by applying ideal condition of PCR.

Using the primers, availability of DNA in feces of both species was detected. Visualization of the gel shows bands of amplicons of Binturong (c.a 300 bp) and Common Palm Civet (c.a. 400 bp) and no amplicon for Small Indian Civet (Figure 1). This result is consistent with our primer design that expect amplicons in the length of 261 bp for Binturong, 426 bp for Common Palm Civet (table 1) and no amplicon for other species. As we did not sequence the PCR products and subsequently match it into a database, there is a possibility that the amplicon do not represent DNA of the targeted species. We minimize the possibility by visual recognition screening. We select only feces that morphologically represent feces of targeted species based on literature (Chame, 2003). Besides form, length and diameter of feces, presence of remaining seed fragments, feathers, hairs, bones or any other indigestible materials in the feces can be used as an indicator of the feces defecator.

Table 1. Sequences of primers designed specifically for *Paradoxurus hermaphroditus* and *Arctictis binturong*

Species	Sequence (5' → 3')	Size[a] (bp)	Tm[b] (°C)
Paradoxurus hermaphroditus	F:TTCCATTCATCATCTCCGCC	426	63
	R: TTCAGAATAGGCATTGGCTGAGTG		64
Arctictis binturong	F: GGCCTATTCTTAGCCATACACTACTCAT	261	62
	R: TGGTAGAACATAACCTATGAAGGCTGTAG		63

a). Length of PCR product
b) Melting temperature

Primer designing is critical also for minimizing the probability of misrepresentation of DNA in feces. We designed primer from cyt-b sequences covering 62 individual of all closely related species or species most probably occurred in the research site. Availability of software (Villard and Malausa, 2013) that is capable of handling this task enable us to involve almost all relevant cyt-b sequence. *In silico*, by involving the wide variety of DNA sequences we expect to have primers that flank sequence unique to targeted species. In an ideal condition, PCR should produce only amplicons that represent targeted species. *In vitro*, we applied high melting temperatures (62-64°C; table 1) to reduce the possibility that the primer anneal unspecifically during PCR. To support successfulness of PCR with high melting temperature, we provide pure DNA and remove PCR inhibitor. We prefer to use high-quality DNA extraction kit and perform additional treatment to obtain DNA with such quality.

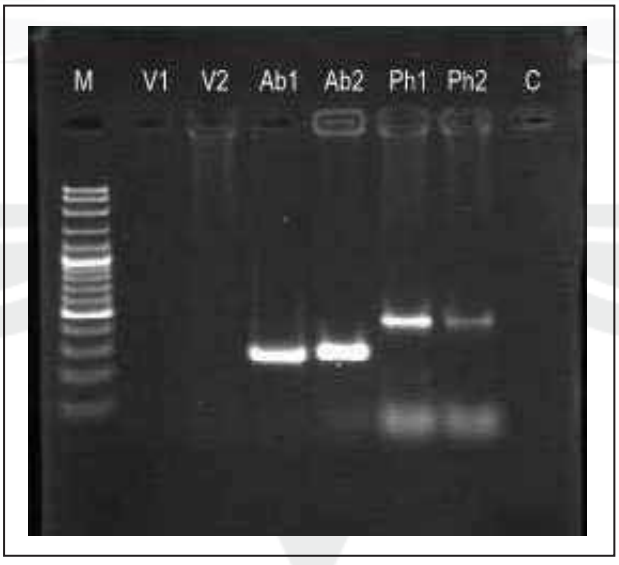

Figure 3. DNA fragments were amplified as expected. M:100 bp Marker;V1& V2:Small Indian civet; Ab1&Ab2: Binturong, Ph1&Ph2: Common Palm Civet, C: negative control

This DNA detection technique may work locally. We suggest using this primer with caution for research with different environmental settings. As we considered the presence of species in a research site for designing primers, this technique may not work in for genetic samples originated outside from proposed the research site. There is a possibility that a genetic sample may contain DNA from species that is

closely related but not considered during primer design. Conducting PCR using that DNA template may result in no amplicon or misdetection. Prior reliable information on species presence in an area is needed during primer designing process.

4. CONCLUSIONS

This study showed that using morphological recognition and species-specific amplification result in reliable detection of DNA availability in feces using only PCR and agarose gel electrophoresis. This technique was simple and inexpensive but need prior information on species presence in proposed research area. This technique support efficient molecular genotyping therefore facilitating more research on conservation genetics.

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Antioxidant and Antibacterial Activity of Humped Bladderwort Extract (*Utricularia gibba*)

*Shanti Dwita Lestari[1], Siti Hanggita Rachmawati[1], Ivan Andeska Marpaung[1]
[1]Program Studi Teknologi Hasil Perikanan, Universitas Sriwijaya, Ogan Ilir, Indonesia
shantidwita_thi@unsri.ac.id

Abstract

The crude methanolic extract of humped bladderwort (*Utricularia gibba*) was evaluated for its phytochemical compounds, antioxidant and antimicrobial activity. The antioxidant activity was assessed through the ability of the extract in inhibiting the stable DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical. Based on the Thin Layer Chromatography (TLC) and quantitative analysis, two bioactive compounds constituents found in the extract were phenolic and tannin with the content of 36.81 ppm and 62.41 ppm respectively. These two compounds contributed to the inhibition of DPPH radicals with the IC_{50} value of 179.02 ppm. Determination of antimicrobial activity using the Kirby-Bauer disc diffusion method showed that the crude extracts of the whole part of the plant inhibited the growth of *Vibrio cholera* and *Bacillus subtilis* but no inhibition effect was shown on *Listeria monocytogenes*. At the concentration of 60mg/mL, the inhibition zone on *Vibrio cholera* was 22 mm while for *Bacillus subtilis*, the recorded clear zone diameter was only 8 mm. This finding indicates that the extract was considered as narrow spectrum antibiotics. However, the activity of the extract was still consistently less than the conventional antibiotic, amoxicillin.

1. INTRODUCTION

Indonesia's aquatic territory is a habitat for diverse aquatic plants that have potential as producers of bioactive compounds. Recently, the interest in the exploration of natural antioxidant and antimicrobial compounds from aquatic sources has increased due to their potential applications in food and pharmaceutical industries and as an alternative of synthetic antioxidant and antibiotic. The characterization of the aquatic plants may also yield more insight into their functionality as well as increasing their economic value.

Some aquatic plants from Utricularia genus that grow endemically in tropical areas have been investigated and reported to have antioxidant and antibacterial potency (Ruangdej and Laohavisuti 2010; Rajagopal *et al*., 2012). The other type of bladderwort which allegedly has antioxidant and antibacterial activity is humped bladderwort (*Utricularia gibba*). This plant is found as weed in natural lake and can be found in several regions in Indonesia. It is usually used as a woof or bait on the fishhook by local people. However, less is known about the phenolic and tannin content of this plant. Plants extracts with high antioxidant and antibacterial activity may be useful for food preservation to prevent lipid oxidation and bacterial contamination. Three species of bacteria, *Vibrio cholera, Bacillus subtilis* and *Listeria monocytogenes* are usually found in the contaminated fish products, therefore, it is necessary to evaluate the ability of humped bladderwort methanolic extract in inhibiting the growth of those bacteria. The dietary antioxidant supplements are also

needed to prevent the premature damage of human cell. Therefore, the aims of this research were to identify and quantify bioactive compounds of the whole part of humped bladderwort collected from Indralaya, Indonesia, and to assess their antioxidant and antibacterial activity.

2. METHODS

2.1 Chemicals

Chemicals used in this study were methanol, toluene, ethyl acetate, ethanol, Na_2CO_3, Folin-Ciocalteau reagent, tannic acid, $FeCl_3$ and K_3Fe $(CN)_6$, DPPH and commercial vitamin C. To asses the antibacterial activity, three species of bacteria including *Vibrio cholerae, Bacillus subtilis* and *Listeria monocytegenes* were used. Microbiological media used were tryptic soy agar, thio citrate bile salt agar, Listeria oxford formula and nutrient broth.

2.2 Procedures

2.2.1 Sample collection

The plant sample was collected from the lentic water body in Tanjung Putus, Indralaya, South Sumatera in September 2014. It was filled into plastic containers and immediately transported to the laboratory. The sample was washed under tap water to separate it from impurity components such as wood, twigs, other types of plants and other foreign objects. After being cut into smaller pieces, it was then sun dried for 48 hours to dry and coarsely powdered.

2.2.2 Preparation of plant crude extract

As much as 125 g of sample and 1000 mL of methanol were placed in an erlenmeyer glass, stirred with a magnetic stirrer for 1 hour at room temperature (26-30 °C) forming a ratio of material and solvent 1: 8 (w/v) and then allowed to stand for 24 hours before being filtrated through Whatman 01 filter paper. The residual solids were subjected to twice re-extraction using the same method, and all filtrates were collected for evaporation with a rotary evaporator at 45 °C. The crude extract was then stored in a refrigerator (4 °C) in dark bottles until analysis.

2.2.3 Identification of total phenolic and tannin using Thin Layer Chromatography

The crude extracts were characterized by means of thin layer chromatography on silica gel plates (Merck) measuring 1 cm x 7 cm using the mixture of toluene: ethyl acetate: methanol in the ratio of 8: 1: 1 (v/v/v) as an eluent. Following developing of plates, the plates were sprayed with an aqueous solution of ferric chloride ($FeCl_3$) to visualize phenolic compounds (Barton *et al*., 1952). Tannins were visualized on plate by spraying with an aqueous solution of glacial acetic acid. Detection of tannin can be performed without the UV rays, shown as yellowish-green color on TLC plate (Hayati *et al*., 2012).

2.2.4 Quantification of total phenolic and tannin

The phenolic content of humped bladderwort extract was determined in accordance with a modified protocol described by Septiana *et al*., (2002). Fifty milligram of sample was mixed with 2.5 mL of ethanol 95% and centrifuged at 3500 rpm for 10 minutes. One milliliter of supernatant was transferred to reaction tube and mixed with 1 mL etanol, 5 mL aquadest and 5 mL of Folin-Ciocalteu reagent then allowed to

stand for 5 min. One milliliter of sodium carbonate (Na_2CO_3) 5% was added to the mixtures, homogenized using vortex and incubated for 60 min. in dark, at room temperature. The absorbance was measured at 725 nm with UV-Vis spectrophotometer after stand for 10 min. A standard curve with serial gallic acid solutions was used for calculation. Total phenol values are expressed in terms of gallic acid equivalent (mg/g of extracted compounds).

Quantification of total tannin was performed according to Suryaningrum (2007). As much as 0.2 g of the extract was added with 10 mL of methanol and then stirred using a mechanical shaker for 1 hour. One milliliter of the supernatant was mixed with distilled water and 0.3 mL $FeCl_3$ 0.1 M. The mixture was shaken well and 0.3 mL of $K_3Fe(CN)_6$ with a concentration of 0.008 M was added and allowed to stand for 10 min. at room temperature. Absorbance was measured at 725 nm. Blank was prepared with water instead of the sample. A set of standard solutions of gallic acid is treated in the same manner as described earlier and read against a blank. The results of tannins are expressed in terms of gallic acid mg/g of extract.

2.2.5 Antioxidant activity test

The ability of the extracts to scavenge the DPPH radical (1,1-diphenil-2-picrylhydrazyl) was evaluated by the method described by Blois (1958). Stock solution of the whole plant extracts was prepared to the serial concentration of 50, 100, 150 and 200 ppm. Ascorbic acid was used as standard with the concentration of 2, 4, 6 and 8 ppm. As much as 4 mL of plant extract solution was reacted with 1 mL methanolic solution of DPPH (1 mM). The reaction mixture is incubated for 30 min at 37^{o}C and the absorbance was recorded at 517 nm.

2.2.6 Antibacterial activity test

The antibacterial activity analysis was performed by Kirby Bauer disc diffusion method (Bauer *et al*., 1966). The respective bacterial culture was spread into its specific agar plates for uniform distribution of microorganisms. Paper discs were dipped in various concentrations of crude extracts (0, 20, 40, 60 and 80 mg/mL) and the loading discs were transferred on to the surface of each inoculated agar plates with sterile tweezers. Commercial antibiotic, amoxicillin, served as standars with the concentration of 0.1 mg/mL. Plates were then incubated for 24 hours and the temperature was set according to the optimum growth condition of respective bacteria. At the end of incubation period, the zone of inhibition was measured in millimeter.

3. RESULTS AND DISCUSSION

In this experiment, extraction using methanol maceration yielded 3.59% crude extract. According to Marcus and Glikberg (1985), as a solvent, methanol is able to dissolve both polar and non-polar bioactive compounds due to its chemical structure that contains a hydroxyl group (OH) and the cluster of carbon (C). The phytochemical test for phenolic compounds and tannins was done qualitatively using thin layer chromatography (TLC) and quantitatively using the spectrophotometric method. Both methods confirmed the presence of phenolic and tannin compounds and showed that the plant is richer in tannin (62.41 ppm) than phenols (36.81 ppm). In TLC plate, phenolic compounds showed gray when visualized on a UV or after spraying with

reagents while tannin gave green color. Fig1 shows the fractionation of phytochemical compounds in the crude extract. The Rf value of phenolic and tannin were 0.94 and 0.78 respectively, indicating that phenolic (Rf5) is less polar than tannin (Rf4) as greater Rf value is related with low polatiry (Spangenberg, 2011). Besides phenolic and tannin, other phytochemicals were also existed as shown as different spots on the plates. Those spots had the Rf values of 0.28, 0.52 and 0.62.

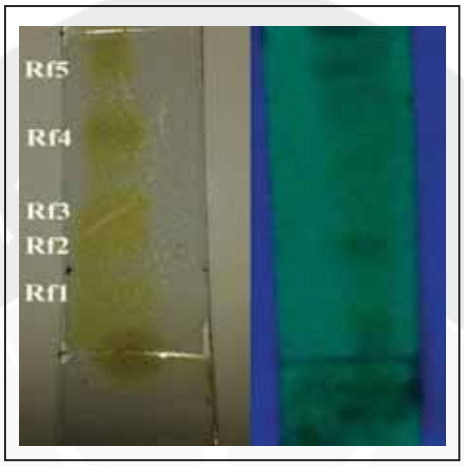

Figure 1. Fractionation oh humped bladderwort crude extract on the TLC plate (left) visualization without the UV light and (right) visualization with UV light

Polyphenols and tannins that were isolated and purified from *Castanea mollissima* has the ability to quench free radicals (Zhao *et al.,* 2011), thus, it contributes to the antioxidative effect of the plant extract. Cook and Samman (1996) also mentioned that antioxidant activity of plants might be due to their phenolic compounds. Antioxidant is a molecule which can quench reactive free radicals and prevents the oxidation of other molecules (Shahidi, 1997). The presence of phenolic and tannin in humped bladderwort crude extract determines its ability to terminate the oxidation process by scavenging free radicals such as DPPH.

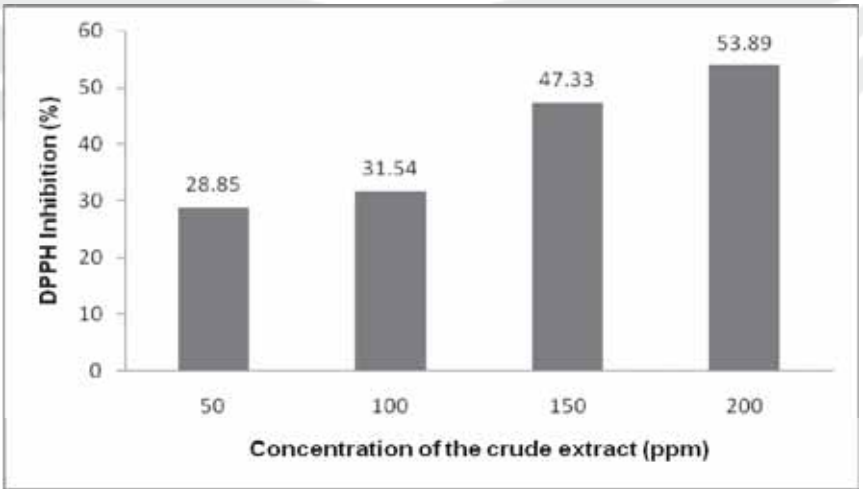

Figure 2. Correlation between concentration of humped bladderwort crude extract and DPPH inhibition

Fig 2 shows that the percent of a DPPH radical inhibitor will increase with increasing concentration of the sample solution. IC_{50} is the concentration of the extract that may lead to a reduction of 50% DPPH activity. Molyneux (2004) stated that the antioxidant activity of a certain compounds can be divided into several categories: very strong (IC_{50}<50 ppm), strong (50 ppm< IC_{50} <100 ppm), medium (100 ppm < IC_{50} < 150 ppm) and weak (150 ppm < IC_{50} <200 ppm). Based on the figures on Fig2, the IC_{50} value of humped bladderwort crude extract was 179.02 ppm, which means that its activities were categorized as weak. This value is still far compared to synthetic antioxidants served as standard which was vitamin C with IC_{50} value of 4.17 ppm. The weak antioxidant activity of the extract was related to the purity of the bioactive compounds.

The assessment of extract's antibacterial activity was done with disc diffusion method. According to Elgayyar *et al.,* (2001), an extract is considered to have a strong antibacterial activity if the diameter of the inhibition zone is wider than 11 mm (d > 11mm), weak (6mm <d <11 mm) and inactive (d< 6mm). Extract solution which has been diluted with varying concentrations produce different clear zone area, as shown in Fig 2.

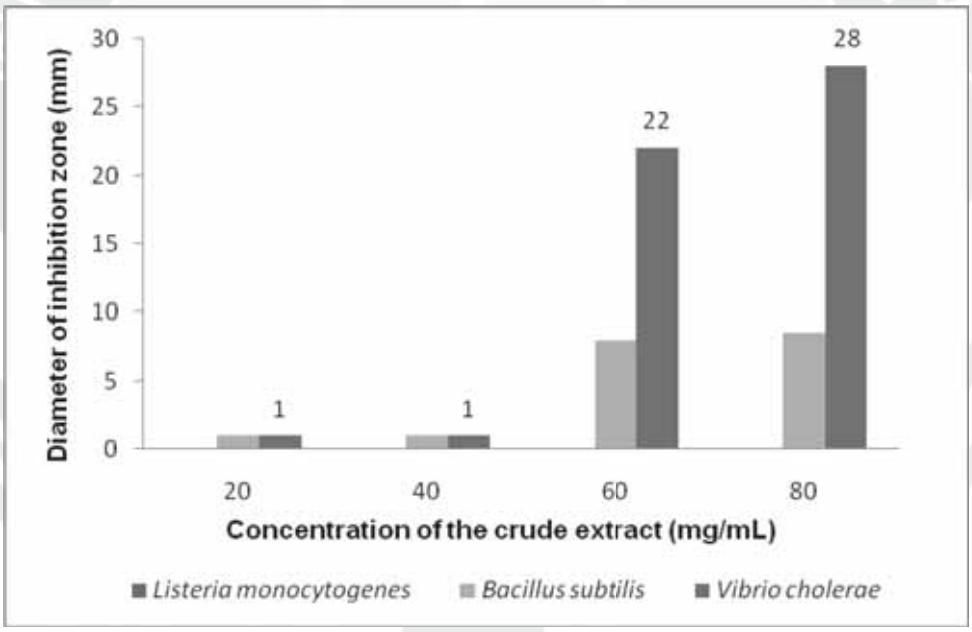

Figure 3. Correlation between concentration of humped bladderwort crude extract and the diameter of inhibition zone of three test bacteria

Figure 3 shows that the methanol extract demonstrated strong antibacterial activity against *Vibrio cholera* and made it the most susceptible of all the test bacteria. The diameter of inhibition zone was 22 mm at 60 mg/mL crude extract concentration. As for *Bacillus subtilis*, the antibacterial activity was categorized as weak, and no inhibition zone was recorded for *Listeria monocytogenes*. The activities of all the extracts were however less than amoxicillin that served as standard. The concentration of 0.1 mg/mL of this antibiotic produced 55 mm diameter of inhibition zone *for V. cholerae*, 10.5 mm for *Bacillus subtilis* and 22 mm for *L. monocytogenes*.

The different result among the three test bacteria was due their specific characteristics. Gram-positive bacteria, such as *Bacillus subtilis* and *Listeria monocytogenes* have a thick cell wall composed of many layers of peptidoglycan, whereas Gram-negative bacteria, represented by Vibrio cholera, have a thinner cell wall consisting of one or two layers of peptidoglycan. One component of Vibrio cholera's membrane proteil is porin which function as entry point of hydrophilic compounds such as sugars and amino acids which are important for the nutrition of bacteria (Purwoko, 2009). In the same way, the polar bioactive compounds in the extract entered into the cell membranes through porin which causes disruption of bacterial cells. Thus, it implicated on the formation of wider inhibition zone.

4. CONCLUSION

Methanolic crude extract of humped bladderwort contains both phenolic and tannin compounds which contributes to its antioxidant and antibacterial activity. Further purification process is needed to improve the activity prior to its application in food or pharmaceutical industries.

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Diversity of Termite Species in Tropical Forest in West Kalimantan

*Yuliati Indrayani[1] , Tsuyoshi Yoshimura[2]
[1]Department of Forestry, Faculty of Forestry, University of Tanjungpura, Pontianak, Indonesia
[2]Research Institue for Sustainable Humanosphere (RISH), Kyoto University,
Gokasho, Uji, Kyoto, 611-0011, Japan
E-mail: mandaupermai@yahoo.com

Abstract

A preliminary survey to determine the diversity of termite species in tropical forest in Pontianak, West Kalimantan was initiated. The survey was conducted according to Jones & Eggleton (2000). The insect were collected from fallen logs, leaf litters, mud trails, etc in three locations in tropical forest in Pontianak, West Kalimantan. A total of 160 samples of termite were collected from the survey. We found a total of 13 species of subterranean termites which belong to 10 genera (*Coptotermes, Schedorhinotermes, Prohamitermes, Dicuspiditermes, Macrotermes, Microtermes, Nasutitermes, Globitermes, Subulitermes, Pericapritermes*) and five subfamilies (*Coptotermitinae, Rhinotermitinae, Macrotermitinae, Nasutitermitinae,* and *Termitinae,*). Of interest, two species were new records for West Kalimantan, i.e. *Prohamitermes mirabilis* and *Dicuspiditermes emorosus*.

Keywords: Species diversity, Termite, Tropical Forest, West Kalimantan

1. INTRODUCTION

Termites are an important group of insects to the natural ecosystem (Sugimoto *et al.,*. 2000). Besides being pests to forest, agriculture and urban structures, termites also play an important role through nitrogen fixation by bacteria present in their gut, accumulation of minerals in their mounds, and improvement of soil texture through their tunneling activities (Lee *et al*., 2003). In addition, Williams (1994) stated that termites are useful recyclers of organic compounds (i.e., cellulose) because their activities accelerate the soil rehabilitation process by (1) breaking up of surface crusts, (2) reducing soil compaction, (3) increasing soil porosity, (4) improving water infiltration into the soil and (5) enhancing water holding capacity of the soil, thereby reducing surface runoff. However in their natural habitat, in many settings, they have severely disrupted the ecological system and/or caused significant economic damage.

Biodiversity studies commonly address insect faunas, which make up the majority of known eukaryote biodiversity and which are associated with the litter and soil rather than the canopy (Stork 1988). Termites play a prominent role in maintaining biodiversity as state by Jones *et al*., 1994; Lavelle 1997; Jouquet *et al*., 2006. Termites are found in wide range of terrestrial environments and are distributed throughout the tropical, subtropical and temperate regions of the world (Khrishna and

Weesner 1970). The number of species and their biomass are especially large in the tropical zone (Krishna and Weesner, 1969; Pearce, 1999). Indonesia is located within the tropical climatic zone with various types of forest ecosystems that are suitable for termite growth and development, and termite nests can easily be found everywhere in the forest, farmland, and rural shelters or even in city buildings. Due to the high diversity of termite species in this region, it is common to find several termite species. Nandika *et al*., (1996) reported 13 species of termites in Java Island. However, little is known about the termite fauna of West Kalimantan.

There has been limited effort to enhance information on termite fauna in West Kalimantan. The aim of this study was to measure termite species richness in tropical forest in West Kalimantan. This study also provides the first insight into the diversity of termite fauna in West Kalimantan.

2. METHODS

2.1 Study sites

Sites were located at or near Pontianak city in West Kalimantan Province. The areas surveyed include three tropical forests represented by secondary forest and dominated by trees from Dipterocarpaceae family, rubber (*Hevea brasiliensis*) and durian (*Durio* sp). Soil types are mostly peat and ultisol.

2.2. Site selection and survey protocol

A transect of 100 x 2 m was marked out for termite survey using the protocols according to Jones and Eggleton (2000). The transect was divided into twenty sections measuring 5 x 2 m, each of which was systematically explored by two collectors for 30 minutes. Species richness is the number of species and morphospecies obtained over the whole transect. Relative abundance is the number of encounters per transect, where the presence of a species in one section represents one encounter. Collection of worker and soldier termites were made on fallen logs, leaf litters, mud tubes, peel-off tree bark etc., at three different locations of forest which located in Anjungan, Teluk Pak Kedai and Pontianak city. The collected termites were kept in 70% alcohol. Termites were identified to species level at LIPI Biology Cibinong.

The transect method is effective because it utilizes collecting expertise within a protocol that standardizes sampling effort and area. The protocol provides a much more rapid and cost-effective method for studying termite assemblage structure than sampling regimes designed to estimate population abundances. The termite transect has potential as a useful addition to any suite of organisms recommended for monitoring functional processes in tropical forests (Jones and Eggleton 2000).

3. RESULTS AND DISCUSSION

A total of 13 species of termites from 5 subfamilies and 10 genera were collected from this study (Table 1).

Table 1. List of termite species collected from tropical forest in West Kalimantan

Family	Subfamily	Species
Rhinotermitidae	Coptotermitinae	*Coptotermes curvignathus* *Coptotermes* sp
	Rhinotermitinae	*Schedorhinotermes malaccensis*
Termitidae	Termitinae	*Globitermes globosus* *Microcerotermes havilandi* *Prohamitermes mirabilis* *Dicuspiditermes nemorosus* *Pericapritermes speciosus*
	Macrotermitinae	*Macrotermes* sp
	Nasutitermitinae	*Nasutitermes* sp1 *Nasutitermes* sp2 *Subulitermes complex* Soil feeder termite (Unidentified)

Table 1 shows the list of termite species collected from tropical forest in West Kalimantan, while the general characteristics of each genus of soldier termites are represented in Table 2. Among the 5 subfamilies, 5 species collected were from subfamily Termitinae, followed by subfamily Nasutitermitinae (4 species), 2 species for subfamily Coptotermitinae and 1 species each of subfamilies Rhinotermitinae and Macrotermitinae.

Two species of genus *Coptotermes* were found in this study. *Coptotermes* is an important genus of subterranean termite in Indonesia with 6 species (*C. curvignathus* Holmgren, *C. havilandi* Holmgren, *C. kalshoveni* Kemner, *C. travians* Haviland, *C. heimi*, and *Coptotermes* sp.) (Nandika *et al*., 1996) and those termite being important structural pests which cause significant economic losses.

One soil feeder termite in this study is unidentified. Soil-feeders, as reported by Wood (1978) are very common and abundant in many tropical rain forests. In the South-east Asian regions, soil-feeders are dominated by the Termitinae with a small number of Nasutitermitinae and Apicotermitinae (Abe, 1987). We also found species of *Prohamitermes* mirabilis and *Dicuspiditermes nemorosus* which were new records for West Kalimantan.

This paper presents an insight into the diversity of subterranean termite species in West Kalimantan, Indonesia. The relatively small number of sites and the lack of replication of sampling do not favor a complete survey. More detailed studies should be executed in the future to further substantiate current findings as well as producing a checklist of termite species of this area.

Table 2. General characteristics of soldier termites of genus (Lee *et al*., 2004)

Genus	General characteristics
Coptotermes	Large and conspicuous fontanelle
Schedorhinotermes	Both mandibles with prominent marginal teeth (2 at left and 1 at right mandible); dimorphic soldiers (minor soldier moves very fast)
Globitermes	Both mandibles with a single marginal tooth at the mid of mandibles; soldier`s abdomen with bright yellow coloration
Microtermes	Head rectangular; Inner margins of mandibles serrated
Macrotermes	Labrum with hyaline tip; meso and metanotum greatly expanded laterally; soldiers distinctly dimorphic
Nasutitermes	Head with nasus; head not constricted behind antennal sockets; left mandible without a rudimentary tooth on apical portion
Subulitermes	Head with nasus; Head somewhat pear-shaped
Prohamitermes	Mandibles long, strongly curved
Dicuspiditermes	Antero-lateral corners of head with pointed projections below antennal sockets with its lateral corners produced into long needle-like projections; anterior margin of labrum deeply concave
Pericapritermes	Labrum with anterior margin straight; anterolateral corners very short; tip of left mandible broad, not strongly bent

4. CONCLUSIONS

A survey of termite species was initiated to determine the diversity in tropical forest in Pontianak, West Kalimantan. We found a total of 13 species of subterranean termites which belong to 10 genera. Two species were new records for West Kalimantan, i.e. *Prohamitermes mirabilis* and *Dicuspiditermes emorosus*.

5. ACKNOWLEDGEMENTS

The authors are grateful to late Dr. Anggoro who assisted identification of termites and we thank the student of Faculty of Forestry for supporting the survey.

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The Effect of Salicylic Acid and Phenylalanineon the Total Phenolic Acid Contentin Cell Suspension Culture of *Moringaoleifera* Lam.

Yunita Permanasari[1], Elvian Haning Pramesti[1], Isdiantoni[2], Maharani Pertiwi Koentjoro[3*], Nurul Jadid[1] and Endry Nugroho Prasetyo[1]
[1]Department Of Biology, Faculty Of Mathematics And Natural Sciences, Sepuluh Nopember Institut Of Technology, Kampus ITS Sukolilo, Surabaya 61111 Indonesia
[2]Agrobusiness Department, Faculty of Agriculture, Wiraraja UniversitySumenep street-Pamekasan Km. 5 Patean, Sumenep, 69451, Indonesia
[3]Laboratory of Environmental Microbiology, Department of Biological and Environmental Science, Faculty of Agriculture - Shizuoka University & Structural Biology Research Center, Inter-University Research Institute Corporation - High Energy Accelerator Research Organization (KEK), Tsukuba-Ibaraki, Japan
*Corresponding author email address: maharanipertiwi@yahoo.com

Abstract

Moringa oleifera is a plant which has high potential to be developed in industrial sectors such as the pharmaceutical industry because of the high content of phenolic compounds. The objective of the present study is to determine the effect of salicylic acid as elicitor and phenylalanine as precursor on the total content of phenolic acids in cell suspension cultures of *M. oleifera*. The research design used was RAL (completely randomized design) with 3 replicates. Test parameters were the estimation ofthe total phenolic acids compounds using UV/Vis spectrophotometer and the dry weight of salicylic acid and phenylalanine treatments. The result was analyzed by one-way ANOVA and Tukey's test. The results showed that varying concentrations of salicylic acid and phenylalanine influenced the increase of the total content of phenolic acids in cell suspension cultures of *M. oleifera*. The highest totalsof phenolic acids treated by 1 ppm salicylic acid and 5 ppm phenylalanine were 1.6067 ± 0.458 ppm and 1,60 ± 0.37 ppm respectively.

Keywords: phenolic acid, salicylic acid, phenylalanine, *Moringa oleifera*

1. INTRODUCTION

*Moringaoleifera*is a plant which has high potential to be developed in industrial sectors such as pharmaceuticals, foods, and cosmetics [1]. The existence of this plant is abundant in Indonesia, including on the island of Maduraand Nusa Tenggara Timur [2, 3]. People in Indonesia are usually called the *M. oleifera* as kelor (Java) and maronggih (Madura) [4]. So far, limited use of these plants only as a food source, plant live fences, boundary or web crawler other plants such as cantilever chilli plants herbs [2, 3].

M. oleifera has been known as a plant that rich of antioxidant compounds [5]. Antioxidant compound are compounds that can neutralize free radicals. Free radicals are unstable molecules and highly reactive because it contains one or more unpaired electrons in their outer orbital. This reaction in the body will cause a variety of

diseases such as cancer and other degenerative diseases [6]. Antioxidant compounds contained in the *M. oleifera* namely polyphenols, flavonoids and alkaloids [5]. One compound that plays an important role as an antioxidant is phenol consisting of quercetin, rutin, catechin and proanthocyanidin [7].

Secondary metabolites that become the main focus in the research are phenolic compounds because of their role as antioxidants to fight cancer, cardiovascular, neurodegenerative [8], anti-bacterial, and anti-tumor [9]. However, the process of extracting secondary metabolites of *M. oleifera* is still not effective because it requires large amounts of material, whereas a long time plantation and cultivation of limited land. Therefore, alternative steps are needed to increase the production of secondary metabolites using suspension cultures treated with elicitor [10]. Other steps that can be used is the technique of precursor feeding [11]. Elicitor used in this process are salicylic acid and jasmonic acid [12]. While the precursor feeding used is phenylalanine [13].

Phenylalanine is a precursor metabolite on the phenylpropanoid pathway [13]. Previous studies cite an increase in the content of total phenolic compounds in wine suspension cultures using elicitor salicylic acid and precursor feeding phenylalanine [8]. Currently, research on the effect of precursor feeding and elicitor to the total content of phenolic acids in *M. oleifera* is still not done. The purpose of this study was to determine the effect of salicylic acid as elicitor and phenylalanine as a precursor feeding to the total content of phenolic acids in cell suspension cultures of *M. oleifera* Lam

2. METHODS

The research was conducted in March until July 2015 at the Laboratory of Botany, Plant Tissue Culture Laboratory, Laboratory of Microbiology and Biotechnology, and Laboratory of Biology Zoology Institute of Technology Surabaya.

2.1 Procedure

The stock solution of phenylalanine 1000 ppm was done by weighing 10 mg of phenylalanine using the analytical balance (Boeco, Germany), and dissolved in 10 ml of distilled water. Then the stock solution was filtered by Whatman filter paper (Whatman International Ltd., England) no. 42 in 100 ml glass bottle, tightly closed, labeled PHE 1000 ppm and stored in the refrigerator [14]. Stock salicylic acid was made in the same manner and labeled SA 1000 ppm and sterilized by autoclave for 20 minutes at a temperature of 121^0C pressure of 1.5 atm.

Medium used for callus induction was MS medium (Murashige and Skoog) with the addition 2,4-D and BAP [15]. 2.4 D 5 ml (0.5 ppm), BAP 10 ml (1 ppm), 30 grams of sucrosethen added 8 grams of gelatin stirred and heated. Once it is poured into a sterile culture bottle and sterilized using an autoclave at a temperature of 121^0C pressure of 1.5 atmfor 30 minutes.

Cell suspension cultures was done by inoculating the callus 200-300 mg in 250 ml sterile bottles containing 100 ml of liquid MS medium then incubated with a rotary shaker speed of 120 rpm at temperature 25 ° ± 2 ^{0}C. After 14 days, the medium plus salicylic acid 0; 0.5; 1; 1.5 ppm to elicitor treatment and phenylalanine 0; 5; 10; 15

ppm for the treatment of precursor feeding [19] and incubated with a rotary shaker for 5 days at 120 rpm. Culture incubation conditions were at25 ° C ± 2^0C.

After giving the elicitor treatment and precursor feeding for 5 days,cell suspension cultures was harvested with filtered cells using Whatman filter paper no. 42. and accommodated weighed using a digital balance for phenol extraction process and calculation of dry weight.

The extraction process was done by the method of [20] that filtered cell suspension culture using Whatman filter paper (Whatman International Ltd., England) no. 42 and weighed as much as 100 mg with digital balance, crushed with a mortar, mixed with 10 ml of 100% ethanol and 5 ml of 0.1% HCl then vortexed with a speed of 2,500 rpm for 10 minutes. Furthermore, settling on a rotary shaker for 30 minutes for maceration process. A mixture of solvent extract and centrifuged for 20 minutes at a speed of 2500 rpm and the supernatant was taken to measure the content of intracellular phenol.

The concentration of total phenolic acids were measured using the FolinCiocalteu technique [20]. Supernatant from the extraction process was taken as 1 ml plus 2 drops of reagent FolinCiocalteu, 1 ml Sodium carbonate 15% (w / v). After 1 hour, the sample absorbance was measured at a wavelength of 760 nm [21] using UV VIS spectrophotometer (Genesys USA). Phenol acid standard curve using tannic acid as standard and plotted between concentrations of 0.03, 0.05, 0.07 and 0.09 ppm [22].

Calculation of the cell dry weight was used cells that were still accommodated in the filter paper and put into oven at a temperature of 80^0C for 1 day. Afterthat, the drycells was weighed in the analytical sheet balance.

2.2 Data analysis

The research design was used RAL (completely randomized design) witha repeat3 times. Parameter observation was the total concentration of phenolic acid sanddry weightof cellsin the treatment of salicylic acid and phenylalanine. The data obtained from the study was analyzed statistic all yusing one-way ANOVA and Tukey's test with aconfidence level of 95% (α =0.05).

3. RESULT AND DISCUSSION

3.1 Callus induction of *M. oleifera* Lam explant seeds

The results of *M. oleifera* seed callus induction on MS medium with combinations of 2.4 D0.5 ppm and BAP 1ppm produce callus yellowish-white friable, and grows after the age of 10 days of inoculation. The first response of the explant seeds are swelling and the appearance of cell mass (callus) on the part around the incision wound explants (Figure 1). This is in line with the statement [23], that the callus will grow on the edges of the explant and callus after a certain time will fill the entire surface of the explants.

Composition PGR concentration used 1 ppm BAP and 0.5 ppm 2,4-D as it aims for continuous callus formation as a source of inoculum for cell suspension cultures. By [24] that the concentration ratio of cytokinin and auxin intermediates will induce the formation of callus. Results of research conducted by [16] on cotyledons of *M.*

Oleifera seeds also showed a significant increase in callus weight in treatment of 0.5 mg/l BAP and 1 mg/l 2.4 D.

The callus showed friable texture and yellowish-white. Absence of color and texture to the callus can be affected by light, PGR and origin of explants. Callus with yellowish-white colour is embryonic callus [25] which formed as a result ofincrease cell division activity [26]. These results are consistent with research [16] on the callus induction cotyledons of *M. oleifera*seed treatment of 0.5 mg/l BAP and 1 mg/l 2,4-D to produce whitefriable callus. Results of callus induction of *Amorphophallusmuelleri* with a balanced combination of 1 mg/l 2,4-D + 1 mg/l BAP also produce white callus [27].

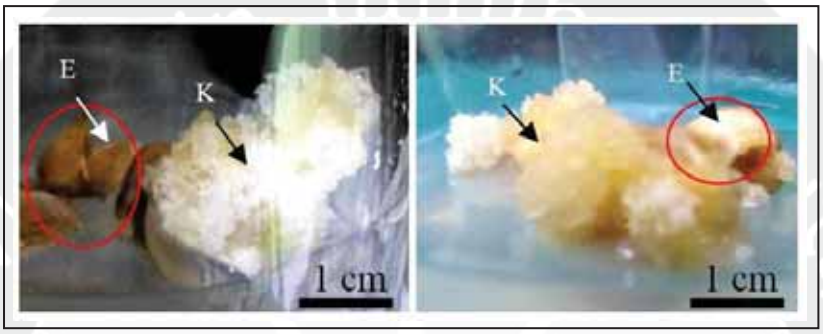

Figure 1. Results of Callus Induction explants Seeds of *M. oleifera* In MS Medium 2.4 D 0.5 ppm and 1 ppm BAP (K: callus, E: explant)

3.2 Qualitative test the presence of phenolic compounds

Callus generated from callus induction process *M. oleifera* seeds were then used to process the cell suspension cultures. After the callus was inoculated on the liquid medium and agitated it will form crumbs cells dispersed in a medium. Cell suspension cultures can be used for the process of improvement of secondary metabolites with the addition of elicitor [20] and the addition of precursor feeding [11].

Results of cell suspension cultures that had been treated salicylic acid and phenylalanine are then extracted using ethanol and generate total phenol extract compounds that are colorless (clear). Callus extracts of varying concentrations of precursor and elicitor treatment after being given the Folin-Ciocalteureagent produces blue chromogen. Chromogen blue color is the result of a reaction between the reagentFolin - Ciocalteu oxidizing the hydroxyl group (OH-) in the alkaline condition. In Figure 2 describes the mechanism of the reaction between phenol compounds can be reduced by the Folin-Ciocalteu reagent to form molybdenum blue complex [28].

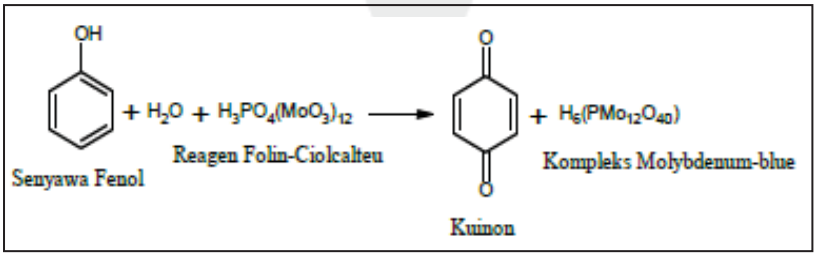

Figure 2. The reactionbetween phenol compounds and the Folin-Ciocalteu reagent

3.3. Effect of salicylic acid in cell suspension cultures of *M. oleifera* Lam.

One Way ANOVA test results p 0.092 (p 0.05) shows that the variation of the concentration of salicylic acid did not affect the total concentration of phenolic acids. This can be due to several factors, salicylic acid concentration range used is too low at 0.5 to 1.5 ppm. In addition, optimization of elicitor on cell suspension cultures depend on specificity, concentration, time elicitation, and plant species so that products which are accumulated by each plant is different elicitation results [30]. Calculation of the total concentration of phenol acid using a regression equation tannic acid standard curve y = 4,091x-0,001. Based on Table 1 is known that salicylic acid elicitor treatment of 0.5-1.5 ppm concentration showed an increase in total acid content of phenolic compounds and a reduction in dry weight compared to control cells. The highest concentration of total phenolic acids contained in elicitor treatment salicylic acid concentration of 1 ppm was 1.6067 ± 0.458 ppm and the lowest concentration found in the control 0 ppm was 0.6304 ± 0.148 ppm. Dry weight is highest in the control treatment was 25.1 ± 0 ppm was 2.42 mg dry weight while the lowest for the treatment of 1.5 ppm was 7.7 ± 1.35 mg.

Table 1. Total Acid Phenol concentration and cell dry weight of *M. oleifera*at elicitor treatment Salicylic Acid

Treatment	Total phenolic acid (ppm)	Cell dry weight (mg)
0 ppm	0,6304 ± 0,148	25,1 ± 2,42
0,5 ppm	1,3613± 0,413	10,0 ± 0,95
1 ppm	1,6067± 0,458	12,5 ± 1,85
1,5 ppm	1,2399 ± 0,211	7,7 ± 1,35

The total phenolic acidincreased in1 ppm concentration of salicylic acid was 1.6067 ± 0.458 ppm but a decline in cell dry weight of 12.5 ± 1.85 mg compared to control, 0.6304 ± 0.148 for total phenol acid and 25.1 ± 2, 42 mg dry weight of cells. Increased total compound total phenolic acid and dry weight of this happening is one response when the plant is in stress condition to defense itself, so the plants produce more secondary metabolites than growth. This is consistent with the statement [31] that one of the compounds that play a role in plant defense mechanisms are phenols compounds. Increased phenol in salicylic acid elicitor treatment allegedly due to an increase in ROS (Reactive oxigen species) which is the initial response elicitor giving salicylic acid [31]. In addition, salicylic acid also affects the formation of the enzyme PAL (Phenylalanine ammonia lyase), which acts in the phenylpropanoid pathway and several enzymes that act to stabilize the ROS such as peroxidase and SOD (superoxide dismutase) [32].

An increase in ROS (Reactive Oxygen Species) which is in line with the increase in the concentration of salicylic acid may cause decreasingcell growth. According to [31] increased levels of ROS in the cells can cause direct binding of the enzyme catalase or other macromolecules such as proteins and lipids. So that the excessive accumulation of ROS can cause oxidative damage and cell death. In addition, salicylic acid also affects the increase in the hormone ABA (abscisic acid) that function in the process of defense against abiotic stress and dormancy. It can be presumed that the increase in ABA can cause a decrease in cell growth because more cells produce compounds such as phenols defense [33]. Results of the study

[31] showed that the concentration of 3.125 to 12.5 mg/L showed a decrease in dry weight and an increase in phenolic compounds on cell cultures of *S. Miltiorrhiza*. At the *Vitisvinifera* plants giving 50 µM salicylic acid methyl decreased dry weight of 16.4 ± 0.5 g/l compared control 17.6 ± 0.9 g/l and the content of phenolic compounds as much as 790 ± 30 mg / l compared to 760 control ± 60 mg/l after a ten-day incubation period [20]. Whereas in plants Chickpea (*Cicerarietinum* L.) addition of 1.5 mM salicylic acid can increase the phenol content <60 mg after 96 hours of incubation [32].

3.4. Effect of phenylalanine in cell suspension cultures of M. oleifera Lam.

Results of one-way ANOVA test, $p \leq 0.010$ ($p \leq 0.05$) showed that the phenylalanine concentration variations affect the total content of phenolic acids. Calculation of the total concentration of phenol acid using a regression equation tannic acid standard curve y = 4,091x-0,001. In the cell suspension cultures of *M. oleifera* precursor feeding was used phenylalanine at concentrations of 0 ppm (control), 5 ppm, 10 ppm and 15 ppm.

Table 2. Total Acid Phenol concentration and celldry weightof *M. oleifera* In Treatment of Precursor Feeding Phenylalanine

Treatment	Total phenolic acid (ppm)	Cell Dry wieght (mg)
0 ppm	$0,63^{a}$ ± 0,15	6,9 ± 0,80
5 ppm	$1,60^{b}$ ± 0,37	7,3 ± 0,61
10 ppm	$1,26^{ab}$ ± 0,31	7,8 ± 0,72
15 ppm	$1,52^{b}$ ± 0,29	9,7 ± 1,46

Based on Table 2, it is known that precursor feeding of phenylalanine treatment of 0-15 ppm concentration showed an increase in dry weight and total phenolic acids compared to control. The highest dry weight contained in the treatment of precursor feeding phenylalanine concentration of 15 ppm is 9.7 ± 1.46 mg and the lowest concentration found in the control 0 ppm was 6.9 ± 0.80 mg. The highest concentration of phenols contained in the treated precursor feeding phenylalanine concentration of 5 ppm was 1.60 ± 0.37 ppm and the lowest concentration found in the control 0 ppm was 0.63 ± 0.15 ppm. Increased cell dry weight due to the addition of exogenous amino acids such as phenylalanine. This is consistent with the statement [34] which states the addition of phenylalanine in the cell suspension culture medium can increase the content of endogenous phenylalanine. So that the excess amount of carbon that can be used for the process of growth [35].

Increased total phenol acid because phenylalanine is the main precursor in the biosynthesis of phenolic compounds. So the more phenylalanine is absorbed the more the total phenolic compounds are produced. This is in line with the statement [36] that the addition of phenylalanine affect the content of phenolic compounds as total acid phenylalanine is the main precursor in the metabolic process through phenylpropanoid, thus increasing the amount of phenylalanine which will be absorbed increase income PAL enzymes that affect the process of formation of

phenol acid. PAL enzyme (phenylalanine ammonia lyase) is a key enzyme in the biosynthesis of phenolic compounds from the amino acid phenylalanine [31]. Results of the study [19] on the plant *Menthaarvensis* showed the addition of phenylalanine concentration of 5 ppm is able to increase the content of phenolic compounds 8.02 ± 1.08 mg/g compared to the control that was 6.24 ± 0.68 mg/g wet weight and increase callus 0, 30 grams compared to the control that was 0.26 grams.

Phenylalanine concentrations of 5 and 15 ppm significant effect on total acid production process phenol compound in cell suspension cultures of *M. oleifera* compared with controls. Improvement of secondary metabolites such as phenolic acid compounds can be due to an increase in PAL enzyme which included the key enzymes before entering cinnamat acid pathway [19]. In the treatment of phenylalanine concentration of 10 ppm decreased phenolic compounds while increasing cell dry weight. This is because in the process of cell suspension cultures, callus *M. oleifera*not completely dispersed so that the cell surface area in contact with the medium are also smaller. This indicates that cell size affects the level of absorption of nutrients in the growth medium. By [37] states that the cell size has an influence on the level of absorption of nutrients, which the smaller cell size has the ability to absorb nutrients faster than larger cells. It has been proved by [37] to study the morphology and size variations of phytoplankton cells and concluded that the level of sinking or absorption of good nutrition is limited to the size of 20µm cells.

4. CONCLUSIONS

Based on the study it can be concluded that the variations in treatment concentration of salicylic acid and phenylalanine affect the increase in the total content of phenolic acids in cell suspension cultures of*M. oleifera* Lam. The highest total phenolic acid in the treatment of salicylic acid concentration of 1 ppm was 1.6067 ± 0.458 ppm compared to controls, which was 0.6304 ± 0.148 ppm and treatment phenylalanine concentration of 5 ppm was 1.60 ± 0.37 ppm compared to the control that was 0.63 ± 0.15 ppm.

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Mangrove Degradation Impacts on Biomass of Intertidal Macrozoobenthic: a Case Study at Sembilang, South Sumatra, Indonesia

Agus Purwoko[1, 2] and Wim J. Wolff[1]
[1] Dept. of Benthic Marine Ecology and Evolution, University of Groningen, P.O. Box 14, 9750 AA Haren, The Netherlands
[2] Dept. of Biology, Sriwijaya University, Palembang, Indonesia
E-mail: agus_purwoko2002@yahoo.com; w.j.wolff@rug.nl

Abstract

Mangrove degradation included abrations and development of mangrove areas such as: plantation estate, transmigration sites and fish ponds. Macrozoobenthic biomass of the intertidal area of the Sembilang peninsula of South Sumatra, Indonesia, has been studied in 1996, 2004 to 2006. Monthly (August – October 1996) 25 core samples were taken at each of plot sampling stations, (March – August 2004) 21 core samples were taken at each of six sampling stations, (March – August 2005) 30 core samples were taken at each of six sampling stations, (June and October 2006) 30 core samples were taken at each of six sampling stations. Macrozoobenthics was identified at the lowest taxonomical level, and counted. Biomass was measured as ash free dry weight (AFDW). Development of shrimp ponds matched the declining biomass of macrozoobenthic significantly (Anova: $p < 0.05$) for long term (1996 to 2006). Three year of observation (2004-2006) show that sites close to the shrimp ponds (pond sites) significantly had lower biomass of macrozoobenthic compare to biomass value at sites further from the ponds (non-pond sites). Mangrove organic matter probably was not the main source of food for macrozoobenthic fauna and pond effluent might not responsible for declining macrozoobenthic biomass. It seemed that disturbance of habitat of macrozoobenthic by fisheries coincided with decreasing biomass.

Keywords: degradations, mangrove, impact, shrimp pond, biomass, macrozoobenthic, intertidal, Indonesia Sumatra, Sembilang,

1. INTRODUCTION

In 1990 Indonesian mangroves occupied 4.25 million ha which represented about 20 % of the world's mangroves. The largest part was found at Papua (2.94 million ha) and 1.31 million ha occurred at more populated areas such as Java, Sumatra, and Kalimantan. South Sumatra counted 195000 ha (Choong *et al*., 1990) with 77500 ha (Danielsen and Verheugt 1990) in our study area Sembilang National Park.

Annual mangrove litter production at some nearby Indo-Pacific locations averaged 1107 g dry wt m^{-2} at Hong Kong (Lee 1989, 1989 a), 497 g dry wt m^{-2} at Sri Lanka (Amarasinghe and Balasubramaniam 1992), and Bunt (1995) found annually on average 1752 g dry wt m^{-2} at Papua New Guinea. Annual litter production at Sembilang, Indonesia, was 1376 g dry wt m^{-2} (Soeroyo 1999). It has been

demonstrated that benthic animals, mostly crustaceans, feed on this resource (Odum and Heald, 1972, 1975). In mangrove forest crabs (Lee 1989), gastropods and polychaetes (Alongi and Sesakumar 1992) feed on mangrove leaves. Lee (1989) found that crabs consumed 50 % of mangrove leaves.

Part of the net canopy production of mangrove forest is exported. Odum and Heald (1972, 1975) suggested that this may amount to about 50% of the production and Alongi and Sesakumar (1992), and Alongi *at al*., (2004) found an export of 40% and 25% respectively. This exported material may enhance the benthic fauna of adjoining aquatic habitats such as tidal flats.

However, in a review Lee (1995) questioned that benthic biomass of the macrofauna had a positive relationship with the availability of detritus originating from mangrove leaves. Indeed, Lee (1999) experimentally found no relationship between mangrove leaves and biomass of macrozoobenthic. Also, Bouillon *et al*., (2002) and Hsieh *et al*., (2002) demonstrated with stable isotopes that many benthic animals in mangrove forests derived their food from other sources than mangrove leaves and litter.

Conversion mangrove forest to ponds involved cutting mangrove forest, dredging and dumping, and discharging water to estuaries. Those activities may have impacts to benthic biomasses at tidal flat. Dredging and dumping are important activities which lead to turbidity and enhanced sediment deposition, and growth of bivalves may be impaired by elevated concentrations of suspended matter (Karel 1999). A three-year study on the effects of reclamation activities on the macrozoobenthic at a Singapore coastal area found that macrozoobenthic abundance significantly decreased over time close to the reclaimed area but it increased again with distance from this area (Lu *et al*., 2002). Kenny and Rees (1994) reported that a dredged site had not fully recovered after 7 months but Lu and Wu (2000) found that the benthic community fully recovered in less than 15 months.

Pond effluents lead to reduced water quality by increasing suspended particles (Jackson *et al*., 2003), total nitrogen and phosphate (Jackson *et al*., 2004; Lemonnier and Faninoz 2006), salinity, acidity (Cowan *et al*., 1999) and biochemical oxygen demand (Trott and Alongi 2000; Cowan *et al*., 1999), dissolved organic matter (Lemonnier and Faninoz 2006), eutrophication (McKinnon *et al*., 2002; Alongi *et al.,*. 2000) and adding antibiotics (Le and Munekage 2004; Le *et al*., 2005). Phytoplankton may benefit of increasing nutrients. However, it will improve shoot biomass of mangrove seedling (Rajendran and Kathiresan 1996).

Two families from Lampung (Southern South Sumatra) Province established 4 ha of shrimp ponds in the Sembilang National Park in 1996. Local government caught the investors, but from 1997 to 2002 the government did not pay attention to Sembilang developments. About 2,000 families established 4,000 ha (traditional) shrimp ponds from 1998 to 2002. They left some mangrove area as a green belt, and their ponds are connected to some small rivers (Fig. 1). Indonesian and Japan restored the ponds area naturally and enriched by human intervention recently in 2014 on JAICA project.

Purwoko (1996) described abundance and biomass of macrozoobenthic at Sembilang before this large-scale pond construction; his site of observation was about 10 km from the first established pond. During and after the pond development at Sembilang peninsula, no study on the impacts of shrimp pond development on the

coastal ecosystems was conducted at Sembilang. Our study objective was to determine the positive or negative impact of pond construction as mangrove degradation and operation on the macrozoobenthic of the tidal flats on that site. We did so by comparing the biomass in the pre-pond construction (1996) and after-pond construction period (2004-2006), and by comparing locations close to the shrimp ponds with sites at further distance after pond construction. The average macrozoobenthic biomass over all was 3.62 g afdw m^{-2}, the highest biomass (14.09 g afdw m^{-2}) found at Sembilang peninsula was due to abundant occurrence of the bivalve *Anadara granosa* (Purwoko and Wolff, 2008).

2. METHODS

2.1. Area description

The Sembilang peninsula (Fig. 1: 1^0 59' to 2^0 15' S and 104^0 45' to 104^0 53' E) is located at the Eastern coast of South Sumatra Province, Indonesia, and it is part of the Sembilang National Park. It is influenced by the Musi River, the Musibanyuasin River and some smaller tributaries. Originally, all land was covered by mangrove and swampy forest. The most seaward belt of the mangrove vegetation is still formed by *Sonneratia* and *Avicennia* where the soil is sandy; however, where the soil is muddy, *Rhizophora* occurs. *Nypa* palms appear where the soil is highly influenced by fresh water. In the mangrove area at the Sembilang peninsula, approximately 200 to 500 meter from the beach, there are 4,000 ha of shrimp ponds. The coastline of the area faces directly Bangka strait and the East China Sea. Two villages are nearby. Sungsang village is at the southern part and Sembilang village at the northern end of the peninsula.

2.2. Description of sampling stations

For our 2004-2006 study we selected 6 sampling stations (Fig. 1) with varied characteristics:

1. Station 1: Tj. (Tanjung) Carat (S: 2^0 16.324' & E: 104^0 55.117' by Gekko 202 Garmin GPS). Located in the estuary of the Musi river; the soil is sandy, and the station is strongly influenced by fresh water.
2. Station 2: S. (Sungai) Bungin (S: 2^0 14.955' & E: 104^0 50.714'). Located in the estuary of the Musibanyuassin river. The surface layer of the sediment is soft and high in organic matter and has a thickness of more than 1 meter. On the sediment young *Avicennia* occur.
3. Station 3: Solok Buntu (S: 2^0 11.063' & E: 104^0 54.764'). Located near the estuary of the Musibanyuasin river. The surface layer of the soil is muddy, and reaches 40 cm depth. Mangrove vegetation consists mainly of *Avicennia*. At sea nearby, there are some pole houses.
4. Station 4: S. Barong Kecil (S: 2^0 9.872' & E: 104^0 54.587'). The site is near the minor estuary of S. Barong Kecil. The soil is muddy and the depth of this layer reaches 50 cm.
5. Station 5: S. Siput (S: 2^0 5.824' & E: 104^0 54.102'). The soil is muddy and the soft layer reaches 40 cm; the adjacent mangrove vegetation is mostly *Avicennia*; close to the site young trees grow. At this station we made also our 1996 observations.

6. Station 6: S. Dinding (S: 2^0 1.924' & E: 104^0 50.573'). The sediment is muddy and contains many dead shells. The depth of the soft layer reaches 40 cm. The site is facing directly the South China Sea, so it is exposed to high waves.

Stations 1, 2 and 6 were further removed from the shrimp ponds; and stations 3, 4, and 5 relatively close to ponds or at least close to estuaries of small rivers that served as an outlet of pond discharges (Fig. 1). So, our after-construction comparison has been made by comparing stations 1, 2 and 6 on the one hand with stations 3, 4 and 5 on the other hand.

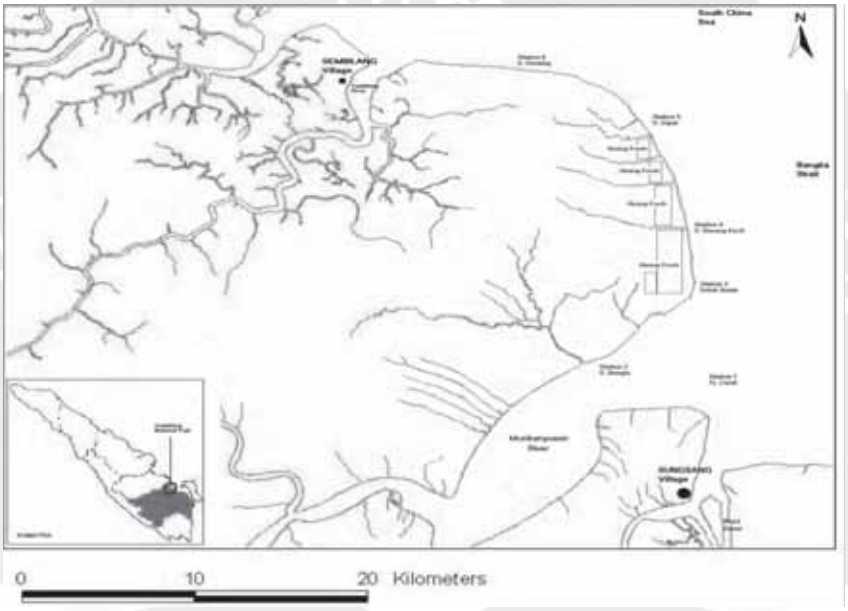

Figure 1. Sampling stations along the coast of Sembilang peninsula. Each station consists of three transect each with seven (2004), ten (2005 &2006) sampling points, plot 1 & 1 (100*100 m, 1996) at station 5.

2.3. Sampling procedure

In 1996, two plots (100 * 100 meters) were established at station 5. Plot x was situated at the upper half of the shore and plot y was set at the lower shore. Sampling started in August and took place three times; the periods between two samplings were four weeks. There were 25 core samples collected at random guided by random numbers at each plot. Samples were taken with a circular corer. Core diameter was 15 cm and the sampling depth 30 cm. The core samples directly were extracted by 5 mm sieve in the field and the animals were collected from the sieve by hand. Macrozoobenthic collected was preserved in 70 % alcohol mixed with 3 % of formalin. Sampling activities took place during low tide at Sungsang. The fauna in the samples was identified to the lowest taxonomic level possible (Dharma 1988, 1992), counted and Ash-Free Dry Weight (AFDW) (Winberg and Duncan 1971) was measured at the laboratory at Palembang.

In 2004-2006 each station consisted of 3 parallel lines transects each consisting of 7 sampling points in 2004 and 10 sampling points for 2005 and 2006. The distance between the lines transects varied from 5 to 10 m; the distance between the

sampling points was randomly chosen and varied between 3 and 20 m. The first core sample of the first transect was taken at the lowest water level and the second till the seventh one were at further distance towards the mangrove. Circular core diameter and sampling depth were the same as for the 1996 core samples. At each sampling point 1 core sample was taken. The core samples directly were extracted by double layers of 1 mm sieve in the field and the animals were collected from the sieve by hand. Macrozoobenthic collected was preserved in 70 % alcohol mixed with 3 % of formalin. Sampling activities took place during low tide at Sungsang in March, May, June, July and August 2004, March, April, May, June, July and August 2005, and June and October 2006. The animals in the samples were identified to the lowest taxonomic level possible (Dharma 1988, 1992); Computer Program: Poly Key), counted and Ash-Free Dry Weight (AFDW) measured like 1996's samples.

In order to compare years, data sets were taken from the same month of each year. In this case, data sets for June in 2004, 2005 and 2006 were comparable. The statistical calculations such as Analysis of Variance (ANOVA), Duncan test and T-test were carried out by the Statistics 7 Program.

3. RESULTS

3.1. *Comparison of pre-construction and post-construction period*

Table 1 from Purwoko (1996) gives the biomasses found at the two plots at station 5 in 1996 that is in the pre-construction period of ponds at Sembilang peninsula. Higher biomass of macrozoobenthic found at plots x and y in October 1996 differs significantly from the biomasses in August and September 1996. However, biomass of macrozoobenthic animals at plot x was not significantly different from those at plot y from August to October 1996 (no letters shown). In 1996, 17 taxa were identified and the average density at the plots was 690 individuals m^{-2}.

Table 1. Average biomass in g ash-free dry weight (g m^{-2}) of macrozoobenthic at plots x and y of station 5 at Sembilang peninsula in different months. Different letters (in brackets) after biomass values in the same row indicate significant differences (ANOVA: p<0.05).

AFDW macrobenthic fauna at station 5 (S.Siput) in 1996			
Treatments	August	Sept	Oct
plot x	16.33 (a)	14.71 (a)	37.74 (b)
plot y	20.44 (a)	15.32 (a)	33.38 (b)

Table 2 present the biomass data per station averaged over the period 2004-2006. Generally, total biomasses of macrozoobenthic at stations 1, 3 and 5 were significantly lower (ANOVA: p<0.05) than those at stations 2, 4 and 6. The same differences occurred for total biomass of bivalves. The highest biomass of polychaetes was found at station 1, differing significantly from the other stations. However, each family of polychaetes had a different pattern of distribution of biomass compared to the total biomass of all polychaetes. Biomass of gastropods at station 1, 3, 4 and 5 was significantly lower than biomass values at stations 2 and 6.

Table 2. Average biomass in g ash-free dry weight (g m^{-2}) of macrozoobenthic taxa at six sampling stations at Sembilang peninsula in 2004 to 2006. Different letters (in brackets) after biomass values in the same row (species of macrozoobenthic) indicate significant differences (ANOVA: p<0.05)

	WAFDW (gram/m^2)	**Location:** Sembilang peninsula in 2004-2006					
	Bivalves	Station 1	Station 2	Station 3	Station 4	Station 5	Station 6
1	*Anadara granosa*	0.0144	0.0000	0.3704	4.1691	0.0000	0.8413
2	*Hecuba scortum*	0.0041	0.0358	0.1490	0.0085	0.2251	0.1136
3	*Solen sp.*	0.0588 (b)	0.0774 (b)	0.0425 (b)	0.0377 (b)	0.0039 (a)	0.0410 (b)
4	*Tellina remies*	0.0184 (a)	0.0801 (a)	0.2533 (a)	0.5155 (a)	0.7711 (a)	8.5615 (b)
5	*Tellina timorensis*	0.1112 (a)	4.3512 (b)	0.2700 (a)	0.1933 (a)	0.1908 (a)	0.3260 (a)
	Total Bivalves	0.2069 (a)	4.5445 (b)	1.0852 (a)	4.9241 (b)	1.1908 (a)	9.8833 (c)
	Gastropods						
1	*Clinthon oualaniensis*	0.0117	0.0576	0.0039	0.0250	0.0059	0.0153
2	*Littorina melanostoma*	0.0000	0.0001	0.0000	0.0001	0.0000	0.0002
3	*Nassa serta.*	0.0176	0.0956	0.0128	0.0221	0.0164	0.0907
4	*Thais buccinea.*	0.0037	0.0066	0.0118	0.0032	0.0016	0.0115
	Total Gastropods	0.0329 (a)	0.1599 (b)	0.0286 (a)	0.0504 (a)	0.0240 (a)	0.1176 (c)
	Decapods (Crabs)						
1	Ocypodidae	0.0373 (a)	0.0184 (a)	0.1885 (a)	0.0412 (a)	1.5910 (b)	0.0271 (a)
2	Leucociidae	0.0799	0.0174	0.0346	0.0193	0.0189	0.0076
	Total decapods	0.1171 (a)	0.0358 (a)	0.2231 (a)	0.0605 (a)	1.6099 (b)	0.0347 (a)
	Polychaetes (Worms)						
1	Nereididae	1.6800 (b)	0.3009 (a)	0.1455 (a)	0.3151 (a)	0.1716 (a)	0.2186 (a)
2	Maldanidae	0.0039 (a)	0.0445 (b)	0.0069 (a)	0.0158 (a)	0.0176 (a)	0.0301 (a)
3	Lumbrineridae	0.0065 (a)	0.0154 (b)	0.0196 (b)	0.0288 (b)	0.0456 (c)	0.0416 (c)
4	Capitellidae	0.0009	0.0143	0.0114	0.0026	0.0022	0.0036
5	Sternaspidae	0.0041 (a)	0.0394 (a)	0.0839 (b)	0.2346 (b)	0.0344 (a)	0.0469 (a)
6	*Unidentified worms*	0.0189 (a)	0.0370 (a)	0.0051 (a)	0.0041 (a)	0 (a)	0.2609 (b)
	Total Polychaetes	1.7145 (c)	0.4034 (a)	0.2724 (a)	0.6009 (b)	0.2715 (a)	0.6017 (b)
	Total macrob. Animals	**2.0715 (a)**	**5.1436 (b)**	**1.6093 (a)**	**5.6359 (b)**	**3.0962 (a)**	**10.6374** (c)

3.2. Comparison of sites in post-construction period

Table 2 gives the biomass data for the period after pond construction. It is, however, based on averages for different months and different numbers of months. To better compare the different years Table 3 compares the biomass values obtained at each station in June of each year. We found in general low biomass values, but with significant differences between years. Also the stations differ significantly from each other (ANOVA: $p<0.05$; not shown in Table) per year.

Table 3 Average biomass in g ash-free dry weight (g m^{-2}) of macrobenthic fauna in different stations at Sembilang peninsula in June 2004 to 2006. Different letters (in brackets) after biomass values in the same row indicate significant differences (ANOVA: $p<0.05$) between years

AFDW of macrobenthos fauna at Sembilang peninsula in June from 2004 to 2006 over 6 stations			
Locations	2004	2005	2006
Station 1	2.23 (a)	3.45(a)	5.34 (b)
Station 2	0.62 (a)	1.48 (b)	2.24 (b)
Station 3	1.35 (a)	1.90 (b)	1.42 (a)
Station 4	47.45 (b)	1.05 (a)	1.08 (a)
Station 5	0.87(a)	0.49 (a)	9.95 (b)
Station 6	0.94 (a)	2.82 (b)	2.83 (b)

Table 4 gives biomasses per species averaged over all stations in June of each year. At the Sembilang peninsula, total biomass of macrozoobenthic was significantly lower in 2005 compared to 2004 and 2006. There were also significant differences among the stations from 2004 to 2006 (Table 3). For example, at station 5, biomass of macrobenthic animals in 2006 was significantly higher than in 2004 and 2005.

Table 4. Average biomass in g ash-free dry weight (g m^{-2}) of macrobenthic fauna taxa at Sembilang peninsula in June 2004 to 2006. Different letters (in brackets) after biomass values in the same row indicate significant differences (ANOVA: p<0.05)

	WAFDW (gram/m2)	Sembilang peninsula in June …		
	Bivalves	**2004**	**2005**	**2006**
1	*Anadara granosa*	7.7037	0.1163	0.0000
2	*Hecuba scortum*	0.0269	0.1131	0.0007
3	*Solen sp.*	0.1278 (a)	0.1854 (b)	0.0725 (a)
4	*Tellina remies*	0.4092 (b)	0.3645 (a)	0.0283 (a)
5	*Tellina timorensis*	0.1996	0.1688	0.1012
	Total Bivalves	8.4672 (b)	0.9481 (a)	0.2027 (a)
	Gastropods			
1	*Clinthon oualaniensis*	0.0673	0.0000	0.0000
2	*Littorina melanostoma*	0.0009	0.0000	0.0000
3	*Nassa serta.*	0.0853 (b)	0.0019 (a)	0.0134 (ab)
4	*Thais buccinea.*	0.0375	0.0000	0.0000
	Total Gastropods	0.1911 (b)	0.0019 (a)	0.0134 (a)
	Decapods (Crabs)			
1	Ocypodidae	0.0640 (a)	0.2985 (a)	1.6395 (b)
2	Leucociidae	0.0000	0.0000	0.0000
	Total Decapods	0.0640 (a)	0.2985 (a)	1.6395 (b)
	Worms			
1	Nereididae	0.0494 (a)	0.5594 (a)	1.4588 (b)
2	Maldanidae	0.0269	0.0094	0.0126
3	Lumbrineridae	0.0269 (a)	0.0251 (a)	0.0430 (b)
4	Capitellidae	0.0180	0.0003	0.0000
5	Sternaspidae	0.0314 (a)	0.0220 (a)	0.1571 (b)
6	*Unidentified worms*	0.0135 (ab)	0.0011 (a)	0.2828 (b)
	Total Worms	0.1661	0.6174	1.9543
	Total macrob. Animals	8.91 (b)	1.87 (a)	3.81 (b)

Bivalves, gastropods, crabs and some polychaetes species were significantly different between 2004 and 2005. Biomasses of bivalves and gastropods reached significantly higher values in 2004, but crabs (Ocypodidae) in 2006. Most polychaetes (Nereididae, Lumbrineridae, Sternaspidae and unidentified worms) showed higher values of biomass in 2006 (Table 4).

Generally, in 2004 to 2006 the pond sites had significantly lower biomass of macrobenthic animals compared to non-pond sites. All species of macrozoobenthic which had a significant difference had lower biomass at pond sites (Table 5).

Table 5. Average biomass in g ash-free dry weight (g m^{-2}) of macrobenthic fauna taxa at non-pond (station 1, 2 and 6) and pond sites (station 3, 4 and 5) at Sembilang peninsula in 2004 to 2006. Different ** after biomass values in the same row (species of macrobenthic animals) indicate significant differences (ANOVA: p<0.05)

		WAFDW (gram/m^2)	
	Bivalves	Non-pond sites	Pond sites
1	*Anadara granosa*	1.8032	1.5132
2	*Hecuba scortum*	0.1920	0.1275
3	*Solen sp.*	0.1325 **	0.0280
4	*Tellina remies*	3.4328 **	0.5133
5	*Tellina timorensis*	3.3016 **	0.2180
	Total Bivalves	8.8621 **	2.4001
	Gastropods		
1	*Clinthon oualaniensis*	0.0628	0.0116
2	*Littorina melanostoma*	0.0002	0.0000
3	*Nassa serta.*	0.1228 **	0.0171
4	*Thais buccinea*	0.0163	0.0055
	Total Gastropods	0.2020 **	0.0343
	Decapods (Crabs)		
1	Ocypodidae	0.6530 **	0.6069
2	Leucociidae	0.0917 **	0.0242
	Total Decapods	0.7447 **	0.6312
	Worms		
1	Nereididae	1.6042 **	0.2107
2	Maldanidae	0.0558 **	0.0134
3	Lumbrineridae	0.0598	0.0314
4	Capitellidae	0.0168	0.0054
5	Sternaspidae	0.1623 **	0.1176
6	*Unidentified worms*	0.1273 **	0.0031
	Total Worms	1.9941 **	0.3816
	Total macrob. Animals	11.8030 **	3.4471

4. DISCUSSION

4.1. Pre-construction compared to post-construction period of ponds at Sembilang peninsula

In August and October 1996, biomass of macrobenthic animals at station 5 was significantly higher than in August and October 2004-2006. This biomass of macrobenthic fauna gained mainly from *Anadara* biomass followed by *Tellina remies*. Biomasses at plot x and plot y were not significantly different in 1996, meaning that the macrobenthic fauna biomass was not influenced by the time of inundation, and beach steepness (Jiang and Li 1995; Ricciardi and Bourget 1999)

Due to the larger sieving mesh in 1996 (5 mm) compared to the sieving mesh applied in 2004 to 2006 (1 mm), the biomass values in 1996 are supposed to have been even higher than the biomasses actually recorded. Small polychaetes apparently passed through the sieve since we did not find any Maldanidae, Capitellidae and Sternaspidae in 1996. These animals approximately contributed average 2 percent to the total biomass value in 2004-2006 (Table 4).

In 1996 macrobenthic animals were larger in numbers of taxa and density than in 2004, 2005 and 2006. Species found in 1996 and absent in 2004, 2005 and 2006 were *Cerithium sp., Clypeomorus sp*., *Vexilles sp*., and *Natica sp*.

We conclude that macrobenthic animal biomass at Sembilang decreased by nearly an order of magnitude between 1996 before the construction of shrimp ponds and 2004-2006 well after the pond construction.

4.2. Comparison of stations after-pond construction period

A three-year observation (2004 to 2006) showed a significantly lower value of macrozoobenthic biomass at the stations 1, 3 and 5 compared to the stations 2, 4 and 6 (Table 2). This detailed evaluation does not support our hypothesis that the established ponds caused the decrease of the biomass of macrozoobenthic. However, based on a comparison of non-pond and pond sites, we could show that biomass of macrozoobenthic at pond sites on average was significantly lower compared to the biomass value at non-pond sites (Table 5). Also all macrozoobenthic species which showed significant differences had lower biomass at the pond sites (Table 5). This supports our hypothesis that biomass of macrozoobenthic declined significantly due to pond establishment.

Mangrove litter fall were believed to be a source of organic matter for macrozoobenthic both in the mangrove area tidal flat nearby (Odum and Heald 1975; Lui *et al*., 2002; Bosire *et al*., 2004). In a review, however, Lee (1995) questioned that benthic biomass had a positive relationship with the availability of detritus originating from mangrove leaves. This is supported by the results of Bouillon *et al*., (2002) and Hsieh *et al*., (2002) who demonstrated by means of stable isotopes that most benthic organisms do not feed on mangrove-derived organic matter. This is in line with the conclusion that macrozoobenthic biomass does not depend only on food availability (mangrove litter fall) but also food quality. Mangrove litter contains tannin which is not palatable for most macrozoobenthic species (Alongi and Sesakumar 1992) and Lee (1999) found negative relationship between solube tannin and biomass of macrozoobenthic in sediment. These conclusions affect the credibility of our hypothesis and force us to look into other explanations.

Discharge water contains nutrients, organic carbon and suspended solids which might have antibiotics. Options to prevent and reduce those pollutants entering the environment are to provide settling ponds (Jackson *et al*., 2003; Gautier *et al*., 2001; Lee 1993; Halide *et al*., 2003), ponds with vegetation (Sansanayuth *et al*., 1996; Halide *et al*., 2003) and mangrove forest as a filter (Nielsen *et al*., 2003). The latter of these options has been put into practice at Sembilang. However, whenever pond effluent was discharged directly into creeks, by 1 km down-stream and within 1-2 months after discharge ceased, water quality was recovered (Trott and Alongi 2000). Trott *et al*., (2004) found that discharge of pond waste carbon (C) and nitrogen (N) during shrimp harvest periods did not cause eutrophication further downstream. Hence, it is unlikely that at Sembilang waste water from shrimp ponds has been responsible for a difference between pond sites and non-pond area.

Hence, we consider the possibility that fishing may have influenced the biomass of the intertidal macrozoobenthic. Fishing at Sembilang aims at shrimp, fish and bivalves. The number of active fishing vessels amounts to about 1970 of which daily 40-60 are active in our tidal flat area (Djamali and Sutomo, 1999). Before 1999, fishermen used gill nets to catch shrimps for export abroad. They hardly harvested shrimps near the intertidal areas (Djamali and Sutomo 1999). After 1999, almost all fishing boats have been equipped with modified mini trawls to catch shrimps (Purwoko, unpubl. obs.). These boats operate at the intertidal areas during high tide. The trawls move over and through the muddy top layer of the sediment, supposedly disturbing the macrozoobenthic habitats. Since shrimp vessels operate at all stations they may have contributed to the general decrease of biomass over time.

Comparing June 2004, 2005 and 2006 (Table 3), a significantly lower average biomass was found at Sembilang peninsula in June 2005. Further, stations 1, 4 and 5 had lower biomass of macrozoobenthic, too. This was probably due to disturbance of the habitat of macrozoobenthic by a fishery for bivalves. Starting in March 2005, fishermen harvested *Anadara* at the intertidal flats of Sembilang peninsula. At station 2, they collected *T. timorensis* instead of *Anadara*. In May 2005, fishermen harvested *Anadara* at station 4 and in June 2005 they collected *Anadara* at station 5, after June, they moved to intertidal areas further North (station 6). It is assumed that a month after massive disturbance, biomass value of macrozoobenthic could not have recovered completely, but it could be recovered after 2 months by showing significantly higher biomass of macrozoobenthic at station 3 which was harvested in March and April 2005. At least three fishing boats operated by shoveling the sediment and collecting *Anadara* every day at high tide. However, no fishing boats operated on station 1. Kenny and Rees (1994) reported that the benthic community at a dredged site had not fully recovered after 7 months and Lu and Wu (2000) supported by demonstrating that the benthic community fully recovered in less than 15 months.

Mesh width of the net of fishermen boat was 2 cm, and they collected *Anadara* bigger than 2 cm (Djamali 1999). In addition, a group of on average 20 people manually collected *Anadara* at intertidal areas at low tide. In 1999/2000 (Djamali and Sutomo 1999) fishermen caught 130 ton wet weight of *Anadara* which is equivalent to about 3 ton afdw at intertidal and subtidal areas; in 2005 we estimate a catch of 32 ton wet weight (0.8 ton afdw) per month from the stations 3 to 6 in the period March to June (Purwoko, unpubl. obs.). This can be compared to the average biomass of *Anadara*

at our stations (0.898 g afdw m^{-2}; Table 2) which implies a standing stock for the Sembilang tidal flats of about 24 tons afdw.

Further, *Tellina remies* also showed no significant difference of biomass value from 2004 to 2006. Field observation showed that *T. remies* migrated in August 2005. We observed about 15 to 20 individual animals m^{-2} floating at the water surface during high tide moving North. Massive harvesting of *Anadara* probably was the triggering of *T. remies* migration. This behaviour might explain why *T. remies* showed no significant differences over time.

We concluded that converting mangrove to ponds coincided with decreasing biomass of macrozoobenthic from 1996 to 2006, and from 2004 to 2006 the nearby presence of shrimp ponds coincided with low biomass of the macrozoobenthic. However, loss of mangrove organic matter and discharge of shrimp pond water might not be responsible for decreasing biomass of macrozoobenthic. Habitat disturbance by fisheries was probably the most reasonable explanation for declining macrozoobenthic biomass. Further research required to evaluate the impacts of mangrove restoration and mangrove development such as harbors and industrial area, on macrozoobenthic biomass in 2015 and 2016.

5. ACKNOWLEDGE

First AP would like to thank to Sriwijaya University, Palembang, Indonesia for sponsoring my study. AP thanked to Ake and Usup for their assistance during sampling and Ismail for acting as a speedboat driver. AP was also glad that Edi P. assisted me on the measuring the AFDW. Finally, special thanks are addressed to Dr. Harry ten Hove who guided AP to identify the macrozoobenthic.

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The Effect of Elicitors (Salicylic Acid and NaCl) on Total Flavonoid and Flavonol Content in *Moringa oleifera* Lamk. Cell Suspension Culture

Elvian Haning Prameisti[1*], Nurul Jadid[1], Isdiantoni[2], Maharani Pertiwi Koentjoro[3], and Endry Nugroho Prasetyo[1]
[1]Department of Biology, Institut Teknologi Sepuluh Nopember, Gedung H 1st floor, Kampus ITS Keputih Sukolilo Surabaya, 60111 Indonesia
[2]Agrobusiness Department, Faculty of Agriculture, Universitas Wiraraja Jalan Raya Sumenep-Pamekasan Km. 5 Patean, Sumenep, 69451, Indonesia
[3]Laboratory of Environmental Microbiology, Department of Biological and Environmental Science, Faculty of Agriculture - Shizuoka University & Structural Biology Research Center, Inter-University Research Institute Corporation - High Energy Accelerator Research Organization (KEK), Tsukuba-Ibaraki, Japan
*Corresponding author email address: elvian_haning@yahoo.com

Abstract

Moringa oleifera Lamk. is highly abundant in Indonesia. *M. oleifera* contains flavonoid and flavonol which are beneficial for health since these compounds act as antioxidants. However, study on increasing flavonoid and flavonol in *M. oleifera* cell suspension culture is still scarce. The objective of the present study is to determine the effect of elicitors (salicylic acid and NaCl) on total flavonoid and flavonol content in *M. oleifera* cell suspension culture. The parameters measured were flavonoid and flavonol content. The methods were callus induction, cell suspension culture, elicitation, extraction, and quantification using UV/Vis spectrophotometer. The data were analyzed using ANOVA and Tukey test. The result of this study showed that the total flavonoid content was influenced by the concentration of elicitors (salicylic acid and NaCl). However, the flavonol content was not influenced by the concentration of elicitors (salicylic acid and NaCl). The highest content of total flavonoid was the treatment by 0.5 mM salicylic acid and 1.5 mM NaCl of 71.56±2.24 mg/L and 52.67±1.71 mg/L respectively. On the other hand, the highest content of flavonol was the treatment by 0.5 mM salicylic acid and 1.5 mM NaCl of 83.41±2.77 mg/L and 60.41±3.10 mg/L respectively.

Keywords: salicylic acid, total flavonoid, flavonol, *M. oleifera*, NaCl.

1. INTRODUCTION

Moringa oleifera Lamk. is highly abundant in Indonesia (Suhartini *et al*., 2013). *M. oleifera* is used as barrier plant in farm field and few is eaten as vegetable in Indonesia (Sarjono, 2008; Mutiara, 2011). Globally, *M. oleifera* is seen as one of the world's most beneficial trees because of its nutritional and medicinal properties. However, the tree hasn't received the same kind of attention in Indonesia and was never utilized optimally (Luqman *et al*., 2012). The medicinal properties have been attributed to phytochemical compositions of the tree's various parts (the roots, bark, leaves, flowers, fruits, and seeds) (Anwar *et al*., 2007; Kumar *et al*., 2010).

Phytochemical analysis of *M. oleifera* ethanolic leaf extract showed the presence of tannins, carbohydrate, saponins, glycosides, reducing sugars, terpenoids, steroids, flavonoids, and alkaloids. Based on the analysis, flavonoid was the highest content (Okechukwu *et al*., 2013). Flavonoid in *M. oleifera* may act as antioxidant (Charoensin, 2004). The most widespread flavonoid is flavonol (Jaganath and Crozier, 2010). In a recent study by Sultana and Anwar (2008), flavonol level was determined in 22 plant materials (9 vegetables, 5 fruits, and 8 medicinal plants) with *M. oleifera* as the highest concentration of flavonol.

Flavonoid and flavonol may act as antioxidants and also regulate blood flow, maintaining healthy heart (Coppin, 2008). These compounds are highly beneficial for health and food industries (Deligiannakis *et al*., 2012). One of the methods for producing secondary metabolites is by plant cell suspension culture. A plant cell suspension culture is a sterile system normally initiated by aseptically placing callus fragments into a suitable sterile liquid medium. Cell suspension culture contains a relatively homogenous cell population, allowing rapid and uniform access to nutrition, growth hormone and signal compounds for the cells (Mustafa *et al*., 2011). Different strategies for the metabolic engineering are constructed by the scientist for the improvement of secondary metabolites compound and one of them is addition of elicitors (Mendhulkar and Vakil, 2013). Elicitors function as defense response signal which trigger and activate the synthesis of secondary metabolites (Patel and Krishnamurty, 2013).

Several studies have demonstrated that the production of secondary metabolites is increased after the addition of elicitors. Salicylic acid functions to increase the production of flavonoid in cell suspension cultures of *Andrographis paniculata* (Mendhulkar and Vakil, 2013). On the other hand, Chen *et al*., (2014) stated that NaCl increased the production of flavonol in cell suspension cultures of *Ginkgo biloba*. However, the study regarding the effect of elicitors (salicylic acid and NaCl) on *M. oleifera* is still scarce. Hence, this study determines the effect of elicitors (salicylic acid and NaCl) on total flavonoid and flavonol content in *M. oleifera* cell suspension culture.

2. METHODS

2.1. Callus Induction

M. oleifera seeds were collected from Sumenep Regency, Madura Island, Indonesia. Samples were washed in flowing water for 10 min, soaked in the detergent solution for 5 min and followed by rinsing in flowing water for 10 min. Then, samples were soaked in the antifungal solution for 5 min and rinsed in flowing water for 10 min. Samples were then soaked in the 2% chlorox solution followed by rinsing twice with aquadest. Samples were then immersed into 96% alcohol, burned 3 times until seeds' coats were peeled. Samples were then cut into 3-4 parts and inoculated in MS medium supplemented with 0.5 ppm 2,4-D and 1 ppm BAP.

2.2. Cell Suspension Culture and Elicitation

Cell suspension culture was conducted by transferring 0.3 g fresh callus into a bottle containing 100 ml liquid MS medium supplemented with 0.5 ppm 2,4-D and 1 ppm BAP. These cultures were incubated on a rotary shaker 100 rpm at room

temperature for 14 days. Elicitors (salicylic acid and NaCl) with the concentrations 0 mM; 0.5 mM; 1 mM; and 1.5 mM were added into the cell suspension cultures. These cultures were incubated on a rotary shaker 100 rpm at room temperature. The cell suspension cultures were harvested 5 days after elicitors treatment.

2.3. Analysis of Total Flavonoid

Extraction of total flavonoid was carried out following the method of Biradar and Rachetti (2013) with few modifications. About 0.15 g sample was extracted repeatedly with 1.5 ml 80 % aqueous methanol at room temperature. The whole solution was filtered through Whatman® filtration paper Grade 42. The filtrate was later evaporated using a water bath. About 1 ml extracts and 1 ml standard quercetin solution (100, 200, 400, 600, 800, 1000 ppm) were positioned into test tubes and 0.3 ml of 5 % sodium nitrite solution was added into each. After 5 min, 0.3 ml of 10 % aluminium chloride was added. At the sixth minute, 2 ml of 1 M sodium hydroxide was added. The absorbance was measured at 510 nm using spectrophotometer (Genesys 10S UV Vis, USA) (Kamtekar *et al*., 2014).

2.4. Analysis of Flavonol

The analysis of flavonol was conducted following the method of Harborne (1987) with few modifications. About 0.15 g sample were hydrolyzed with HCl and boiled. The solution was cooled and extracted twice using ethyl acetate. The filtrate was later evaporated using a water bath. About 1 ml extracts and 1 ml standard quercetin solution (10, 20, 40, 60, 80, 100 ppm) was mixed with 3 ml of 95% ethanol, 0.2 ml 1 % aluminium chloride, 0.2 ml of 1 mol/L potassium acetate. After incubation at room temperature for 30 min, the absorbance was measured at 415 nm spectrophotometer (Genesys 10S UV Vis, USA) (Humadi and Istudor, 2008).

3. RESULT AND DISCUSSION

3.1. Callus Induction and Cell Suspension Culture

Callus in this study was formed 7-10 days after inoculation of the explant. The emergence of callus was characterized by swelling and the presence of irregular cells in the explant. Figure 1 presents the callus obtained in this study.

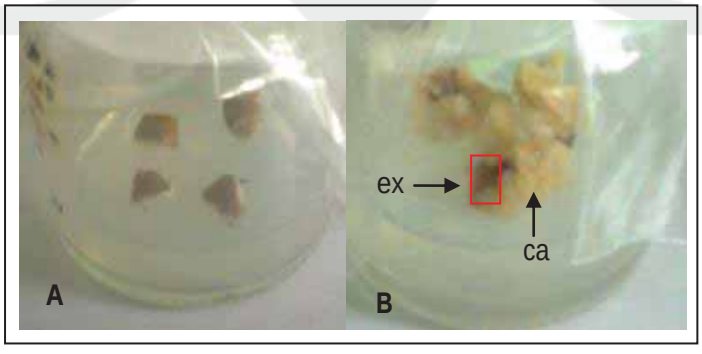

Figure 1. Callus induction of *M. oleifera* in MS medium supplemented with 0.5 ppm 2,4-D and 1 ppm BAP (A: explant; B: callus 29 days after inoculation of explants; ex: explant; ca: callus)

Callus of *M. oleifera* was white brownish in color. The callus still carried the original color of the explant. Furthermore, callus induction in this study was conducted in dark room to avoid photo-oxidation (Salisbury and Ross, 1995). The dark condition also influences the color of callus since the dark condition may inhibit the formation of chlorophyll (Mohr and Schopfer, 1995). The texture of *M. oleifera* callus was compact and friable. Vasil (1985) stated that the accumulation of secondary metabolites in compact callus is higher than friable callus. Callus obtained from the induction process was used as the cell suspension culture material. Callus was inoculated in MS liquid medium supplemented with 0.5 ppm 2,4-D and 1 ppm BAP. Callus in the present study was not entirely separate into cells (Figure 2).

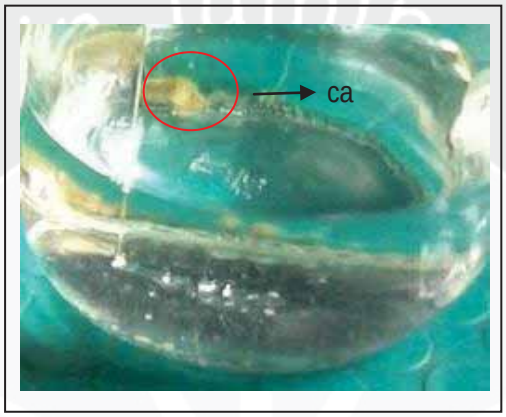

Figure 2. Cell suspension cultures of *M. oleifera* in MS liquid medium supplemented with 0.5 ppm 2,4-D and 1 ppm BAP (ca: callus)

3.3. Phytochemical Test

Phytochemical test was conducted to examine the presence of total flavonoid and flavonol on the samples of *M. oleifera* cell suspension cultures after elicitation treatment. The extraction of total flavonoid and flavonol was carried out using maceration. Result showed that all samples contained flavonoid and flavonol (Figure 3). Aluminium chloride forms acid stable complexes with the C-4 keto groups and either the C-3 or C-5 hydroxide group of flavones and flavonols. Orange yellowish color was developed (Kamtekar *et al*., 2014).

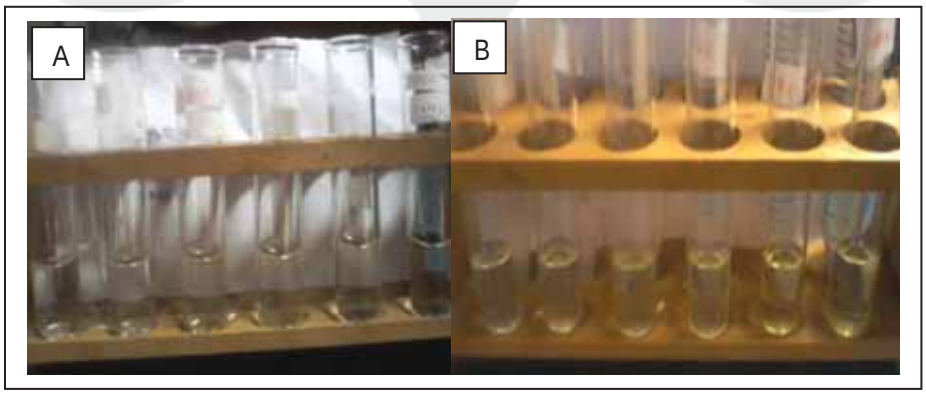

Figure 3. Phytochemical test
(A. extracts before $AlCl_3$ addition; B. extracts after $AlCl_3$ addition)

3.3. The Effect of Elicitors on Total Flavonoid Content

The total flavonoid content of the samples was measured using quercetin as standard. Standard curve of quercetin is presented in Figure 4.

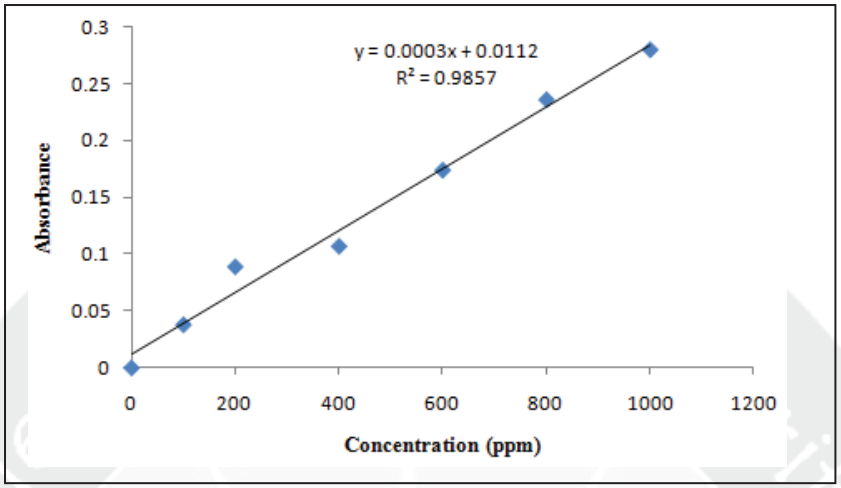

Figure 4. Standard Curve of Quercetin on Wavelength 510 nm

Regression equation y = 0,0003x + 0.0112 shows a linear relationship between absorbance and concentration measurement with a correlation coefficient 0.9857 (Figure 4). Figure 5 presents the results of the average concentration of total flavonoid in each treatment after elicitation treatment.

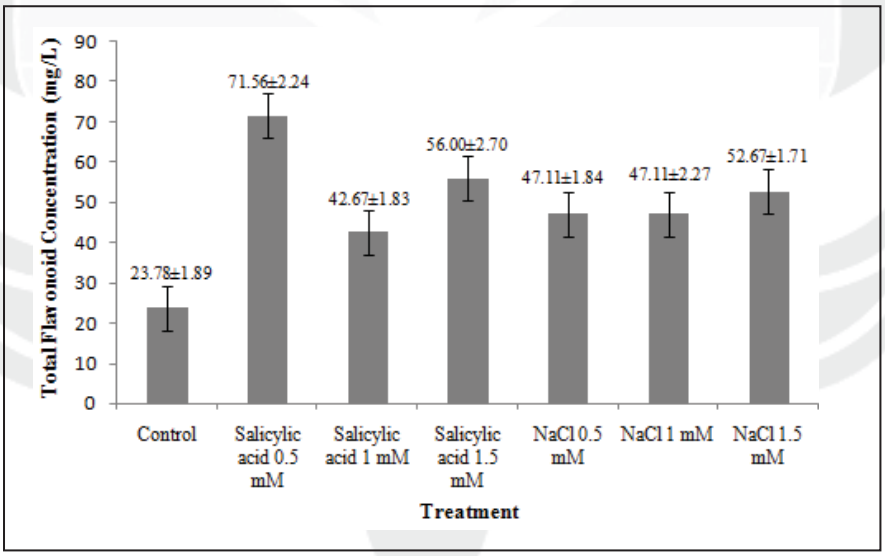

Figure 5. Average concentration of total flavonoid

The highest average concentration of total flavonoid (71.56±2.24 mg/L) was 0.5 mM salicylic acid treatment whereas the lowest total flavonoid concentration (23.78±1.89 mg/L) was the control. The total flavonoid concentrations in each replicate are shown in Table 1. Data were analyzed using ANOVA One Way with the acquisition of P<(0.05) which means that the concentration of elicitor (salicylic acid and NaCl) significantly influenced the total flavonoid content. Furthermore, Tukey test was conducted to determine the significance of the treatments.

Table 1. Total Flavonoid Concentration

Treatment	Total Flavonoid Concentration (mg/L)			
	R1	R2	R3	Average±SE
Control	19.33	16	36	23.78±1.89[b]
Salicylic acid 0.5 mM	56	72.67	86	71.56±2.24[a]
Salicylic acid 1 mM	42.67	32.67	52.67	42.67±1.83[ab]
Salicylic acid 1.5 mM	32.67	59.33	76	56.00±2.70[ab]
NaCl 0.5 mM	49.33	56	36	47.11±1.84[ab]
NaCl 1 mM	29.33	56	56	47.11±2.27[ab]
NaCl 1.5 mM	46	62.67	49.33	52.67±1.71[ab]

The same letter in the column shows no significant difference by Tukey's test with a confidence level of 95%. R1: Repetition 1; R2: Repetition 2; R3: Repetition 3; SE: Standard Error.

The total flavonoid concentration of 0.5 mM salicylic acid treatment was significantly different from control. Salicylic acid triggers the increase of genes expression involved in flavonoid biosynthesis, thus accumulating the total flavonoid (e.g. *CHS*, *CHI*, *F3H* and *ANS*) (Tounekti *et al*., 2013). Furthermore, salicylic acid may trigger a signaling pathway that regulates the expression of genes encoding enzymes associated with phenylpropanoid pathway, e.g. chalcone synthase, the first enzyme for producing flavonoid compounds in the phenylpropanoid pathway (Ghasemzadeh *et al*., 2012).

The total flavonoid concentration of salicylic acid treatment decreased at concentration of 1 mM and increased at concentration of 1.5 mM. Secondary metabolites of most plants are produced during the stationary phase (Malik *et al*.,, 2013). Plant cells treated by 1.5 mM salicylic acid were presumed to have longer stationary phase than plant cells treated by 1 mM salicylic acid. Therefore, the production of total flavonoid was also higher in the former. High concentration of salicylic acid may also activate pathways of other secondary metabolites, such as terpenoids (Tounekti *et al*., 2013).

The total flavonoid concentration treated by NaCl 1.5 mM was higher than NaCl 0.5 mM and 1 mM, although the results of these treatments were not significantly different. The result obtained was in accordance with the study conducted by Hussein and Aqlan (2011) in which the addition of NaCl increased the total flavonoid production of *Trigonella foenum-graecum* L. NaCl causes perturbation of ionic steady state not only for Na^+ and Cl^- but also for K^+ and Ca^{2+} in the plant cells. External Na^+ negatively impacts intracellular K^+ influx, attenuating acquisition of this essential nutrient by cells. High NaCl causes cytosolic accumulation of Ca^{2+}, a signal stress response of cells (Hasegawa *et al*., 2000). Plant subjected to environmental stresses produce reactive oxygen species (ROS). ROS can damage vital cellular macromolecules, e.g. proteins, DNA, and lipids. To reduce excess ROS, plants have developed an antioxidant defence system, which comprises enzymatic and non-enzymatic components. Non-enzymatic response is mainly linked with overproduction of phenolics, including flavonoid (Swiecha, 2015).

3.4. The Effect of Elicitors on Flavonol Content

The flavonol content of the samples was measured using quercetin as standard. Standard curve of quercetin is presented in Figure 6.

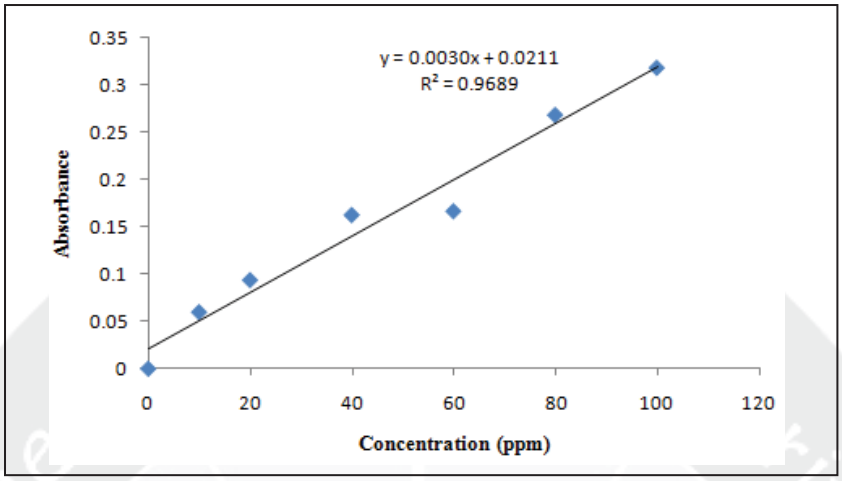

Figure 6. Standard curve Quercetin on Wavelength 415 nm

Regression equation y = 0,0030x + 0.0211 shows a linear relationship between absorbance and concentration measurement with a correlation coefficient 0.9689 (Figure 6). Figure 7 presents the results of the average concentration of flavonol in each treatment after elicitation treatment.

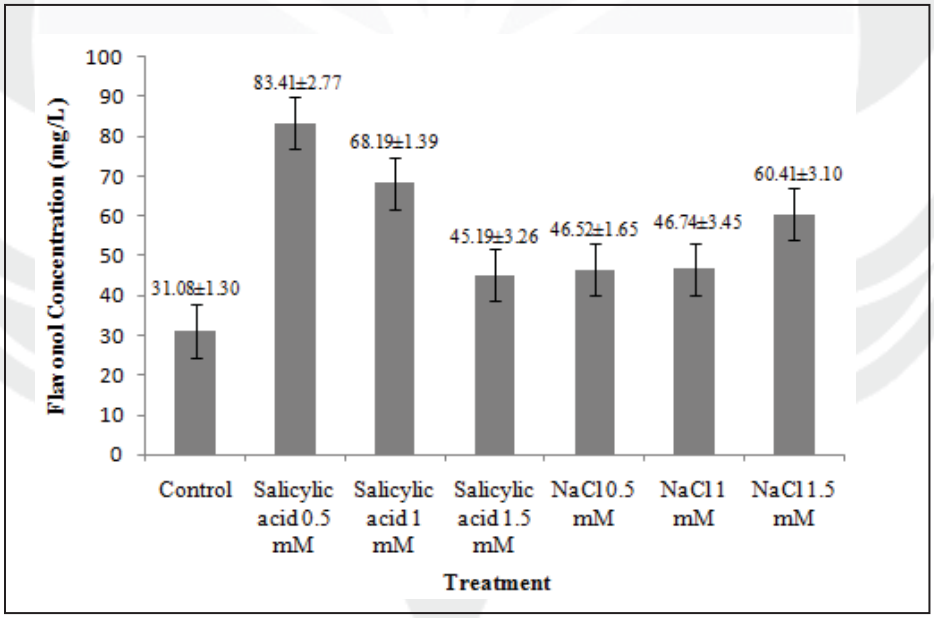

Figure 7. Average concentration of flavonols

The highest average concentration of flavonol (83.41±2.77 mg/L) was treated by 0.5 mM salicylic acid whereas the lowest flavonol concentration (31.08±1.30 mg/L) was the control. Flavonol concentrations in each replicate are shown in Table 2. Data were analyzed using ANOVA One Way with the acquisition of P>(0.05) which means that the concentration elisitor (salicylic acid and NaCl) did not significantly influenced the flavonol content.

Table 2. Flavonol Concentration

Treatment	Flavonol Concentration (mg/L)			
	R1	R2	R3	Average±SE
Control	36.63	26.63	29.97	31.08±1.30
Salicylic acid 0.5 mM	83.30	57.97	102.97	83.41±2.77
Salicylic acid 1 mM	62.30	68.30	73.97	68.19±1.39
Salicylic acid 1.5 mM	58.97	8.63	67.97	45.19±3.26
NaCl 0.5 mM	37.63	48.30	53.63	46.52±1.65
NaCl 1 mM	68.63	5.63	65.97	46.74±3.45
NaCl 1.5 mM	34.30	91.30	55.63	60.41±3.10

R1: Repetition 1; R2: Repetition 2; R3: Repetition 3; SE: Standard Error.

The highest flavonol concentration was treated by 0.5 mM salicylic acid (83.41±2.77 mg/L). Badrhadad *et al*., (2013) mentioned that salicylic acid is recognized as a signal in plat defense response for secondary metabolite production in plant cells. Salicylic acid can induce regulation of genes related to the biosynthesis of secondary metabolites in plants (Taguchi *et al*., 2001; Tounekti *et al*., 2013). Furthermore, Fang and Huang (2013) stated that salicylic acid can enhance the biosynthesis of flavonol by increasing the activity of flavonol synthase (FLS) that plays a role in the formation of flavonol from dihidroflavonol in the phenylpropanoid pathway. Flavonol concentration decreased at concentration of 1 mM and 1.5 mM salicylic acid (68.19±1.39 and 45.19±3.26 respectively).

The flavonol concentration treated by 1.5 mM NaCl was higher than NaCl 0.5 mM and 1 mM. Flavonol concentration increased with the increase of NaCl concentration. These results are in accordance with the study demonstrated by Chen *et al*., (2014) in which high salinity can stimulate the production of secondary metabolites in some cell suspension cultures, but have an impact on cell growth and ultimately inhibit cell viability. NaCl addition in the medium causes stress and generally induces the formation of ROS. Therefore, plant cells produce anti-oxidative defense system, e.g. flavonol production (Chen *et al*., 2014).

4. CONCLUSION

This study demonstrated the effect of elicitors (salicylic acid and NaCl) on total flavonoid and flavonol content in *M. oleifera* cell suspension culture. Hence, it was concluded that the total flavonoid content was influenced by the concentration of elicitors (salicylic acid and NaCl). However, the flavonol content was not influenced by the concentration of elicitors (salicylic acid and NaCl). The highest content of total flavonoid was the treatment by 0.5 mM salicylic acid and 1.5 mM NaCl of 71.56±2.24 mg/L and 52.67±1.71 mg/L respectively. On the other hand, the highest content of flavonol was the treatment by 0.5 mM salicylic acid and 1.5 mM NaCl of 83.41±2.77 mg/L and 60.41±3.10 mg/L respectively.

5. ACKNOWLEDGEMENT

The authors are grateful to *Deutscher Akademischer Austauschdienst* (*DAAD*) / *German* Academic Exchange Service for funding and supporting this study. Special

thanks are also given to Mrs. Kristanti Indah Purwani, S.Si., M.Si. and Mrs. Indah Trisnawati Dwi Tjahjaningrum, M.Si., Ph.D. for reviewing the manuscript; Nur Shabrina, S.Si. for proof-reading the manuscript; and also local people in Poteran Island, Sumenep Regency, Madura, Indonesia for the help in the field. We thank to Student Research and Development Team (SR&DT) Batch 1, Laboratory of Botany Group, Moringa Team (Yunita Permanasari, Hanum Laila Rizkia, and Alfi Rahmawati), Biomaterial and Enzyme Technology Research Team (2014/2015).

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Development of *Cecal Coccidiosis Immunized Chicken* for Controling on *E. tenella* Infection by Administration of attenuated *E. tenella*

*Muchammad Yunus[1], Endang Suprihati[1], Suryanie Sarudji[2]
[1]Department of Veterinary Parasitology, Faculty of Veterinary Medicine,
University of Airlangga, Surabaya, Indonesia,
[2]Department of Veterinary Microbiology, Faculty of Veterinary Medicine,
University of Airlangga, Surabaya, Indonesia
muhyunus_99@yahoo.com

1. INTRODUCTION

Coccidiosis is a disease that causes a lot of huge economic losses for poultry breeders, among others because the mortality rate is quite high, growth retardation, decreased egg production and feed efficiency and high treatment costs and labor costs [1]. One of pathogenic *Eimeria* species is *E. tenella* that cause cecal coccidiosis. The fact that there is the poultry industry, the control of coccidiosis is mostly associated with the use of routine anti-coccidiosis (coccidiostat). To overcome these conditions the option to conduct immunization approach (immunization) against chicken in controlling the disease more intensive and planned indispensable. Immunization against chickens to control coccidiosis more promising in avoiding or decreasing the reliance on the use of chemicals and other coccidiostat The previous study already constructed attenuated *E. tenella* isolate by serial passages in naïve chicken. The present study is to prove reduction of pathogenicity of those isolate.

2. METHODS

Twenty broiler chickens at two weeks old were divided into two groups, with each group consisting of 10 individuals. The first group was a positive control group, a group of chickens were administered with unattenuated *E. tenella* at a dose of 1 x 10^4 (UG). The second groups of chickens were inoculated with attenuated *E.tenella* at the same dose of 1 x 10^3 (AG). From both groups were observed characterization include: testing reproductive potential of parasite (oocyst production), pathogenicity (clinical symptom and the productivity of chickens administered with unattenuated and attenuated *E. tenella*, respectively). The data of both groups were analyzed t test.

3. RESULTS AND DISCUSSION

The pattern of daily oocyst production in chickens of each group (UG and AG) during infection was seen different (Fig. 1). In the first group (UG), oocysts were first seen at feces 168 hours after inoculation and then reached a peak of 240 hours after inoculation and decreased drastically to 288 hours and 312 hours after inoculation had no detectable oocysts in the feces. The temporal pattern of oocyst output per day confirms those previously reported [2] with this isolate of *E. tenella*. Oocyst firstly appeared on the 7 days pi, the reached peak on the 10 days pi before numbers declined rapidly and the fewest oocysts were detected on 12 days pi. On the first

group (UG), clinical signs such as blood diarrhea, appetite, lethargy were seen 5 days post infection with a prepaten period of 168 hours, whereas the second group showed consistency and colour feces appeared normal without any changes. Feed consumption, body weight gain and feed conversion were significantly different among groups (Table 1). There was significantly difference at the mean of total oocyst production between the first group and the second group ($p<0.01$) (Fig.2). Basically, the same pattern of daily oocyst output was seen in both groups.

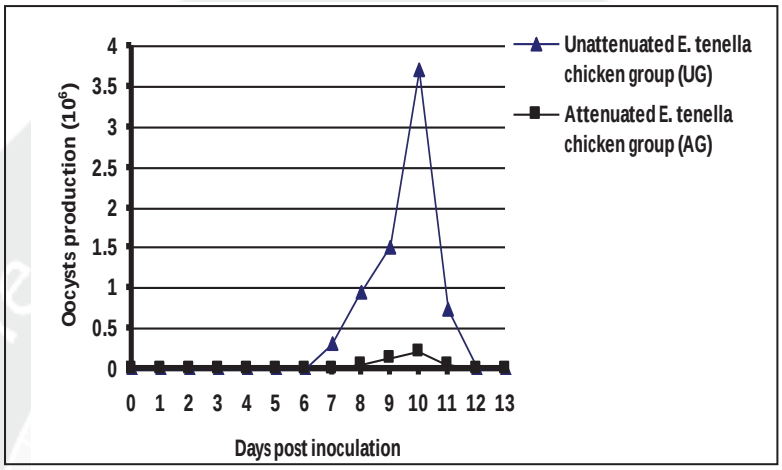

Figure 1. Pattern of daily oocyst production of Unattenuated *E. tenella* chicken group (UG) and Attenuated *E. tenella* chicken group (AG).

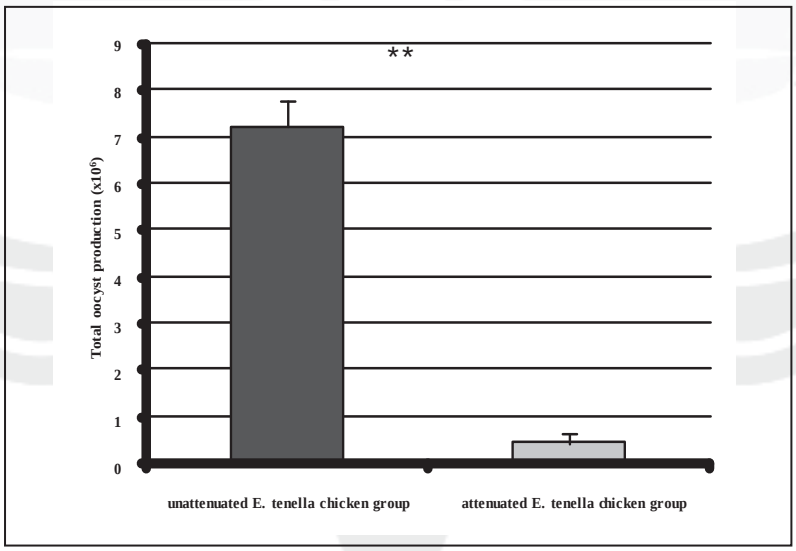

Figure 2. The comparison of total of oocysts production of Unattenuated *E. tenella* chicken group (UG) and Attenuated *E. tenella* chicken group (AG). **, p<0.01

Table 1. The Comparison of Effect of Administration of Unattenuated *E. tenella* and Attenuated *E. tenella* Oocysts on Productivity aspects of broiler (Feed Consumption, Body Weight Gain and Feed Conversion) during 6 weeks

Treatmen Groups	Fcs	Rw	ECv
		Means+SD	
UG	658.7^{b} ± 98.81	316.7^{b}±77.63	2.08^{b}±0.51
AG	922.2^{a} ± 370.58	506.7^{a} ±62.74	1.82^{a}±0.38

Superscript different same column represented significantly

The number of intracellular parasitic stages present within the cecal epithelia were in largest number at approximately 7 to 8 days pi especially for unattenuated *E. tenella*, parent strain [3] so that oocyst production of unattenuated *E. tenella* administered chicken group (UG) was very significantly higher than attenuated *E. tenella* administered chicken group (AG). Field strain of parasite isolated show high pathogenicity, well developed and completely life cycle perform in hospes. While capacity of development and proliferation of attenuated *E. tenella* is significantly reducted by serial passages in naive chicken. Therefore, serial passages is proved to reduce pathogenicity and ability of development and multiplication in the hospes .

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The Character of Biogas Fermentation on Simple Sugars by *Enterobacter ludwigii* Mutants

*Mariana Wahjudi, Bryant Roossel Macciano, Junus Rangan and Mangihot Tua Goeltom[1]
[1]Department of Purification and Molecular Biology, Faculty of Biotechnology,
University of Surabaya, Surabaya, Indonesia
mariana_wahyudi@staff.ubaya.ac.id

Abstract

Bio-hydrogen is one of promising alternative energy nowadays. Pure-wild type bacteria commonly produce biohydrogen in a low level. In this research, we aim to carry on transposon mutagenesis of *Enterobacter ludwigii*, the isolate obtained from sediments of Kalimas River Surabaya, to produce more biohydrogen. The second aim was to characterize the properties of wild type and its mutants in biogas fermentation growing on simple sugars. Fermentations were performed anaerobically at 37^{o}C and shaken at 200 rpm under dark condition. There were two target conjugant obtained, mutant A24-16 and B24-37. The *E. ludwigii* wild type, mutant A24-16 and B24-37 grew on M9 minimal medium containing glucose, xylose or arabinose without any differences between strains. All strains grew faster on glucose and arabinose compare to growth on xylose. Biogas was appeared since late logarithmic phase for glucose and arabinose or early stationary phase for xylose. The mutant A24-16 and B24-37 produced more biogas on all these sugars compared to its wild type. At the end of fermentation, sugars content of the cultures were diminished, which were determined as total reducing sugars. The pHs were decreased. It gave impressions that the sugars were consumed. The biogas produced during fermentations contained hydrogen. Ethanol and acetate were detected in the cultures at the end of fermentation. It can be concluded that the transposon mutagenesis of *E. ludwigii generated* two mutants, A24-16 and B24-37. The wild type and both mutants' cells could grow and produce biohydrogen on glucose, xylose or arabinose substrates in M9 minimal medium.

Keywords: *Enterobacter ludwigii*, mutant A24-16 and B24-37, biohydrogen, glucose, xylose, arabinose.

1. INTRODUCTION

Nowadays, the availability of conventional energy stock in the world is delimited, especially petroleum, is only enough for 30 – 50 years (Nugroho, 2006). In the other side, the demand is increased 7% each year (Kementerian Energi dan Sumber Daya Mineral, 2012). If there is no effort to decelerate, it was predicted that this energy sources will be deprived in 18 years. In spite of its usefulness, the utilization of fossils and petroleum for energy can cause pollution which then resulted to global warming.

The global warming and energy deprivation issues push the experts to seek the alternative, renewable and eco-friendly energy. Bio-hydrogen, H_2, is one of promising alternative energy nowadays. The energy generated by hydrogen is large without

gaseous pollutant during its combustion; the end-product during energy creation is merely water (Vazquez *et al*., 2008).

Bio-hydrogen is commonly produced by obligate anaerobes such as *Clostridium* (Debabrata, 2001), facultative anaerobes such as *Enterobacter* (Tanisho, 1998), and aerobic bacteria, such as *Alcaligenes* and *Bacillus* (Vazquez *et al.*, 2008). However, a pure-wild type strain commonly produces bio-hydrogen in a low level.

Most widely-studied model strain for bio-hydrogen producers is *Enterobacter aerogenes* (Tanisho *et al*., 1983; see review by Zhang *et al.*, 2011). Many members of the genus *Enterobacter* have been investigated regarding its ability to produce H_2 (review by Zhang *et al.*, 2011). *Enterobacter sp.* possess a supremacy characters over other genus as H_2 producers due to their fast growing, wide substrate range, resistance to oxygen and resistance to pressure of high H_2 concentration (Ito *et al*., 2004). Other *Enterobacter sp.* studied for hydrogen production include *Enterobacter asburiae* (Shin *et al*., 2007), *Enterobacter cloacae* (Nath *et al*., 2006).

Although fermentation technology of E. aerogenes enhanced hydrogen production, bioengineering method was more effective to increase the biohydrogen production. As investigated by Rachman *et al.,* (1997), chemically-random mutation of *E. aerogenes* generated *E. aerogenes* AY2 strain with double productivity. Second mutation using *ethyl methane sulfonate* (EMS) resulted to *E. aerogenes* ADH43 mutant with higher H_2 yield than AY2 strain (Said *et al*., 2007).

In this research, we used *Enterobacter ludwigii*, a local isolate obtained from river bank of Kali Mas River, Surabaya. This strain has never been investigated its features others than Cu resistance characteristic (Wiradinata, 2013). In order to exploited the strain and enhance the hydrogen production, we attempted to mutate the strain using transposon cassette reside on pUTmini-Tn5-luxCDABE-Km plasmid. Because the transposition would be occurred randomly, it is important to investigate the characteristic of the hydrogen fermentation by mutants, at first by growing on simple sugars.

Main carbon sources for hydrogen producer bacteria are simple sugars (Kapdan, 2005), rarely from complex compounds such as amylum, celluloses or others big polymers. To avoid the affordability of fermentation substrates with the food-and-feed life stocks requirements it is important to consider the usage of forestry and agricultural waste. Forestry and agricultural waste are available abundantly in Indonesia, mainly consists of celluloses and hemicelluloses. Upon hydrolyzes of all these components, monomers are released, frequently to be glucose, xylose and arabinose, etc.

The aim of this research was to characterize the properties of *Enterobacter ludwigii* wild type and transposon mutants in biogas fermentation growing on simple sugars. In this report we presented the possibility of E. ludwigii cell to be mutated to produce biogas during fermentation on simple sugars, especially on glucose, xylose and arabinose.

2. METHODS

2.1 Chemicals and Strains

Media for culturing bacterial cells were Luria-Bertani (LB) (Merck), Simmon's Citrate

Agar (Merck), and Phenol Red broth (Merck). For hydrogen fermentation, medium M9 was used, with compositions as described by Sambrook *et al*., (2001) containing 0.1% yeast extract and 0.4% of simple sugar i.e. glucose, xylose or arabinose. If it was needed, kanamycin (Sigma) was added at final concentration of 30 µg/ml. The transposon donor cells were *Escherichia coli* S17-1λpir bearing Tn5 transposon on the pUTmini-Tn5-luxCDABE-Km plasmid, and as a recipient was *Enterobacter ludwigii*, culture collection, obtained from

2.2. Procedures

Mutagenesis process. The *E. ludwigii* were **freshly** grown overnight in **LB** and then conjugation with Escherichia coli S17-1λpir bearing the Tn5 transposon in pUTmini-Tn5-luxCDABE-Km plasmid (de Lorenzo *et al*., 1990). The selection process was performed by growing conjugant on agar plates containing 100 ug/mL of kanamycin, firstly on Luria-Bertani agar and then Simmon's Citrate Agar. Finally, conjugants was selected by the ability to produce more gas however less acids compared to original cell, the *E. ludwigii* wild type cells (Rangan, 2014).

Bacterial growth curve. This step was performed to explore the cells ability to grow and to produce biogas during fermentation on the simple sugars. The biogas fermentation of *E. ludwigii* wild type and mutants' cells was performed in 30 mL of M9 medium containing 0.4% glucose, xylose or arabinose in 100 mL-serum bottles. Fermentation bottles were saturated with N_2 gas following the inoculation. The cultures were shaken at 200 rpm in 37^{o}C incubator, under dark condition. Samples were taken at interval time for measuring the cells density, biogas volume, pH, total reducing sugars and ethanol and acetic acid quantification.

Cells density was determined using spektrofotometer (Uvikon) at λ_{600}. The volume of biogas was measured using glass flask after flowing it through CaOH solution in a respirometer. The pH culture was detected using pH meter (Accumet® BASIC). The presence of hydrogen was detected by fuel cells. The total reducing sugar was measured using dinitrosalicylic acid (DNS) method according to Miller (1959).

3. RESULTS AND DISCUSSION

Mutagenesis result

Transposon mutagenesis of *Enterobacter ludwigii* was done to improve its ability to produce biohydrogen. The *Enterobacter ludwigii* used in this study is a bacterium isolated from river bank of Kali Mas, Surabaya. This bacterium has a characteristic of being resists toward up to 100 ppm Cu.

Although, the ability of this bacterium has never been known yet, it was predicted that it could be improved by bioengineering technology. *Enterobacter* in common is capable to produce biohydrogen.

Screening and selection were carried on to discrete the presence of E. ludwigii conjugant which confer the ability to produce biohydrogen. Since the process is complex involving many genes, the mutation is quite complicated to be done by site directed mutagenesis. Selection is based on the deficiency in acids production and increasing biogas production. There were hundreds of conjugant obtained and underwent selection processed; two of them, A24-16 and B24-37, showed the lowest acids production however highest gas production. Although both mutants produced

biogas more than the wild type however it is important to determine whether the transposition changed its character in carbohydrates metabolism.

Growth of E. ludwigii cells *on simple sugars*

In M9 minimal medium containing 0.4% of glucose, xylose or arabinose, all of E. ludwigii cells grew well, without any significant differences between wild type and mutants. The result exhibited that it might be no defect in metabolism of these sugars, caused by transposition, (Figures 1). All of cells grew faster in arabinose and glucose than in xylose. Arabinose seems to be the most suitable carbon sources for cells metabolism. Logarithmic phase were achieved faster on arabinose than on glucose. It might be caused by intrinsic factor of this genus. Study by Stringer *et al.,* (2013) revealed that family member of *Enterobacteriaceae* have a conserved regulatory network comprising 10 genes associated with arabinose transport and metabolism. Xylose might be metabolized slowly because this C5 sugar undergone catabolism through pentose-phosphate pathway into gliseraldehid-3P which then entered the glycolysis pathway (Bi, 2009). However, both glucose and arabinose as C6 sugars entered directly into glycolysis pathway.

In general, it can be said that no differences in cells growth between wild type and both mutants, except the growth of B24-37 on xylose showed longer adaptation time.

Biogas production

During cells growth, biogas was produced by all cells since late logarithmic phase for glucose and arabinose or early stationary phase for xylose (Figure 1). It means that biogas was produced by cells as secondary metabolites, as revealed by other studies of *Enterobacter* sp. (Shin *et al.,* 2006).

Although the growth rate of both mutants were not differ significantly, both mutants produced more biogas compared to wild type on these simple sugars (p value < 0.05%).

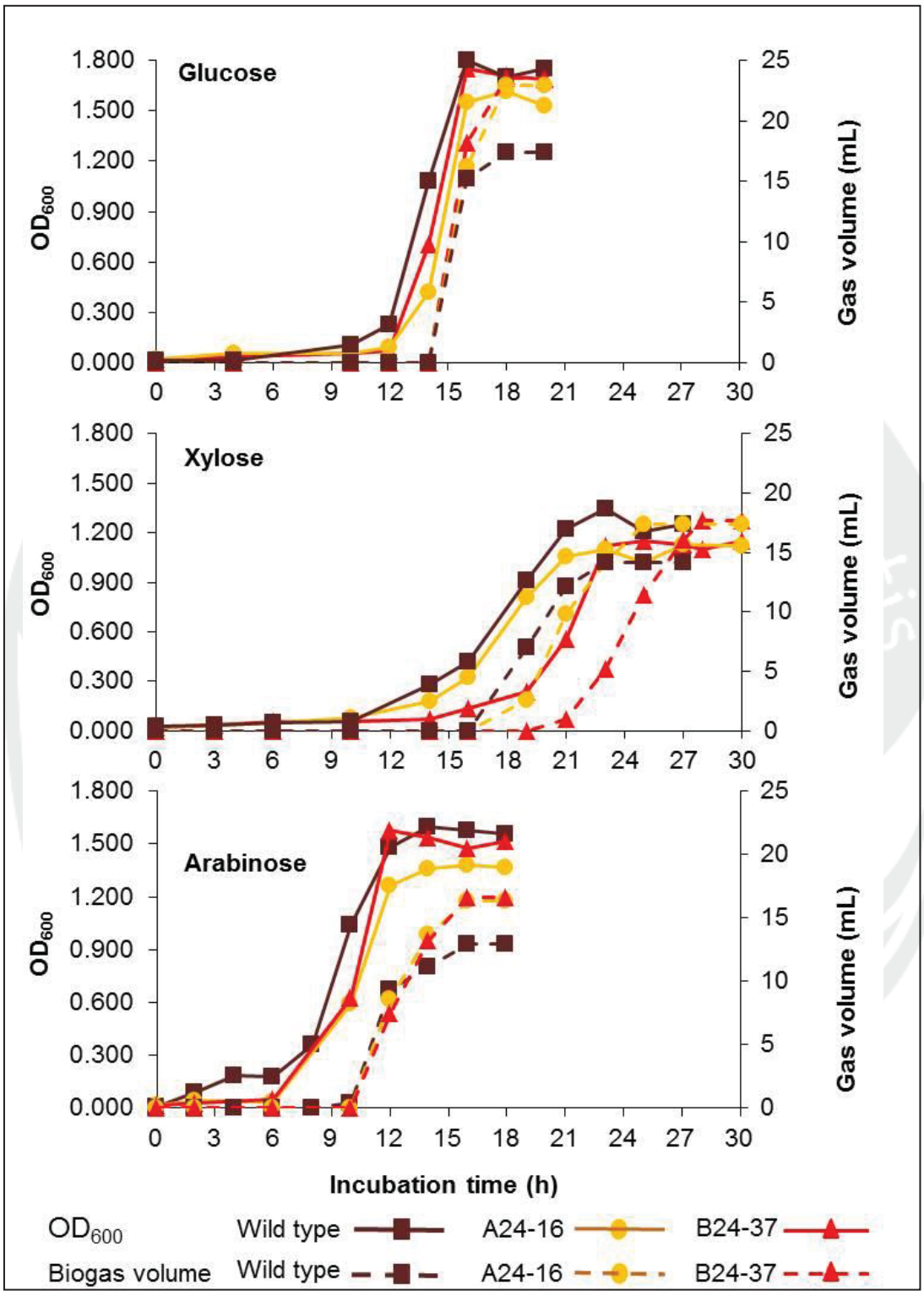

Figure 1. Biogas production during growth stages of *E. ludwigii wild* type (blue), mutant A24-16 (pink) dan B24-37 (yellow) cells in M9 medium containing 0.4% of glucose, xyose and arabinose.
Cells were cultured anaerobically at 37°C and 200 rpm.

The volume of accumulated biogas by both mutants on glucose is approximately 23 mL, whilst wild type is ~17 mL. The volume were higher compared to biogas in xylose or arabinose fermentations.

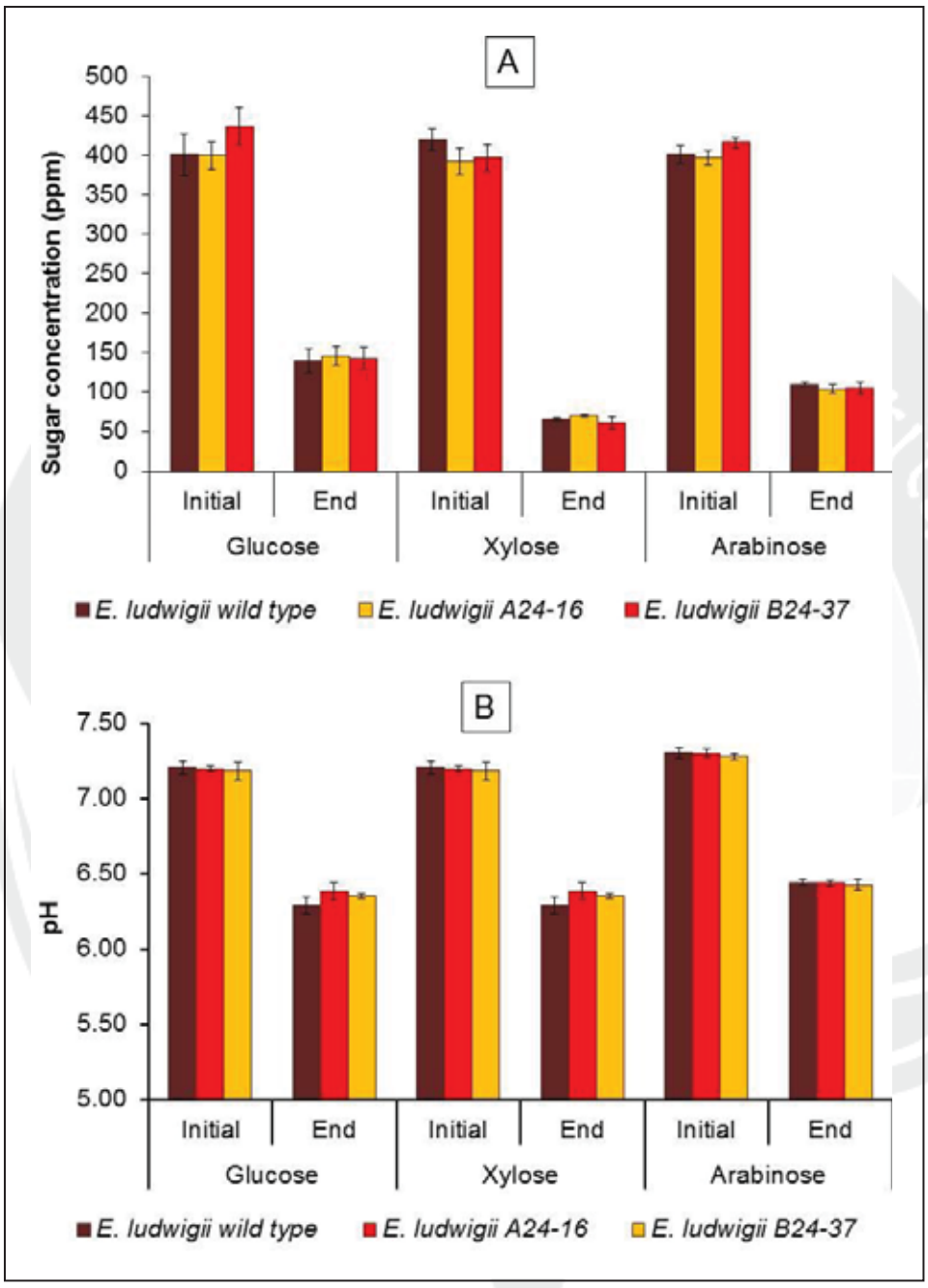

Figure 2. Biogas production during growth stages of *E. ludwigii wild* type (blue), mutant A24-16 (pink) dan B24-37 (yellow) cells in M9 medium containing 0.4% of glucose, xyose and arabinose. Cells were cultured anaerobically at 37°C and 200 rpm.

The amount of biogas could not be accomplishing higher than these level until the end of fermentation. It might be caused by diminishing sugars in the medium.

Determination of total reducing sugar of each fermentation culture showed that all of the sugars were deprived at the end of fermentations (Figures 2A). To verify the relation between biogas production and sugars metabolism, determination of sugars content and pH value we performed at the end of fermentation. At the end of fermentation, total sugars in all fermentation bottles were decreased (Figures 2A) as well as the pHs (Figure 2B). The initial pHs of cultures were 7.1-7.3 and became 6.2-6.4 at the end of fermentation.

Hydrogen detection

Shin *et al.,* (2006) investigated the content of biogas produced by *Enterobacter asburiae*. The strain produced hydrogen via formate-lyase pathways. Furthermore, the metabolic flux of well-studied *E. aerogenes* strain in anaerobic fermentation showed that hydrogen can be produced from several intermediate compounds and pathways (Zhang *et al.*, 2011).

In accordance to pH decrement of the cultures, the existence of ethanol and acetate in end-cultures were determined. This step was conducted to investigate whether the biogas production was generated via the ethanol pathways. The determination ethanol and acetate was detected in all cultures at the end of fermentations (Table 1). However, confirmation has to be done to compare the amounts of both compounds between wild type strains and the mutants.

Table 1. The determination of ethanol and acetate in *E. ludwigii* cultures at the end of fermentation on glucose, xylose and arabinose substrates

		Wild type	A24-16	B24-37
Ethanol (%)	Glucose	0.054±0.037	0.100±0.042	0.078±0.029
	Xylose	0.064±0.015	0.44±0.017	0.054±0.004
	Arabinose	0.374±0.48	0.107±0.03	0.098±0.032
Acetate (%)	Glucose	0.83±0.111	0.03±0.019	0.074±0.071
	Xylose	0.033±0.029	0.044±0.01	0.054 ± 0.003
	Arabinose	0.109 ± 0.071	0.186 ± 0.015	0.14 ± 0.038

The metabolic pathways of hydrogen production by this strain and both mutants have not been investigated yet. This mutation was initial investigation that leads out better understanding of hydrogen production by this strain.

Although the volume of biogas exhibited higher in mutants compared to wild type, it is important to verify the hydrogen content in it. Using *Fuel Cell*, the hydrogen in biogas was detected which drive the voltmeter of this apparatus to move. The presence of hydrogen, simultaneously with ethanol and acetate excavated the facts that the mutation process was succeeded as expected (Pandey *et al.*, 2013; del Campo *et al.,* 2013; Abreu *et al.,* 2012; Zhang *et al.*, 2011; Zhang *et al.*, 2009; Shin *et al.*, 2007); Shin *et al.*, 2006).

4. CONCLUSIONS

It can be concluded that, the transposon mutagenesis of *Enterobacter ludwigii generated* two mutants, A24-16 and B24-37. The *Enterobacter ludwigii* wild type and both mutants could grow and produce biohydrogen on glucose, xylose or arabinose substrates in M9 minimal medium.

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Selection of Natural Antimicrobial in Poteran Island Based Ethnobotany

Fanindya Citra Ayu Ardian[1], Isdiantoni[2], Maharani Pertiwi Koentjoro[3*], and Endry Nugroho Prasetyo[1]
[1]Biotechnology Laboratory, Department of Biology, Institut Teknologi Sepuluh Nopember, Gedung H 1st floor, Kampus ITS Keputih Sukolilo Surabaya, 60111 Indonesia
[2]Agrobusiness Department, Faculty of Agriculture, Universitas Wiraraja Jalan Raya Sumenep-Pamekasan Km. 5 Patean, Sumenep, 69451, Indonesia
[3]Laboratory of Environmental Microbiology, Department of Biological and Environmental Science, Faculty of Agriculture - Shizuoka University & Structural Biology Research Center, Inter-University Research Institute Corporation - High Energy Accelerator Research Organization (KEK), Tsukuba-Ibaraki, Japan
*Corresponding author email address: maharanipertiwi@yahoo.com

Abstract

Antibiotics treatment using synthetic chemicals are less efficient due to bacterial resistance. The use of plants extract as an agent has been one of approaches for less using synthetic chemicals antibiotics. Further, ethnobotany study which is taken from ethnic experiences curing the diseases would be easier to collect information concerning antimicrobials extracted from the plant. Therefore this study aim to explore the antimicrobial from plants like *Azadirachta indica*, *Piper retrofractum*, *Strychnos* ligustrina, *Musa acuminata*, and *Lannea coromandelica* by referring the local people experience using for remedy *againts Staphylococcus aureus, Salmonella typhi, and Candida albicans.* Collecting data of etnobotany with interview to local people in Poteran island randomly. Extraction using etil asetat with maseration method. Antimicroba activity using extract with concentration 100% using diffusion method dan the dilution method using concentration of *1000*µg/ml *,500*µg/ml *,250*µg/ml*, 12*5µg/ml, and *62.5*µg/ml. The result of this research show that all of tester plant have all same fitochemistry like flavonoid, alkaloid, saponin, steroid. The antimikroba activity with diffusion method forming the largest clear zone in leaves mimba extract of 14mm in bacteri *S.aureus*, 17mm in *Salmonella typhi*, and 7.67mm in *Candida albicans*. Value of MIC in mimba leaves extract is 1000µg/ml to *S.aureus* dan *Salmonella typhi,* while for *Candida albicans* in concentration 500µg/ml. Value of MBC just in Candida *albicans* with concentration 1000µg/ml.

Keywords: Antimicroba, ethnobotany, plants extract

1. INTRODUCTION

Various kind of diseases caused by microbes pathogen, common to find in tropical countries including in Indonesia [1]. Some of microorganism pathogen such as *Escherichia coli* causing infection of the intestines, *Staphylococcus aureus*, and *Candida albicans* causing infection in skin [2]. The prevention and treatment of disease using synthetic antibiotics less effective because it could trigger increase kinds of bacteria that is resistant to the antibiotics and have price is very expensive

[3]. The use of traditional antimicroba is an alternative synthetic substitute because antibiotics has side effect relatively few [4]. The use of plant as a source of natural antimicroba agent has been known for a long time and hereditary [5]. In Indonesia, the plant that serves as a source of natural antimicroba is very empirical [6], so that the need of the study of links between experience the local community for generations to use of natural effect antimicroba as a drug. Etnobotany approach is one method to know and efficacy studies of plants containing antimicroba [7].

For example in the community of Sumenep, Madura use plant to antimicroba Mimba leaf is an effective hinder the growth of *Saalmonella typhosa* because it contains a compound Azadiractin (8). Studi of etnobotany exploration natural antimicroba has not been done in Poteran island. Therefore information etnobotany from habits of the community on the Poteran island making use of local plants as a medicine for research to determine new natural antimicroba that can be developed as a source of alternative for change the synthetic antibiotics.

This research to know contents phytochemical from leaf of daun *Azadirachta indica*, *Piper retrofractum* Vahl, *Strychnos ligustrida* Bl, *Andrographis paniculata* Nees, *Lannea coromandelica* and bark of *Musa sp* and to test rate of force antimicroba from plant extract to *Staphylococcus aureus*, *Candida albicans*, and *Salmonella* sp and diffusion, MIC and MBC method.

2. METHODS

2.1 Data Collection of Study Ethnobotany

Collecting data of study ethnobotany by interview regularly and observation directly. Responden from local people in Poteran island randomly.

2.2 Extraction with Maseration Method

Extraction use etil acetat. Sampel leaf use dry wind about 7 days. Not only leaf but also bark of *Musa sp* to. Maseration of bark *Musa sp* about 2 days then remaserasion 2 days again to get more extract. Extraction for 100 gram sampel leaf *A. indica*, *P. retrofractum* Vahl, *S. ligustrida* Bl, *A. paniculata* Nees, dan *L. coromandelica* 100 gram with 750 l etil acetat about 2 days. Then, continue step remaseration is residu maseration again with 250 ml etil acetat about 2 days. Filtrate from first and second extraction to combined, the solvent is evaporates using vaccum rotary evaporator then continue with an oven with temperature of 40^0C for 15 minutes until tickened.

2.3 Components Identification

Phytochemical test have 4 testing, there are saponin test, steroid test alkaloid test and flavonoid test. This testing to know what the chemical compound. Sampel aquaous from 300mg extract add 25 ml etil acetat.

a. Saponin test
   Sampel aqueous for 5 ml in reaction tube then shake it for 10 minutes. If there are have foam about 1-10cm, it can identification have saponin.
b. Steroid test
   Steroid testing based Lieberman-Burchad. Sampel solution about 2 mlin porselin dish. And then add 0,5 mL klorofrm, kemudian 0,5 mL acetat acid anhidrat and 2

mL sulfat acid. Formed brown ring or violet showed have triterpenoid, if formed blue-green ring showed have steroid.

c. Alkaloid test
   Alkaloid test based Mayer, Wagner and Dragendorff method. Tickned extract about one spatula add 1ml klorofom, add 2ml HCl and 2 sprinkel Dragendroff. Positif result will have yellow-sediment or yellow-ring
d. Flavonoid test
   Flavonoid test use sample aquaous about 1ml and add 1ml NaOH. Result positif will get yellow aqueous

2.4 Antibacteria Disk Duffusion Test

S.aureus and *S.typhii* inoculating about 1-2 ose in reaction tube where have NaCl 0,85% aquous until get turbidity based 0,5 Mc.Farland (same with total bacteria 1,5 x 10^8 CFU/ml) [9]. Antibacteria test use extract from leaf of *A. indica*, *P. retrofractum* Vahl, *S. ligustrida* Bl, *A. paniculata* Nees, *L. coromandelica* and bark of *Musa sp* with disk diffusion test method. Disk paper put in Petri dish with steril pinset. Concentration bacteria wiped all in Petri disk where have Mueller Histon Agar (MHA) medium. Disk paper (whatman no 1) have diameter 10mm where have extract, put in that medium (bacteria medium). And then incubation that medium at room temperature. Checking that test for 24 and 48 hour. Wide of persue area have classification like tabel 1 (10).

Table 1. Clasification persue responses [10]

Clear zona diameters	Responses
>20mm	strong
16-20mm	rather
10-15mm	weak
<10mm	nihil

2.5 Antifungi of Disk Diffusion Test

Before disk diffusion test, make mikroba suspension based o,5 Mc.Farland. Take *C.albicans* about one ose and inoculating that in NaCl 0,85% until have turbidity same with 0,5 Mc.Farland (10^8 CFU/ml) [11]. Antifungi testing use extract from leaf of *A. indica*, *P. retrofractum* Vahl, *S. ligustrida* Bl, *A. paniculata* Nees, *L. coromandelica* and bark of *Musa sp* with disk diffusion test. Disk diffusion use spread plate methods. First step pour the PDA medium in steril Petri disk about ±12 ml and waiting until to be solid. After be solid, give 1ml *C.albicans* in that medium and spread that inoculum with driglasky. The paper disk is whatmann paper no.1 have 10mm diameters. Put that paper in extract and move in Petri dish where have been medium in there. Petri dish incubating in incubator with tempaerature ±30 °C for 24 and 48 hours. And then check the clear zone. The clear zone have classification like tabel 1.

2.6 Minimum Inhibitor Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Method

Antimicroba solution has choosed where have big clear zona, and continue to another step are MIC (Minimum Inhibitor Concentration) and MBC (Minimum Bactericidal Concentration). First, make master extract solution about 10000 µg/ml from 100mg sampel extract then add 2ml DMSO and steril aquades until 10ml.and

the make serial dilution with steril aquades until get 10000 µg/ml, 500 µg/ml, 2500 µg/ml, 1250 µg/ml, and 625 µg/ml [12]

a. Concentration of 100 µg/ml: make from solution of concentration 10000 µg/ml and enter it to tube and add NB (Nutrient Broth) medium about 9ml. Then shake it with vortex and decide it to be 3 part for antimicroba test to 3 test microba.
b. Concentration of 500 µg/ml: make from solution of concentration 5000 µg/ml and enter it to tube and add NB (Nutrient Broth) medium about 9ml. Then shake it with vortex and decide it to be 3 part for antimicroba test to 3 test microba.
c. Concentration of 250 µg/ml: make from solution of concentration 2500 µg/ml and enter it to tube and add NB (Nutrient Broth) medium about 9ml. Then shake it with vortex and decide it to be 3 part for antimicroba test to 3 test microba.
d. Concentration of 125 µg/ml: make from solution of concentration 1250 µg/ml and enter it to tube and add NB (Nutrient Broth) medium about 9ml. Then shake it with vortex and decide it to be 3 part for antimicroba test to 3 test microba.
e. Concentration of 62.5 µg/ml: make from solution of concentration 625 µg/ml and enter it to tube and add NB (Nutrient Broth) medium about 9ml. Then shake it with vortex and decide it to be 3 part for antimicroba test to 3 test microba.
f. Positive Control: in positive control 2.5g kloramfenikol and add DMSO 2ML then enter it to tube where there are 9ml NB medium. Shake it with vortex and decide it to be 3 part for antimicroba test to 3 test microba.

For MIC add 0,1ml suspense microba. The MBC method from most clearness in MIC tube. Take 0,1ml suspens and then growing in solid medium with pour plate method and incubation in room temperature for 24 hours. Continue with measure the coloni what growing in that medium.

3.RESULTS AND DISCUSSION

3.1 Studi Ethnobotany

The plant which as sampel of research are leaf of mimba (*Azadirachta indica)*, cabe jamu (*Piper retrofractum* Vahl), bidara laut (*Strychnos ligustrida* Bl), sambiloto (*Andrographis paniculata* Nees), kayu jaran (*Lannea coromandelica*) and bark of pisang (*Musa sp*) like Figure 1.

Figure 1. Kind of herbal

In Poteran Island, Mimba tree have big abundant. Local society use mimba leaf for heal of hurt, skin disease, diarrhoea, and pneumonia. First step make mimba leaf aa drug is choose fresh leaf and pound that with pounder (alu). And then filter that solution, so will get extract water.

Cabe jamu tree, many found it in local society's house and that trees have plantation. The local people in there believe what cabe jamu leaf can heal to fever and cough. Usually cabe jamu leaf boiled and drinking that water. Based information from local people what cabe jamu leaf as material for tradisonal herbal because to get warn in body.

Bidara laut tree n Poteran Island can found in every road as wild tree. In Poteran island using bidara tree is rare but many of herbalis using bidara tree for bathing of corpse, blood of cought and ambeien. Obey with [13], bidara laut tree can heal another disease like skin disease and blood diseases.

Different with mimba, cabe jamu, and bidara tree, sambiloto's tree have little population in Poteran island. Obey with [4] sambiloto tree have advantages, there are for care diarrhea and malaria. That tree's also famous for fever. Kayu jaran's tree many use it for to preserve a siwalan sugar. Obey of local people said if kayu jaran leaf can care to skin diseases, diarrhea and panas dalam. Befor use kayu jaran leaf, must be pound that leaf and then drink that water extract. That same with other studi what kayu jaran leaf can heal to ill in mounth, coroner, and disentry caused infection by *Salmonella sp* bacteria.

Banana tree is wild tree in Poteran island. Banana leaf usually for food packaging. Not only leaf but also bark of banana as medicine of diarrhea with pound that leaf and drink that water.

3.2 Phytochemical Analisis

Phytochemical of extract use kualitatif test to several of chemistry, there are alkaloid, steroid, saponin and flavonoid. Princepal this test based visibility from colour formed of between chemical and cromogenik reagen in Figure 2.

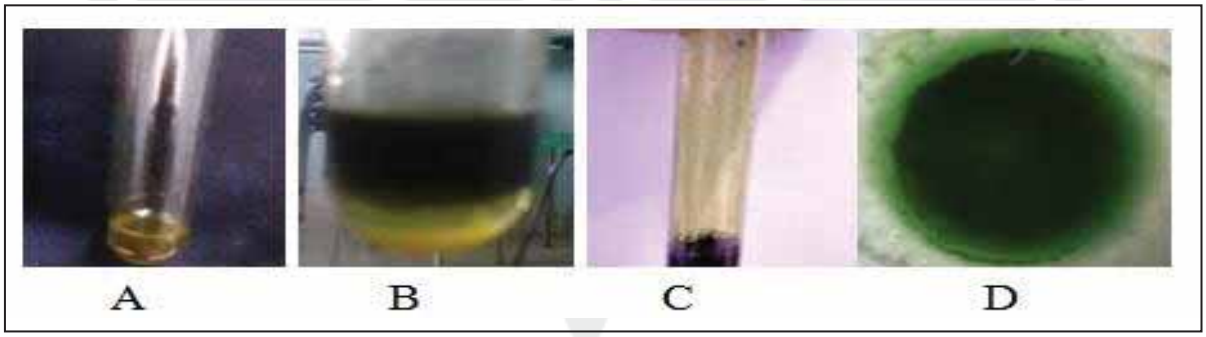

Figure 2. Positif result pytochemical. A. flavonoid B.Steroid. C. Alkaloid. D. Saponin

Table 2. Tabel content phytochemical in sampel

Tumbuhan	Kandungan Fitokimia			
	Flavonoid	Steroid	Alkaloid	Saponin
Mimba	+	+	+	+
Cabe jamu	+	+	+	+
Bidara	+	+	+	+
Sambiloto	+	+	+	+
Kayu Jaran	+	+	+	+
Pisang	+	+	+	+

This result agree with [14] mimba's leaf known have content chemical such as terpenoid, flavonoid, alkaloid, saponin, tannin, steroid and triterpenoid. [15] also said mimba leaf have another chemical such as Azadirachtin, paraisin, and atsiri oil where have sulfide. Positive result of cabe jamu like mimba leaf. This result agree with [16] confirm to what cabe jamu leaf contents alkaloid, saponin, tannin, flavonoid, steroid, tetraterpenoid dan glikosida, but also have another chemical is piperine (about 4-6%) what known as anti strest[17]

Bidara laut extract show positive result in all phytochemical testing. Obey with [18] *Strychnos ligustrina* Bl, containing striknin (strychnine) ($C_{21}H_{26}O_4N_2$) and brusin (brucine) ($C_{23}H_{26}O_4N2$), both are alkaloid kuinolin. Another chemical such as tannin, alkaloid, and steroid. Another research said if bidara laut have saponin where that have foam [19].

Dominan component in sambiloto leaf is steroid. This result same with [20] if not only steroid but also saponin, flavonoid, alkaloid, tannin, diterpenoid lactones (andrographolide), paniculides. Kayu jaran extract and bark of banana tree have positive result too. This result agree with reference if kayu jaran containing alkaloid, tannin and flavonoid [21].

3.3 Antimicroba Activity

The formed clear zone caused antimicroba activity every extrat with concentration 100% like Figure 3.

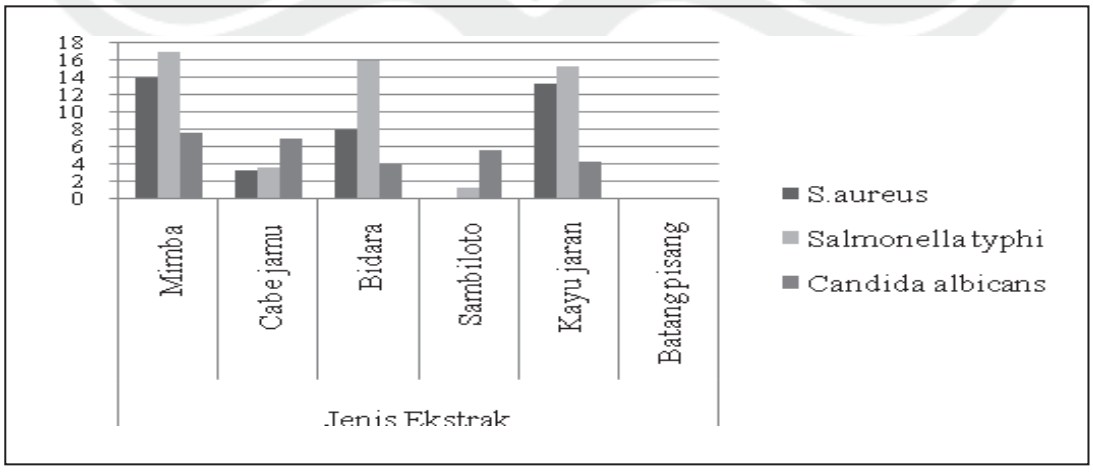

Figure 3. Clear zone diameters every extract

Figure 3 show what the biggest clear zone in all sample microba is mimba extract, whereas bark of banana extract have little clear zone. clear zone formed in mimba extract have category is weak, in *Salmonella typhi* have rather category, but in *C.albicans* have not respons category.

In cabe jamu extract have 3 clear zone amount not respons category because have small diameters about 2-6mm [10]. Clear zona formed because cabe jamu have alkaloid, saponin, tannin, flavonoid, steroid, glikosida, and trefrepenoid what can stop bacteria growing [16], and have other chemical, there are piperine, sitosterol, sitral, piperlonguminine, sylvatine. Piperin in cabe jamu amoung alkaloid is less base amida what can formed salt and stronge mineral acid so can stop bacteria growing with destroy component in peptidoglican in bacteria cell, so can formed cel wall and make that died [22].

Antimicroba in bidara laut extract have clear zone what category not respon in *Staphylococcus aureus*, clear zone in *Salmonella typhii* have weak category, and clear zone in *Candida albicans* have category not respon [10]. Active chemical are (strychnine) ($C_{21}H_{26}O_4N_2$) and brusin (brucine) ($C_{23}H_{26}O_4N_2$) what formed clear zone, both is alkaloida kuinolin (Dediwan *et al*., 1993). alkaloid mechanism in bidara and other sampel can change amino acid structure by nitrogen base in alkaloid. So can destroyed cell wall and DNA [23].

Sambiloto extract have not respon antimicroba to *S. aureus*, but have clear zone to *Salmonella typhii* and *Candida albicans*. That clear zone have not response. Saponin,flavonoid, alkaloid, tannin, andrographolide, paniculides can stop *Salmonella typhii* and *Candida albicans* growing [20]. The saponin in sambiloto will give hydrogen bonded [24] so can destroy cell wall permeability [25]. Clear zone in kayu jaran extract have weak respon to *S.aureus* dan *Salmonella typhii*, and have not respon category to *Candida albicans*. Whereas in banana extract have not respon in all microba. Observation in this research have 24 and 48 hours. Every observation do measure of clear zone. In tabel 3 show incubation time 24 hours give influence to *S.aureus* growth is have more efetivity that observation in 48 hours. Clear zone formed in incubation 24 hours have biggest diameter clear zone that 48 hours because possible to antimicroba have been decomposition or microba have been resisten. Antimicroba in sample have cateristics is bacteriostatik except sambiloto and bark of banana extract. Agree with [26] because that bakteriostatik caused chemical just stop growth that microba not died that microba. But if bacteriosida caused that chemical can died that microba.

Table 3. Clear zone diameter with all type extract with 100% concentration to *S. aureus*

Jenis Ekstrak	Zona Bening (mm) dalam inkubasi		Bakteriostatik/Bakteriosida	Kategori Respon
	24 jam	48 jam		
Mimba	14	13.3	Bakteriostatik	Lemah
Cabe jamu	3.3	3.3	Bakteriostatik	Tidak ada
Bidara	8	7	Bakteriostatik	Tidak ada
Sambiloto	0	0	Tidak ada	Tidak ada
Kayu jaran	13.3	12.3	Bakteriostatik	Lemah
Batang Pisang	0	0	Tidak ada	Tidak ada

Based that antimicroba test use diffusion method have result to Staphylococcus aureus, Salmonella typhii, Candida albicans is mimba extract so continue to dillusion method. Dillusion method to know about Minimum Inhibitor Concentration (MIC) and Minimum Bactericidal Concentration (MBC) in Tabel 4.

Table 4. MIC an MBC value to all micoba sampel

Jenis Bakteri	CFU/ml					
	Kloramfenikol	1000 µg/ml	500 µg/ml	250 µg/ml	125 µg/ml	62.5 µg/ml
S.aureus	0	$60x10^{2*}$	$>60x10^{2}$ ^	$>60x10^{2}$ ^	$>60x10^{2}$ ^	$>60x10^{2}$ ^
Salmonella typhii	0	$67x10^{2*}$	$77x10^{2}$ ^	$125x10^{2}$^	$>125x10^{2}$^	$>125x10^{2}$^
Candida albicans	0	0^{+}	$6x10^{2*}$	$9x10^{2}$^	$9x10^{2}$^	$76x10^{2}$ ^

Keterangan:
***** : menunjukkan nilai MIC (Minimum Inhibitory Concentration)
$^{+}$: menunjukkan nilai MBC (Minimum Bactericidal Concentration)
^ : menunjukkan tidak memiliki respon

MIC value in mimba extrat (Tabel 3) to *S.aureus* dan *Salmonella typhi* in concentration 1000µg/ml whereas *Candida albican* have concentration 500µg/ml with method of measure all coloni what grow in medium PCA. Obey with [27] if MBC value certain from have noth microba growing in medium. It's same if MBC value just to *Candida albicans* with concentration 1000µg/ml. Mimba leaf have caracteristick bakteriostatik to *S. aureus* and *Salmonella typhi*, and have bakteriocidal to *Candida albicans.*

Antimicroba from mimba extract caused have complete phytochemical, there are yaitu saponin, alkaloid, flavonoid anf steroid also have azadiractin [28], [29]. The mimba advantages can destroy of a growth microba patoghen like *Salmonella thyposa* (caused tipus) and *Staphylococcus aureus* (caused gastroenteritis) [30]

4. CONCLUSIONS

All sample tree have positive result in pytochemical testing. There are have chemical such as alkaloid, saponin, flavonoid, steroid. All sampel have clear zone except bark of banana extrat to all sampel microba and sambiloto extract to *S.aureus.* The strongest clear zone is mimba extract about 14 mm to *S.aureus* and about 17 mm to *Salmonella typhii*, there are rather category and about 7,67 to *C. albicans.* MIC value in mimba extract is 1000µg/ml to *S.aureus* and *Salmonella typhi* whereas in Candida sp have concentration about 500µg/ml. MBC value have concentration 1000µg/ml only to *Candida albicans.*

5. ACKNOWLEDGEMENT

The thanks Ir. Sri Nurhatika, MP. and N. Dwianita Kuswytasari S.Si., M.Si., for reviewing the manuscript; Isdiantoni, S. P., M. P. for useful advice and assistance; Special thanks are also given to Biomaterial and Enzyme Technology Research Team (2014/2015) for assistance and support

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Improvement of *Growol* As a Probiotic-Functional Food (Case Study at Kalirejo, Kokap, Kulon Progo, DIY)

Chatarina Wariyah* and Sri Luwihana
Faculty of Agroindustry, Mercu Buana University of Yogyakarta, Jl. Wates Km 10 Yogyakarta 55753, Indonesia
*E-mail: chatarina_wariyah@yahoo.co.id

Abstract

Growol is a steamed-fermented cassava which was used as a staple food in Kulon Progo, especially at Kalirejo village, Kokap District, Kulon Progo Regency-DIY. Recently, *growol* consumption at Kalirejo is getting decreased. This condition due to a change in land use from cassava plantation into *albasia* tree (*Albizia falcataria* (L) Fosberg), which is more profitable economically. Impact of this situation is the decrease of *growol* production and the increase of rice consumption as a staple food. Whereas, *growol* contains of *Lactobacillus* sp bacteria which has functional property as a probiotic. The purposes of this research were to increase of the Kalirejo' communities awareness on the benefits of *growol* as a functional food, to improve the processing technology and the packaging method of *growol*, to evaluate the potency of *growol* as a probiotic staple food. The Kalirejo communities were trained and practiced the modified technology of making *growol*. The processing of *growol* was improved by prolong fermentation time to increase the number of *Lactobacillus* sp. and packaged a *growol* with banana leaf combined with cardboard. The *growol* resulting from these process were analyzed their proximate composition and the *Lactobacillus sp* content. The research showed that processing of *growol* with fermentation during 4-5 days resulted *growo*l with high *Lactobacillus* sp content. The result of the proximate analysis showed that the moisture content of *growol* was 56.64 $\pm$ 0.69%, protein 1.04 $\pm$ 0,02%, fat 0.19 $\pm$ 0.02%, ash 0.18 $\pm$ 0.01% and carbohydrate was 41.95% (by different). *Growol* was packaged by using cardboard with label was more exciting than using of banana leaf only, because it was more hygienic and the consumer knew about *growol* identities. Increasing of the *growol* demand from the residents and the rural communities surrounding Kalirejo village was indicated by the increase of the production from 6.0 quintal per month into 12.0 – 15.0 quintals per month. Total *Lactobacillus* sp. in *growol* was 4.7 x 10^3 cfu/g. and the beneficial of *growol* was understood by the communities, hence lead to increase of *growol* production.

Keywords: fermented-cassava, probiotic, functional-food.

1. INTRODUCTION

Cassava (*Manihot esculenta*) is the third carbohydrates source food in Indonesia after rice and maize. The cassava starch content is between 13.94 to 19.79% depends on the growth site conditions (Susilawati *et al*., 2008). Indonesia cassava production reached 23.436 tons in 2014 (BPS, 2015). Cassava is an important food crop in Indonesia, because some areas use cassava as a staple food such as *a tiwul*

in Gunung Kidul Regency and *growol* in Kulon Progo Regency. *Growol* is the staple food in Kulon Progo Regency-DIY, especially at Kalirejo village, Kokap District. Everyday, in the morning and the afternoon, the main food of the communities is *growol*, while rice is consumed once in the afternoon. *Growol* was made through stages of: peeling of the cassava, slicing, soaking (spontaneous fermentation) for 3 days, screening and crushing, washing, pressing, enumeration and steaming (Anonymous, 2015). The *growol* was weighed about 5 kg and was molded in cone-shaped and then packaged in a banana leaves. Kalirejo's *growol* was known as a Sangon *growol* according to the name of the potential hamlet which produce *growol*. The g*rowol* taste is sour and a bit bland with no salty or sweet; however, *growol* is usually ate with complementary foods that are called *ketak* and *srundeng* both food is made from coconuts. Business of *growol* in Kalirejo village was began in 1950 and was the family business which was managed hereditary. Recently, *growol* consumption and number of growol producer are getting decreased and shifting the staple food to rice. However, at least there are two hamlets in the Kalirejo village who still produce *growol*, namely Sangon I and Sangon II. This matter due to the land use change from cassava plantation into land of albasia tree (*Albizia falcataria* (L) Fosberg) that is more profitable economically. Whereas, *growol* as the local staple food has a potential beneficial as a probiotic-functional food.

According to the Drug and Food Monitoring Agency /BPOM (2005), a functional food is a natural or processed food containing one or more compounds which have a beneficial specific physiological functions to health. Moreover, a functional food does not leave contraindications and side effect at the recommended usage amount on the metabolism of other nutrients. The compound group which are considered to have certain physiological functions in the functional food include food that contain of lactic acid bacteria. These functional food are called probiotics that are living organisms which can provide the host health beneficial effects when consumed in a sufficient quantities (FAO/WHO, 2002) by improving the balance of the intestinal microflora at the digestive tract (Shitandi *et al*., 2007). A probiotics are generally from the lactic acid bacteria (LAB), particularly the genus *Lactobacillus* and *Bifidobacterium*, which are part of the normal flora in the human gastrointestinal tract (Sujaya *et al*., 2008). *Growol* has physiological effect for preventing diarrhea (Lestari, 2009), which is caused by lactic acid bacteria cell activity (*Lactobacillus casei subsp. Rhamnosus TGR2*) and the secondary metabolites produced against pathogenic bacteria cells. The growth of LAB in *growol* occurs in a spontaneous fermentation during soaking. The dominant LAB in *growol* are *Lactobacillus plantarum* and *Lactobacillus casei subsp. Rhamnosus* (Putri *et al*., 2012). In view of the high potency of *growol* as a functional food, it is important for conducting research to enhance communities awareness to make *growol* as the local staple food and improvement the processing technology of *growol* to increase the potency of probiotics.

2. MATERIALS AND METHODS

2.1. Raw materials

Materials used for making *growol* were cassava which were obtained from the farmers in the Kalirejo village and the surrounding with the variety of Martapura. The cassava in a fresh condition with a period less than 48 hours after harvested. The chemicals used for proximate analysis (analysis of moisture content, protein, fat and

ash) with the qualification pro analysis from Merck, while the media for testing the total lactic acid bacteria (LAB) and total plate count bacteria in *growol* were obtained from Ovoid Ltd.

2.2. Methods

The study was conducted by using method of: education, implementation or application and test in the laboratory. This study was divided into two stages: 1) education to increase Kalirejo' communities knowledge about the benefits of *growol*, g*rowol* processing technology improvement as a probiotic food and application of processing technology modifications of *growol*, 2) proximate analysis, determined of total lactic acid bacteria and total bacteria on *growol*.

2.2.1. Education of the benefits of *growol* and *growol* processing technologies

Education for the participants were carried out by counseling and training with the material of : beneficial of *growol* as a probiotics and processing of *growol* with the correct way to produce high probiotic effect. The participants of this program were the *growol* producers from Sangon I and Sangon II hamlets in Kalirejo village with the number of 20 people. The meeting was done in 4 times, followed by mentoring. The participants were trained and practiced the modified technology of making *growol.* The processing of *growol* was improved by prolong fermentation time to obtain the optimal number of the *Lactobacillus* sp. and the packaging of *growol* was improved by combine the use of banana leaves and cardboard.

The improvement of *growol* processing technology was referred to Sutanti *et al.,*. (2013). The cassava was peeled, cut about ± 5 cm, washed 2 - 3 times, then was soaked with water at a ratio of cassava/water 1: 3 (w/v) during 4 days. The fermented cassava was washed 2 times, filtered and pressed. The resulted flake was crushed into small size, while the filtrate was settled and the starch was blended with the flake. Finally, the flake was steamed for 20-25 minutes. *Growol* resulting from this process was determined its total lactic acid bacteria and total plate count bacteria and then was compared with original growol.

2.2.2. Proximate analysis and total bacteria in *growol*

The *growol* resulting from the modified process was analyzed their proximate composition, total *Lactobacillus sp* content and total plate count bacteria. Proximate analysis of growol consist of: moisture content with gravimetric static method, protein was determined by mikro Kjeldhal, fat with Soxhlet and ash with muffle furnace (AOAC, 1990). The total lactic acid bacteria and total plate count bacteria were determined with solid agar (Maturin and Peeler, 1995). The fresh *growol* was stored at room temperature during 0, 3 and 5 days to determine their shelf-life which was indicated by the number of total plate count bacteria.

2.2.3. Design Experiment

The research was conducted by the Completely Randomized Design with the factor of *growol* processing method. The resulted data was processed statistically with descriptive analysis (Sugiyono, 2004).

3. RESULTS AND DISCUSSION

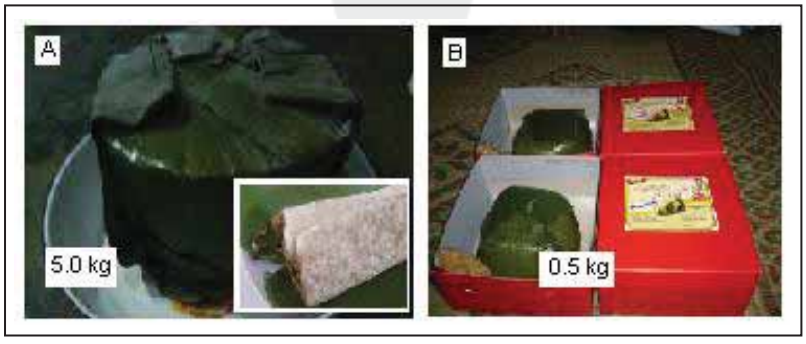
3.1. The increase of production and *growol* processing method

The training program resulted data that indicated increasing of growol production and processing technology method, as shown in Table 1.

Table 1. The increase of production and processing quality of *growol*

No.	Criteria	Before a training	After a training
1.	Production per month (quintal cassava)	6.0	12.0 – 15.0
2.	Fermentation duration	2 - 3 days	4 – 5 days
3.	Filtrate	was not pricipitated	precipitated to increase the rendemen
4.	Washing of fermented-cassava	4 times	2-3 times
5.	Packaging	banana leaves	banana leaves and cardboard
6.	Shelf-life	2 days	3 days
7.	Marketing	-	Designated manager

The Sangon I and Sangon II hamlets at Kalirejo village have seven active *growol* producers, but the training scouted 20 producers (included the former producers). Slightly *growol* produced from Sangon was used for consumption, mostly *growol* was sold in the nearby markets. At the previous, the production of *growol* at those hamlets were about 6.0 quintals per month. After the training and improvement of the processing and packaging technology, the production increased into 12.0-15.0 quintals per month. The producers were put together in a group namely "Growol***ku*lon** Progo Hebat". Marketing of *growol* extends to the village around Kalirejo and many middlemen come to buy *growol*. The fascination of Kalirejo *growol* are their small size ("*growol kecil*"), because a little *growol* was packed in banana leaves combined with cardboard (with weight 0.5 kg growol/pack) with sweet or savory taste (Luwihana and Wariyah, 2014). The *growol* price was Rp 2,500, - (for savory *growol*) – and Rp 3.000,- (for sweet *growol*). The increase of *growol* price was from Rp 16,000,-/5kg (home industry) into Rp 25,000, - (*growol* of the group), hence increase the profits. Demand of *growol* was much more because the flavor and the appearance of *growol* was more attractive. Figure 1 shows the changes in appearance of the *growol* before and after training.



Gambar 1. *Growol* (A. before training, B. after training)

3.2. *Growol* composition and the potential of probiotic

Growol is a staple food as a source of carbohydrates and Table 2 shows the results of the proximate analysis of *growol*.

Table 2. Composition of *growol*

Components (% wb)	Kalirejo's *Growol*	*Growol*[*)]
Moisture	56.64 ± 0.69	56.74 ± 0.06
Protein	1.04 ± 0.02	8.56 ± 0.06
Fat	0.19 ± 0.02	1.23 ± 0.10
Ash	0.18 ± 0.01	1.03 ± 0.10
Carbohydrate (by different)	41.95	32.44

*)Rukmini, 2003.

Carbohydrate is a main component of *growol.* The carbohydrate content of Kalirejo' *growol* is higher than common *growol* which was processed by craftsmen (Rukmini, 2003), but the protein content is lower than Kalirejo' *growol*. According Susilawati *et al*., (2008), the composition of cassava as a raw material of *growol* was influenced by the type, age and place of harvest growth, hence there are difference between Kalirejo's *growol* and common *growol*. While the total plate count bacteria in *growol* as shown in Table 3.

Table 3. Total bacteria in *growol* during storage

Soaking time (days)	Storage time (days)	Total bacteria (cfu/g)	Lactic Acid Bacteria (cfu/g)
3	0	8.3×10^3	0
4		4.3×10^2	4.7×10^3
3	3	4.9×10^7	-
4		4.4×10^4	
3	5	5.9×10^7	-
4		5.5×10^5	

Table 3 shows the total bacteria in a *growol* made by soaking (fermentation) for 3 and 4 days, and storage at various of 0, 3 and 5 days. The purpose of this treatment was to determine the shelf-life of the *growol*. The result showed that the longer the fermentation time, the lower total plate count bacteria. Storage of *growol* during 5 days increased the total bacteria, however the total bacteria of *growol* which was fermented for 4 days had lower total bacteria than 3 days fermentation. It means that the fermentation *growol* for 4 days resulted *growol* with longer shelf life.According to Anonymous (2009), the requirements of total bacteria in food such as uncooked pasta or noodles are as much as 1×10^6 colonies/g, therefore, analog with those

food, *growol* with fermentation during 4 days still suitable for consumption, while *growol* was fermented for 3 days the total bacteria is quite high (5.9 x 10^7 cfu /g) and exceeds the maximum allowable requirement. It was caused the *growol* with 3 days fermentation had no lactic acid bacteria, while fermentation for 4 days the total lactic acid bacteria was as much as 4.7x 10^3 cfu / g. the lactic acid bacteria are capable of producing metabolites such as lactic acid and bacteriocins which can inhibite the growth of pathogenic bacteria such as *E. Coli* (Rachmawati *et al*., 2005), therefore the *growol* had longer shelf life.

4. CONCLUSION

The research showed that the use of banana leaf combined with carboard for packaging of *growol* was more attractive and more favourlable, because the *growo*l was more hygienic and there was a complete *growol* identity. *Growol* demand from the residents and rural communities around Kalirejo increased as shown by the high production of *growol* from 6.0 quintals per month into 12.0-15.0 quintals per month. The proximate analysis of *growol* was obtained that the water content was 56.64 $\pm$ 0.69%, protein 1.04 $\pm$ 0.19, fat 0.19$\pm$0.02%, ash 0.18 $\pm$ 0.01% and carbohydrate by different was 41.95%. Processing of *growol* with fermentation during 4 - 5 days resulted high *Lactobacillus* sp content and the shelf-life was longer than the original *growol*. The total *Lactobacillus* sp. in *growol* was 4.7 x 10^3 cfu/g. The beneficial of *growol* was understood by the communities, hence leads to increase of the *growol* production.

5. ACKNOWLEDGEMENTS

The authors would like to thank Directorate General of Higher Education, The Ministry of Education and Culture, Republic of Indonesia for financing the project from IbM Grant Program in 2015.

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Biogrouting : Urease Production From Carbonat Presipitation Bacteria (*Oceanobacillus* sp.)

*Sidratu Ainiyah[1] , Endry Nugroho[2],Puspita Lisdiyanti[3] ,Maharani Pertiwi[4]
[1]Biology Department of Mathematic and Science Faculty of Sepuluh Nopember Institute of Surabaya, Indonesia
[2]Biotechnology of Institute Science Centre, Cibinong-Bogor, Indonesia
Sidratu.marinebiology@gmail.com

Abstract

Grouting is the process of pore filling with grout material (construction material). Biogrouting is a technology that simulates the process of diagenesis, namely the transformation of sandgrain into sandstone (calcarinite). Calcite ($CaCO_3$) formed from biogrouting process. The research problem is how to optimize the product with the urease activity test, isolation, purification, characterization urease and applying it as a grout material. Optimization activities test carried out by growing isolates Oceanobacillus sp. in 2 variations medium (urea and B4 urine), 5 variations pH (4-8) and 2 variations of temperature (25 ° C and 29 ° C). Then, the optimal isolates purified using ammonium sulfate (Test Bradford) and identified isoelectric point. The product of protein precipitates were characterized using SDS-PAGE. Based on the research results revealed that the highest urease activity was 203.32 units / ml. Optimal urease produced in isolates grown in medium B4 urine pH 8 (25 ° C), while in medium B4 urea at pH 7 (25 ° C). The molecular weight of urease were characterized using SDS-PAGE was 440 kDa, and the isoelectric point at pH 6. Urease can be used as grouting material because it gives positive response to simple application biogrouting cementation.

Keywords: Biogrouting, diagenesis, Oceanobacillus sp., urease

1. INTRODUCTION

Grout is material construction consisting of suspension (cement, ground, loam, pozzolan, bentonite and others) chemical like a silicate of urethane, urea, akrilamide wich used as hardening agent (Van paasen, 2008). Charging pore in the process of construction material and interstices between ground particles by certain depth known as grouting. Groutting can be used to improve the structure of the ground like change physical characters of the ground (xanthakos *et al.*, 1994; karol, 2003). The purpose of grouting to wake up done chemically use presipitation silica (water glass) (van paasen, 2008). Silica can settles when mixed with acid bikarboksilat spontaneously. This reaction is weakness grouting chemically, because only can be applied at the point of injection nearest to ground repaired the structure (hammes *et al.*, 2002). In Indonesia biogrouting begun at five years ago.

Biogrouting is technology that simulates diagenesis process, transformation of sand grains being rocks (Lisdiyanti, 2011). Naturally, this process requires time up to million years. According to Van Paasen (2008) diagenesis process can be speeded

up the use of bacteria producing urease. Aplication marine bacteria as material grout have been conducted (Lisdiyanti, 2011). Limited abiotic factor such as temperature and pH less than optimal cause the process of biogrouting (hames *et al.*, 2003b). Keikha *et al.,* (2012) said that using isolates marine bacterium *Bacillus* sp. shows that application directly on the sand less efficient diagenesis in the process. This caused isolates *Bacillus* sp. difficult to enter into the pores of the land. Kaltwasser (1972) and Ramakhrisnan *et al.,* (2001) states that one way to increase efficiency and minimize the impact on the environtment used urease as grout material.

Urease is enzyme produced by presipitation carbonate marine bacteria. Urease act as catalyst and not toxic (Fujita *et al*., 2000; Mobley *et al*., 1995). The importance of urease in the process of biogrouting and until now there has been no research on the production of material urease as grout (Chu *et al*., 2012).

2. METHOD

2.1. Optimization and Urease Activity Test

Oceanobacillus sp. cultivated in medium production (B4) and urine medium (containing urine). Urine wich used not the urine from pregnant women, 22 years old, and has a pH of 5. Then the culture incubated in rotary shaker 150 rpm at room temperature (30 ° C) for 72 hours. Urease activity was measured using the Weatherburn (1967) method were modified, Na2HPO4 used in alkaline solution of hypochlorite compared with NaOH. The reaction carried out in test tubes containing 100 µL of sample, 500 µL of 50 mM urea and 500 µL of 100 mM KH2PO4 buffer (pH 8.0) so that the total volume was 1.1 ml. The solution was incubated in a water bath at 37 ° C for 30 minutes. The reaction was stopped by adding 100 µL into the tube which contains 1000 µL solution of phenol-sodium nitroprusside. Then a solution of alkaline hypochlorite 1000 mL was added to the tube and incubated at room temperature for 30 minutes. Furthermore, the measured optical density (OD) with a spectrophotometer at a wavelength of 630 nm and compared with standard curves $(NH4)_2\ SO_4$. One unit of the enzyme means the amount of enzyme required to liberate 1 mol NH3 from urea per minute under standard conditions [10].

2.2. Isolation and Purification of Urease

2.2.1 Protein precipitation method using ammonium sulfate

Crude extract protein produced from the process of centrifugation precipitated with ammonium sulfate up to a concentration 100%. After the precipitation process, then resuspended using phosphate buffered saline to obtain protein precipitates as many as 10ml. Protein presipitat concentration is then determined using the method of Bradford [11].

2.2.2 Isoelectric Point

Isoelectric point is the specific protein area which does not have the charge (difference between the number of positive and negative charges), so it does not move when placed in an electric field. At the isoelectric pH, min protein solubility, causing the protein precipitates [12]. First, prepared 9 test tube clean and dry, then put 3 ml of urease in each tube. Then add 1 ml of acetate buffer solution respectively at pH 4; 5 and 6. Then whipped, recorded the degree of turbidity after 0, 10, and 30 minutes. Observed how the tube formed precipitate maximum. Furthermore, all the

tubes were heated over a water bath. After the observed results. The formation of sediment and turbidity fastest or most was the isoelectric point [13].

2.2.3 Characterization Urease

SDS-PAGE Electrophoresis

Protein characterization using SDS-Page aims to determine the molecular weight (MW). Proteins that have been treated with a strong detergent containing ions such as sodium dodesyl sulphate (SDS) and the reducing agent will experience the elimination of structures [14]. The method used in the manufacture of gel is the method Edelstein and Bollag (1991) [15]. Materials for separating gel mixed individually by entering TEMED (Tetramethylethylenediamine) at the end of the mixture. The solution stirred and slowly pipetted into the plate glass up to 1.5 cm from the glass surface and then allowed to stand 15-20 minutes. In this process sought the creation of air bubbles. After the gel solidifies, stacking gel mixture pipetted slowly into the glass plate and then immediately put the comb (10 wells) as places for the samples. Samples were heated at 1000^{o}C for 3 minutes then mixed with sample buffer and loading the sample into the wells as much as 12 µL. Unlike the case with the sample, which in Marker-loading into the wells of 10 µL. Before running done, buffers incorporated into the electrophoresis chamber. Running electrophoresis was performed on a 120 Volt, 28 A in cold conditions. The time required for running electrophoresis approximately 1.5 hours. After separation, the gel removed from the glass plate and then immersed in a fixative (25% methanol + 12% acetic acid) for 1 hour. Furthermore, the gel soaked in a solution of 50% ethanol for 20 minutes and a 30% ethanol solution for 2 x 20 min. Thereafter, the gel soaked in a solution enhancer (Na2S2O3.5H2O solution) for 1 minute. Gel washed with aquabides for 3 x 20 minutes. After washing with aquabides, staining the gel was soaked in a solution of silver nitrate (AgNO3 + formaldehyde solution of 37%) for 30 minutes then rinsed quickly with aquabides for 2 x 20 seconds. Excess color removed by destaining solution (solution of formaldehyde Na2CO3 + 37%) to obtain the protein bands were clearly observed with relatively clear background. The reaction was stopped by using a fixative [15].

2.3. Urease Production

The bacteria wich used for applications biogrouting was a bacterium that has the highest enzyme activity among other isolates. Isolates were grown in liquid media B4 100 mL and 100 mL of urine media. Then incubated using a 250-ml Erlenmeyer flask for 5 days set temperature, pH and optimal medium (according the data optimization urease). The resulting product still contains biomass cells are not needed in the process biogrouting. Urease can be applied after the fermentation centrifuged at 10000-12000 rpm for 15 minutes [5].

2.4. Urease Application On Biogrouting

Applications biogrouting done by preparing urease in 50 mL syringe size. Then prepared sand still in saline conditions (fresh) into the mold, then check mass of sand. Sand was given treatment using direct injection (De Jong *et al.*, 2006), with urease volume of 10 mL each. Furthermore, a mixture of sand and urease incubated at room temperature for 24 hours, every 4 hours check pH of sand, mineral calcite

formation visually and compacting process. Quantitatively measured mass of sand after solidified [16].

2.5. Data Analysis

The General Linear Model (GLM) used to analysis of data. Each treatment was repeated 10 times, each repeat measurements were made three times. A combination that used a variation of the type medium (source B4 urea and urine), pH (4; 5; 6; 7; 8, and 9), and temperature (25 °C and 29 ° C) during measurement of urease activity. Graphic showing the highest value chosen as the most optimum conditions.

3. RESULTS AND DISCUSSION

3.1. Test And Optimization Urease Activity

Test and optimization activities carried out to determined the optimum activity biogrouting bacteria to produce urease. These enzymes will be produced to be applied on a laboratory scale. Bacterial isolates used in this study was isolate P3BG43 code. Based on previous research these isolates were from the collection of the Biotechnology Research Center LIPI Cibinong. According to [5], this P3BG43 isolates taken from altitude (± 4,000 m above sea level), which has low air pressure so that the ambient temperature was too low. Therefore, it was difficult bacteria grown tropics. This bacterium was sensitive to environmental change, temperature and medium. This condition indicated by the number of repetitions performed to adapt. Need 4-5 times of cultivation using medium NB, B4 (marine agar), and NA.

Based on previous research P3BG43 isolates identified as *Oceanobacillus* sp., with phenotypic characteristics include a rod-straight shape (size 0.3-2.2x1.2-7.0μm), motile with flagella lateral-type, heat-resistant form endospores (the amount of not more than one in one cell sporangia) and give a positive reaction on the urease test. *Oceanobacillus* sp. does not have the activity of extracellular enzymes when starch hydrolysis test, trybutirin and casein. Isolates P3BG43 been known to have the highest urease activity based methods Weatherburn (1967) were modified (Figure 1) [5].

Optimization of bacterial growth biogrout done by growing isolates in medium B4 and B4 modified using urine (urine B4). Then the cell density was measured using a spectrophotometer. B4 medium is the medium that contains the nutritional content of minerals and urea. Urea $(NH_4)_2CO_3$ containing ammonium which causes precipitation of calcite [17]. While the source of urea B4 medium replaced with urine (urine B4) containing ammonia (NH_3) [18]. The content of ammonia in the urine causing incomplete reaction to precipitate carbonate. The existence of ammonia in a medium containing water (distilled water) causes a spontaneous reaction. Spontaneous reaction caused by the presence of water (H_2O) convert ammonia (NH_3) into ammonium (NH_4^+) and carbon dioxide (CO_2) will balance the chemical reactions become carbonic acid, bicarbonate ions and carbonate ions. The increase in pH due to hydroxyl ions formed from NH_4^+ production that exceeds the availability of Ca^{2+}. This condition causes an alkaline environment so that the carbonates needed for calcite precipitation.

Optimization was done by conditioning the growth of bacteria on two types of medium, variations in pH range of 3-9 and at a temperature of 25 ° C and 29 ° C. The setting conditions of the medium, pH and temperature was done with the assumption that it still appropriate when applied biogrout products in the environment. Based on data analysis using the General Linear Model was known that at temperature of 25 ° C and pH 7 are the optimum condition biogrouting bacterial growth (Figure 1). P value less than 0.05 ($p < 0.05$) which means pH and temperature affect the growth of bacteria biogrout.

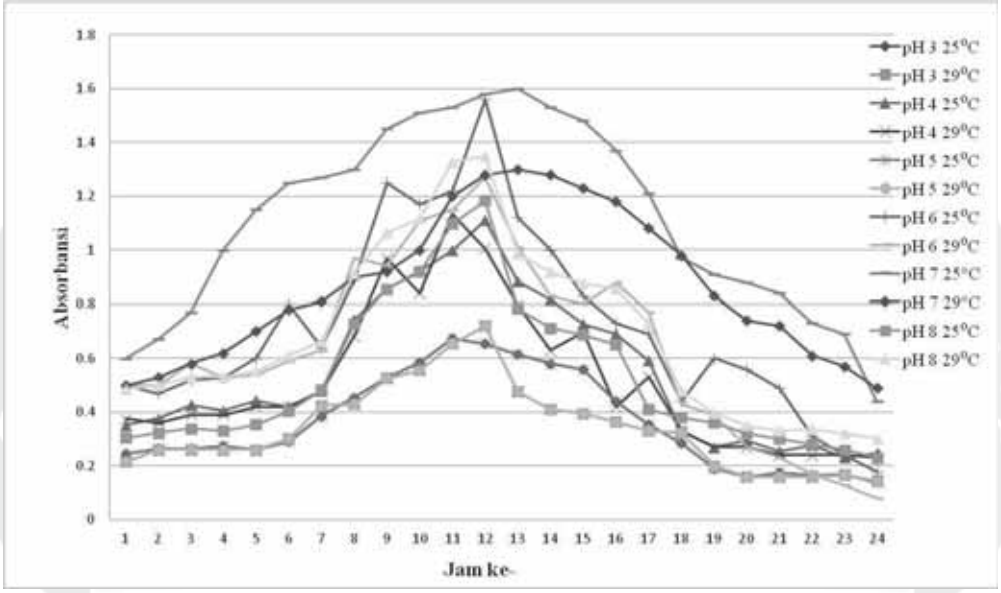

Figure 1. Bacterial growth curve at pH 3-8 and a temperature of 25 ° C and 29 ° C in Medium B4 Urea

While the growth bacteria biogrout optimization test on the urine medium showed a less significant. The treatment was done up to 10x repetition. This was possible due to several factors such as pH range of urine and ammonia levels were not measured. According to Shafiee *et al*., (2003) urine contains more salt waste from the body and a little ammonia [19]. Based on the test, known biogrout bacteria can grow optimally in medium B4 urine pH 8 and a temperature of 25 ° C. Based on data analysis using the General Linear Model, the growth of bacteria in the urine B4 shows the p value less than 0.05 ($p < 0.05$, ā (alpha) of 5%.). The figure shows that the urine contained in the medium B4 significantly affect the growth of bacteria.

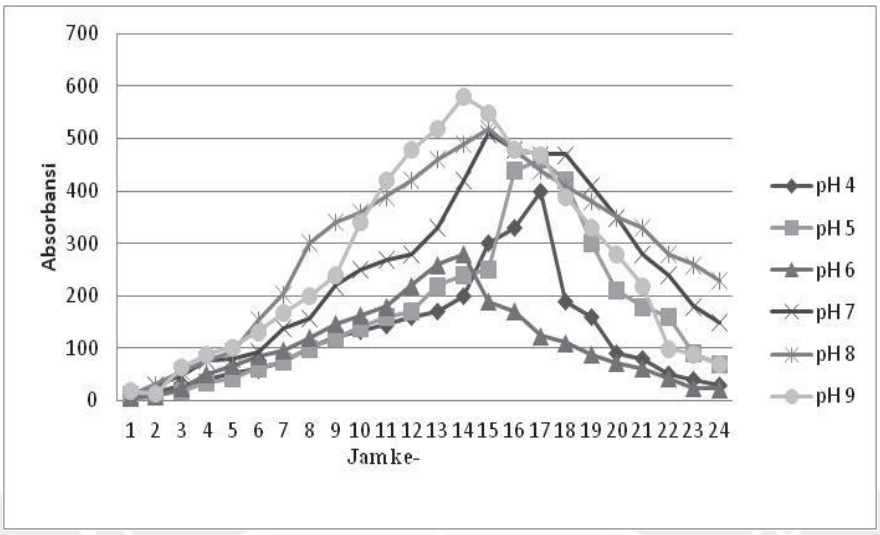

Figure 2. The growth curve Bacteria in Urine B4 Medium

Urease activity was measured based on optimum conditions bacterial growth. Based on the analysis of data, the growth of bacteria known optimal time on 12th and 13th. The time assumed to be a potential to produce urease. Based on the measurement of enzyme activity using Bradford methods known 144 units / ml. While the measurement of enzyme activity using methods Weatherburn (1967) obtained urease activity of 203.32 units / ml.

3.2 Isolation And Purification of Urease

3.2.1. Protein Precipitation Using Ammonium Sulfate

The purpose of precipitation is to separate crude protein containing urease ekstract of compounds other impurities. Protein precipitation method using ammonium sulfate can inhibit the growth of microorganisms. According to Fujimoto *et al*., (2002) protein should be precipitated through centrifugation stage because of the density of a saturated solution of low value [20].

Based on the results ammonium sulfat precipitation, proteins could be separated in the test solution. Urease did not indicate the concentration of ammonium sulfate precipitation at 40%. Urease significant precipitation occurs in ammonium concentration between 40% to 90% (Figure 3). At a concentration of 90% ammonium sulfate precipitation the greatest, but for better separation of these enzymes used concentrations from 40% -80%. The solubility of the protein will continue to increase in line with the increase in salt concentration. The higher the concentration of salt will decrease the solubility of the protein [21]. Having obtained precipitate proteins were tested using the Bradford test. Measurement of protein concentration using the Bradford made based on the value of the maximum absorbance. Reagents used Coomasiie Brilliant Blue G-250. Then the solution was measured using a spectrophotometer with a wavelength of 595nm. Urease activity measurement of results using ammonium sulfate precipitation. Highest urease activity reached 144 units / ml and lows 5,36 units / ml. Urease activity in units / ml means that 1 unit of enzyme needed to liberate 1 mol NH3 from urea per minute under standard conditions [11].

3.2.2. Isoelectric Point

The isoelectric point is a specific condition which the protein does not have the charge or the difference between the number of positive and negative, so it does not move when placed in an electric field. Isoelectric point of the enzyme (crude extract) medium B4 and B4 urine showed at pH 6 (Figure 4). This indicated by precipitation at most after heating.

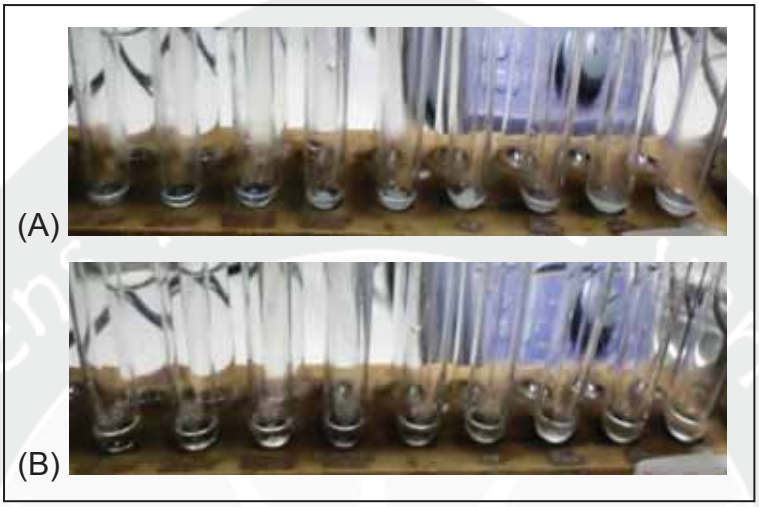

Figure 3. (A) Isoelectric point B4 urea medium (B) Isoelectric point urine B4 medium

3.2.3 Characterization Of Urease

SDS-PAGE electrophoresis

Protein characterization using SDS-Page aims to determine the molecular weight (MW). Proteins have been treated with a strong detergent containing ions such as sodium dodesyl sulphate (SDS) and the reducing agent will experience elimination of structures [13]. After electrophoresis, the protein can be visualized with a dye that binds to protein premises [22].

Based on the protein bands were seen in polyacrylamide gel with 7% separating gel, 3% stacking gel, the power supply voltage of 120 volt 28 A and electropherogram with coomasie blue gel staining obtained nine protein bands with molecular weight of 440-500 kDa (Figure 5). Ribbons were at least identical molecular weight of 440 kDa. 7% acrylamide separating gel used to separate proteins with a molecular weight of 100-500 kDa protein. This was according to research conducted by Jones and Mobley (1989) that the molecular weight urease is 440-480 kDa [23].

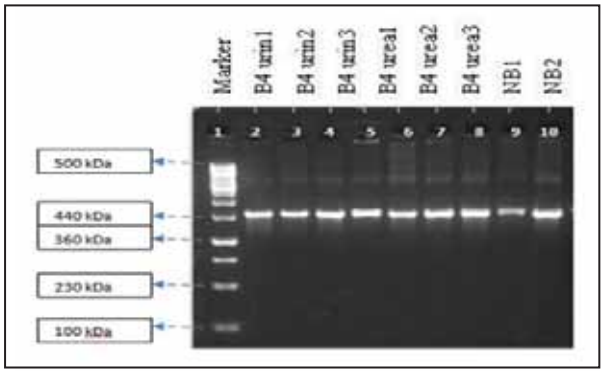

Figure 4. SDS-PAGE

3.3 Urease Production

Enzyme used for applications biogrouting was enzyme that had the highest activity in the optimization phase. Based on the analysis, the highest urease activity produced by bacteria grown on medium urea B4 of 144 units / ml, while the isolates were grown on urine B4 of 51.74 units / ml. Researchers conducted a production by growing isolates in 2 variations medium B4 100ml each medium. Based on the research design isolates were grown in liquid medium B4 1500 mL and 1500 mL of urine medium. Then incubated using a fermenter (5L size) for 5 days set temperature, pH and optimal medium (according the data optimization urease). Fermentor used a modification of Renge *et al*., (2012) [24]. It could not be done because there were damage to the appliance and the limited number of medium fermentor. 100ml scale production conditioned in accordance with the optimum growth of bacteria (Figure 5). In B4 medium, bacteria grew optimally at a pH of 7 temperature of 25 ° C while the medium B4 urine optimal at pH 8 (same temperature).

Figure 5. Production of urease

3.4 Application Urease on Biogrouting

Applications biogrouting done with direct injection method (De Jong *et al*., 2006) (Figure 7) [25]. Total of 10ml urease 200gr sand injected at saline conditions (Figure 6). Based on the observation of the application parameters, sand pH increased from pH neutral (7) to an alkaline pH (11). The formation of the mineral calcite visually, and sand compaction process. Negative controls used are sand without injected urease. Based on observations, sand grain change to sandstone caused by the presence of carbonate results of the activity of bacteria that become the bridge cementation between grains of sand. There is a difference between the results of control (not injected urease). The cementation speeds allegedly caused by the size of the urease activity produced.

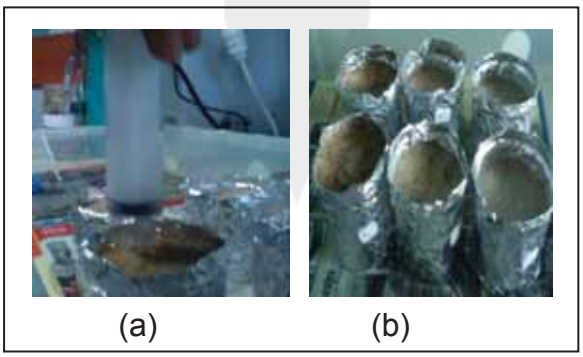

Figure 6. Application biogrouting

4. CONCLUSIONS

Based on the results of this research concluded that bacteria grew optimum at B4 medium in pH 7 and temperature 25 °C, while in the medium B4 urine biogrout bacteria optimum growth at 25 ° C and pH 8. Measurement of urease activity reached 144 units / ml. Based on the characterization of proteins using ammonium sulfate precipitation protein maximal concentration at 90% (203.3 units / ml). Isoelectric point urease known at pH 6 and had a molecular weight of 440-500 kDa. Urease can be used as a grout material because it had the ability of cementation (diagenesis) on simple application biogrouting use sea sand with saline conditions.

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α-Glucosidase Inhibitors from Indonesian Indigenous Plants, *Pluchea indica* L. leaves and *Caesalpinia sappan* Wood

*Ines Septi Arsiningtyas[1] , Eisuke Kato[1] , Jun Kawabata[1]
[1]Division of Applied Bioscience, Graduate School of Agriculture, Hokkaido University, Sapporo, Japan
Email address: inessepti@gmail.com

Abstract

A promising approach for treating diabetes mellitus (DM) is to decrease postprandial hyperglycemiaα by suppressing carbohydrate digestion using - glucosidase inhibitors. This work has demonstrated the effective finding of potential Indonesian medicinal plants, *Pluchea indica* leaves and *Caesalpinia sappan* wood, as alternative for antihyperglycemic source. Aqueous methanol extracts of twenty-eight Indonesian indigenous herbs were used for searching active compounds against intestinal maltase. Among them, *P. indica* leaves and *C. sappan* wood showed high inhibitory activity against intestinal maltase. Thus, *P. indica* and *C. sappan* were chosen for further investigation to identify the active principles responsible for an antihyperglycemic effect through the inhibition of intestinal maltase. Chemical identification of the active principles was done by in vitro assay-guided isolation and extensive instrumental analyses. This study provides basic information for further examination the suitability of *P. indica* and *C. sappan* as a functional food, alternative medicines and complementary therapies for diabetes prevention and management.

1. INTRODUCTION

The goal for the treatment of DM is to restore the normal glucose level in blood of diabetes patient. As the first-line treatment for diabetic individuals with postprandialα hyperglycemia, commercial -glucosidase inhibitors such as acarbose and voglibose considered to be used.[1] It would delay the digestion and absorption of carbohydrates and consequently suppress hyperglycemia.

However,α these -glucosidase inhibitors have prominent gastrointestinal side effects like flatulence, diarrhea, and abdominal discomfort.[2] This warrants the search for alternative natural herbal medicines that have fewer side effects than the available commercial inhibitors.

Pluchea indica (L.) Less (Asteraceae) is widely distributed in SoutheastAsia.[3] Extracts of P. indica parts have anti-oxidant, anti-ulcer, anti-nociceptive, anti-diuretic, and anti-inflammatory properties.[4567] In Thailand, *P. indica* leaves are used as tea because they are believed to have an indigenous remedy due to their anti-diabetic properties. It has been reported for its anti-diuretic and anti-diabetic pharmacological effects in streptozocin-induced rats.[8] Prior chemical investigations of *P. indica* have led to the isolation of several terpenes, lignin glycosides and terpenic glycosides from the aerial part.[9] The methanolic extract of the *P. indica* roots led to the isolation of an

alkynylthiophene derivative, 2- (prop-1-ynyl)-5(5,6-dihydroxyhexa-1,3-diynyl) thiophene, for its anti-amoebic activity.[4]

Much attention has been focused also on the use of *Caesalpinia sappan* L. (Leguminosae) wood as an ingredient in a functional drink with antihyperglycemic properties.[10] The wood of *C. sappan* is native to many parts of Southeast Asia and has been cultivated in Africa and the U.S.A.[11] Owing to its pleasing fragrance and color,[12] *C. sappan* has been used in many traditional cultures; for example, it is used as an ingredient of a refreshing beverage called Bir Pletok in Indonesia.[13]

Recent scientific studies have also shown that it is valuable for use in foods. *C. sappan* wood extract can be used as an additive to prevent lipid oxidation in beef patties[14], to inhibit bacterial growth for up to 6 months in chilli paste.[15] In addition to its use in beverages and foods, a decoction of the bark and wood is used as a natural astringent for the treatment of tuberculosis, diarrhea, and dysentery.[16]

Based on screening experiments, *P. indica* and *C. sappan* was found to be one of αpromising source of -glucosidase inhibitors. However, the active compounds in *P. indica* leaves and *C. sappan* wood responsibleα for - glucosidase inhibition are unknown. Therefore, this study was performed to discover the compounds responsibleα for the inhibition of -glucosidase, specifically of intestinal maltase, using an *in vitro* assay-guided approach.

2. METHODS

2.1 Materials

P. indica leaves (batch number B0107.002) and *C. sappan* wood (batch number CS1314.17) were purchased from Merapi Farma Traditional Herbs Distributor, Yogyakarta, Indonesia. The voucher specimens were deposited with the distributor. Rat intestinal acetone powder (Sigma Aldrich Japan Co.) was used as the enzyme source. Cosmosil 75C18-OPN (Nacalai Tesque. Inc) as reverse phase ODS gel. All chemicals used were analytical grade and were purchased from Wako Pure Chem. Co. unless otherwise stated.

2.2. Procedures

2.2.1αRat intestinal -glucosidase inhibitory activity assay

Rat intestinal maltase inhibitory activity was determined using the described method,[17] with minor modifications. Rat intestinal acetone powder was dissolved in 0.1 M potassium phosphate buffer (pH 7.0) containing 5 mM ethylenediaminetetraacetic acid (EDTA), homogenized, and centrifuged at 10,600 x g for 30 min at 4°C. The supernatant was collected and used as the crude enzyme solution. The maltose-hydrolyzing activity of the crude enzyme solution (2.33 U/mg protein) was designated as maltase activity.

Inhibition of maltose hydrolysis was measured using the following procedure: Test tubesμ containing 350 L maltose solution (3.5 mM) in potassium phosphate buffer (0.1 M, pH 6.3) were preincubated at 37°C for 5 min. Plant extracts were dissolved in 50% dimethylμ sulfoxide (DMSO, 100 L) and added to preincubated tubes. Control tubes containedμ 50% DMSO alone (100 L). The reaction was started by the addition of crude rat intestinal glucosidase solution (50 μL). Test tubesμcontaining 400 L potassium phosphate buffer (0.1 M, pH 6.3) served as blanks. After incubation for 15

min at 37°C, the reaction was stopped by adding 0.75 mL of 2 M Tris HCl buffer (pH 7.0). The reaction mixture was passed through a short ODS column (Cosmosil 75C_{18} -OPN,ϕ×5 55 mm) to remove phenolic compounds that might interfere with the glucose quantification. The amount of liberated glucose was measured by the glucose oxidase method using a commercial test kit (Glucose CII-test Wako, Wako Pure Chem. Co.) in 96-well microplates at 37°C for 30 min. The optical density (OD) of the wells was measured at 490 nm using Synergy™ MX microplate reader (Biotek Instruments,Inc.). Inhibitory activity was calculated using the following equation (1):

$$\text{Inhibitory activity (\%)} = [1- (OD_{sample}-OD_{sample\ blank})/(OD_{control}-OD_{control\ blank})] \times 100 \quad (1)$$

All experiments were performed in duplicate. Acarbose, which had 50% inhibitory activity against maltase at 0.5 μM concentration, was used as the positive control

2.2.2 Instrumental analysis

^{1}H-NMR spectra were recorded using JEOL JNM-EX-270 (^{1}H, 270 MHz; ^{13}C, 67.5 MHz, JEOL Ltd.) and Bruker AMX500 spectrometers (^{1}H, 500 MHz; ^{13}C, 125 MHz, Bruker Biospin Co.). Chemical shifts were calculated from the residual solvent signal (DMSO-d_6, δ_H 2.49, δ_C 39.5; methanol-d_4 , δ_H 3.30; acetone-d_6, δ_H 2.05, δ_C 29.8). Mass spectra were obtained using a Thermo Scientific Exactive spectrometer (Thermo Fisher Scientific K.K.) or a Waters LCT Premier XE spectrometer (Waters Co.). High-pressure liquid chromatography (HPLC) was performed with a JASCO 802-SC system (JASCO Co.).

2.2.3 Isolation of intestinal maltase inhibiting principles from *P. indica* leaves

Compounds were extracted from dried leaves of P. indica (94 g) with 50% Aqueous methanol for 24 h at room temperature and filtered. Evaporation of the solvent under reduced pressure yielded a 50% methanol extract (16.18 g). This extract was partitioned between ethyl acetate (EtOAc) and water. Each layer was evaporated at reduced pressure to produce a EtOAc-soluble fraction (2.98 g) and water-soluble fraction (13.2 g).

The EtOAc-soluble fraction was applied to a silica gel (Silica Gel 60 N, Kanto Chemical Co. Inc. Tokyo, Japan) column (ϕ 3× 42 cm) with a chloroform - methanol gradient and yielded 8 fractions. The eluent (volume and dry weight of eluate) of each fraction was as follows: fraction 1, chloroform (300 ml, 19 mg); fraction 2, 10% methanol in chloroform (150 ml, 27 mg); fraction 3, 10% methanol in chloroform (150 ml, 767 mg); fraction 4, 15% methanol in chloroform (150 ml, 196 mg); fraction 5, 15% methanol in chloroform (150 ml, 128 mg); fraction 6, 20% methanol in chloroform (150 ml, 189 mg); fraction 7, 20% methanol in chloroform (150 ml, 125 mg); and fraction 8, methanol (500 ml, 1.51 g). Fraction 8 was active and further fractionated using ODS column chromatography (Cosmosil 75C18-OPN,φ×**3**0 cm) with a water -methanol gradient and yielded 9 fractions. The eluent of each fraction was as follows: fraction 8-1, 25% methanol in water (200 ml, 110 mg); fraction 8-2, 25% methanol in water (200 ml, 221 mg); fraction 8-3, 25% methanol in water (200 ml, 94 mg); fraction 8-4, 50% methanol in water (200 ml, 342 mg); fraction 8-5, 50% methanol in water (200 ml, 189 mg); fraction 8-6, 50% methanol in water (200 ml, 57 mg); fraction 8-7, methanol (200 ml, 72 mg); fraction 8-8 methanol (200 ml, 63 mg);

and fraction 8-9, methanol (200 ml, 43 mg). Fractions 8-4 and 8-5 were active and further purified by reversed-phase preparative HPLC. Fraction 8-4 was subjected to preparative HPLC (column: Inertsil PREP-ODS,×25020mm,.0 GL Science Inc.; mobile phase, water-methanol 60:40 to 40:60 (0-50 min) containing 0.1% of formic acid; flow rate 5 ml/min; detection: UV 254 nm) and yielded 10 mg of 3,5-di-O-caffeoylquinic acid (1, t_R 32 min), 75 mg of 4,5-di-O-caffeoylquinic acid methyl ester (2, t_R 35 min), 42 mg of 3,4,5-tri-O-caffeoylquinic acid methyl ester (3, t_R 47 min), and 41 mg of 1,3,4,5-tetra-O-caffeoylquinic acid (4, t_R 50 min). Fraction 8-5 was subjected to preparative HPLC in a manner similar to the purification of fraction 8-4, except water:methanol (60:40) containing 0.1% of formic acid was used as a mobile phase. Fraction 8-5 yielded 19 mg of 3,4,5-tri-O-caffeoylquinic acid (5, t_R 17.5 min). The purity of the material was confirmed by HPLC and NMR analyses.

3,5-di-*O*-caffeoylquinic acid (**1**), off-white powder, ESI-TOF-MS (negative) m/z 515.13 [M-H]-; 1H NMR *δ* (methanol-d4): 2.12-2.33 (4H, m, 2 and 6-H), 3.96 (1H, dd, *J* = 7.3 Hz, 3.5 Hz, 4-H), 5.41 (1H, m, 5-H), 5.42 (1H, m, 3-H), 6.26 and 6.34 (each 1H, d, *J* = 16.0 Hz, 2 ×8'-H), 6.77 and 6.88 (each 1H, d, *J* = 8.0 Hz, 2 × 5'-H), 6.95 and 6.96 (each 1H, brd, *J* = 8.1 Hz, 2 ×6'-H), 7.05 and 7.06 (each 1H, d, *J* = 2.2 Hz, 2 × 2'-H), 7.57 and 7.61 (each 1H, d, *J* = 16.0 Hz, 2 × 7'-H). Compound **1** was identified by comparison with literature data.18

4,5-di-*O*-caffeoylquinic acid methyl ester (**2**), light yellow powder, ESI-MS (negative): *m/z* 529.14 [M-H]-; 1H NMR *δ* (methanol-*d*4): 2.07 and 2.32 (2H, dd, *J* = 16.0 Hz, 5.0 Hz, 2-H), 2.28 (2H, m, 6-H), 3.71 (3H, s, OCH3), 4.40 (1H, m, 3-H), 5.12 (1H, dd, *J* = 8.2 Hz, 3.2 Hz, 4-H), 5.54 (1H, ddd, *J* = 13.0 Hz, 8.2, 4.0 Hz, 5-H), 6.16 and 6.28 (each 1H, dd, *J* = 16.0 Hz, 2 × 8'-H), 6.75 (2H, d, *J* = 8.2 Hz, 2 × 5'-H), 6.90 and 6.91 (each 1H, dd, *J* = 8.2 Hz, 2.0 Hz, 2 × 6'-H), 7.00 and 7.02 (each 1H, *J* = 2.0 Hz, 2 × 2'-H), 7.50 and 7.60 (each 1H, d, *J* = 16.0 Hz, 2 × 7'-H). Compound **2** was identified by comparison with literature data.19

3,4,5-tri-*O*-caffeoylquinic acid methyl ester (**3**), light yellow powder, ESI-MS (negative): *m/z* 691.17 [M-H]-; 1H NMR (methanol-*d*4) δ: 2.13 and 2.84 (each 1H, m, 2 × 6-H), 2.39 and 2.45 (each 1H, dd, *J* = 13.5 Hz, 3.8 Hz, 2-H), 3.73 (3H, s, OMe), 5.33 (1H, dd, *J* = 7.6 Hz, 3.0 Hz, 4-H), 5.57 (1H, m, 3-H), 5.64 (1H, m, 5-H), 6.19, 6.23, and 6.29 (each 1H, d, *J* = 16.0 Hz, 3 × 8'-H), 6.71, 6.75, and 6.75 (each 1H, dd, *J* = 8.2 Hz, 3 × 5'-H), 6.86, 6.91, and 6.91 (each 1H, dd, *J* = 8.2 Hz, 1.9 Hz, 3 × 6'-H), 7.00, 7.02, and 7.02 (each 1 H, d, *J* = 1.9 Hz, 3 × 2'-H), 7.52, 7.52, and 7.58 (each 1H, d, *J* = 16.0 Hz, 3 × 7'-H). Compound **3** was identified by comparison with literature data.20

3,4,5-tri-*O*-caffeoylquinic acid (**4**), yellowish white powder, ESI-MS (negative): *m/z* 677.12 [M-H]-; 1H NMR (methanol-d4) *δ*: 2.06-2.47 (4H, m, 2,6-H), 5.31 (1H, dd, *J* = 8.4 Hz, 3.5 Hz, 4-H), 5.67 (2H, m, 3,5-H), 6.22, 6.23, and 6.29 (each 1H, d, *J* = 16 Hz, 3 × 8'-H), 6.68-6.85 (4H, m, 3 × 5'-H, 6'-H), 6.92 (2H, m, 2 × 6'-H), 6.98, 7.02, and 7.06 (each 1 H, br s, 3 × 2'-H), 7.51, 7.53, and 7.60 (each 1H, d, *J* = 16 Hz, 3 × 7'-H). Compound **4** was identified by comparison with literature data.21

1,3,4,5-tetra-*O*-caffeoylquinic acid **(5**), yellowish-white amorphous powder, ESIMS (negative): *m/z* 839.18 [M-H]-; 1H NMR (methanol-*d*4) *δ*: 2.17 and 2.73 (2H, br d, *J* = 13.0 Hz, 6-H), 2.60 (1H, br dd, *J* = 16.0 Hz, 2.0 Hz, 2ax-H), 2.94 (1H, m, 2eq-H), 5.37 (1H, dd, *J* = 10.3 Hz, 3.6 Hz, 4-H), 5.73 (1H, m, 3-H), 5.86 (1H, ddd,

J = 11.5 Hz, 10.3 Hz, 4.5 Hz, 5-H), 6.15, 6.24, 6.37, and 6.37 (each 1H, d, *J* = 15.7 Hz, 4 × 8'-H), 6.05, 6.66, 6.73, and 6.73 (each 1H, d, *J* = 8.2 Hz, 4 ×5'-H), 6.78, 6.78, 6.92, 6.92 (each 1H, dd, *J* = 8.2 Hz, 2.2 Hz, 4 × 6'-H), 6.67, 6.94, 7.01, 7.07 (each 1H, d, *J* = 2.2 Hz, 4 × 2'-H), 7.47, 7.49, 7.57, and 7.65 (each 1H, d, *J* = 15.7 Hz, 4 × 7'-H). Compound **5** was identified by comparison with literature data.22

2.2.4 Isolation of intestinal maltase inhibiting principles from *C.sappan* wood

Dried *C. sappan* wood (200 g) was twice extracted at room temperature

With 50% aqueous methanol for 24 h per extraction. The crude extract was filtered through a filter paper (Whatman No. 5C, 110-mm) and evaporated under reduced pressure below 40 °C to obtain the methanol extract (14.5 g, yield 7.23% based on dried wood). This residue was partitioned between ethyl acetate (EtOAc) and water. Each layer was evaporated under reduced pressure to yield an EtOAc-soluble fraction (11.6 g) and water-soluble fraction (2.4 g). Material from the EtOAc-soluble fraction was found to have maltase inhibitory activity.

The EtOAc-soluble fraction was separated on a silica gel (Silica Gel 60 N,Kanto Chemicalϕ×316cm)Coto., Incyield.) columnfivefractions (The fractions were obtained using the following solvent systems (chloroform:MeOH): 19:1 (Fr 1), 9:1 (Fr.2), 4:1 (Fr.3), 1:1 (Fr.4), and 0:1 (Fr.5).

Fractions 2 and 3 were found to be active and were further separated on a silicaϕ×3gel9cm) column. Eluting (fraction 2 with a 1:1 mixture of EtOAc and hexane yielded three fractions (Fr.2-1 to 2-3), eluting with EtOAc yielded six fractions (Fr.2-4 to 2-9), and eluting with MeOH yielded one fraction (Fr.2-10).

Fraction 3 was fractionated using the same method to give Fr.3-1 to 3-10. Fractions 2-4, 3-4, and 3-5 were found to have inhibitory activity. These fractions were further fractionated by preparative HPLC using a reversed-phase ODS columnϕ (InertSustain C18, 20×250 mm, GL Sciences Inc.) monitored at 220 nm. Fraction 2-4 was purified using a methanol-water solvent system as follows: isocratic 30:70 (0–30 min), 30:100 to methanol (30–60 min) as a gradient system.

Fraction 2-4 yielded brazilin (60 mg; t_R, 18 min), protosappanin C (240 mg; t_R, 19–24 min), and sappanchalcone (20 mg; t_R, 79 min). Fractions 3-4 and 3-5 were purified in a similar manner to Fraction 2-4 to give protosappanin B (1000 mg; t_R, 27 min). Protosappanins B and C existed as a mixture of conformers (B and C) and/or in the hemiacetal form (C) in the HPLC eluates as discussed below. Brazilin (**6**), ESI-TOF-MS (negative): *m/z* 285.08 $[M-H]^-$; ^{1}H NMR (500 MHz, methanol-d_4) *δ*: 2.76 (1H, d, *J* = 15.8, H-7), 2.92 (1H, d, *J* = 15.8, H-7), 3.69 (1H, d, *J* = 11.3, H-6), 3.92 (1H, d, *J* = 11.3, H-6), 3.96 (1H, s, H-12), 6.29 (1H, d, *J* = 2.5, H-4), 6.47 (1H, dd, *J* = 8.3, 2.5, H-2), 6.60 (1H, s, H-11), 6.71 (1H, s, H-8), 7.18 (1H, d, *J* = 8.3, H-1). Compound **6** was identified by comparison with literature data.[13]

Sappanchalcone (**7**), ESI-TOF-MS (negative): *m/z* 285.08 $[M-H]^-$; ^{1}H NMR (500 MHz, methanol-d_4) *δ*: 3.90 (2'-OMe), 6.50 (1H, dd, *J* = 2.2 Hz, 8.5 Hz, H-5'), 6.56 (1H, d, *J* = 2.2 Hz, H-3'), 6.83 (1H, d, *J* = 8.2 Hz, H-5), 7.02 (1H, dd, *J* = 2.2, 8.1, H-6), 7.18 (1H, d, *J* = 2.2, H-2), 7.47 (1H, d, *J* = 15.8, H-α), 7.47 (1H, d, *J* = 15.8, H-β), 7.58 (1H, d, *J* = 8.5, H-6'). Compound **7** was identified by comparison with literature data.[23]

Protosappanin B (**8**), FD-MS: *m/z* 304.11 $[M]^+$; ^{1}H NMR (500 MHz, acetone-d_6) *δ*: 2.58–

2.78 (4H, each H *J* = 13 Hz, H-8), 3.20–4.38 (2H, each H *J* = 11.19 Hz, 7- CH_2OH), 3.20–4.38 (4H, each *J* = 12 Hz, H-6), 6.50–6.58 (2H, d, *J* = 2.5 Hz, H-4), 6.60–6.65 (2H, dd, *J* = 8.0, 2.5 Hz, H-2), 6.73–6.87 (4H, s, H-9 & H-12), 7.02 (2H, d, *J* = 8.0, H-1); ^{13}C NMR (acetone-d_6) *δ*: 42.69/39.98 (C-8), 65.81/68.19 (7-CH2OH), 72.80/72.32 (C-7), 77.29/76.05 (C-6), 108.98/108.17 (C-4),111.91/111.23 (C-2), 117.38/117.51 (C-9), 119.03/119.77 (C-12), 125.28/123.48 (C-8a), 128.45/127.40 (C-12b), 131.86 (C-12a), 134.34/132.45 (C-1),

144.65/144.65 (C-11), 144.80 (C-10), 158.93/159.31 (C-3), 160.68 (C-4a). Compound **8** was identified by comparison with literature data.[24,25]

Protosappanin C (**9**), FD-MS: *m/z* 302 $[M]^+$; ^{1}H NMR (270 MHz, DMSO-d_6, 90°C) *δ*: 3.18 (2H, s, H-8), 3.46–4.14 (2H, m, H-6), 6.94 (1H, d, J=8.3, H-1), 9.67 (1H, s, -CHO). ^{13}C NMR (DMSO-d_6, 90°C, 67.5 MHz) *δ*: 39.80 (C-8), 72.39 (C-6), 75.18 (C-7), 106.80 (C-4), 110.51 (C-2), 116.55 (C-12), 118.75 (C-9), 121.34 (C- 8a), 124.04 (C-12b), 130.30 (C-12a), 131.70 (C-1), 143.40 (C-10), 143.96 (C-11), 157.39 (C-3), 157.73 (C-4a), 202.68 (-CHO). Compound **9** was identified by comparison with literature data.[26]

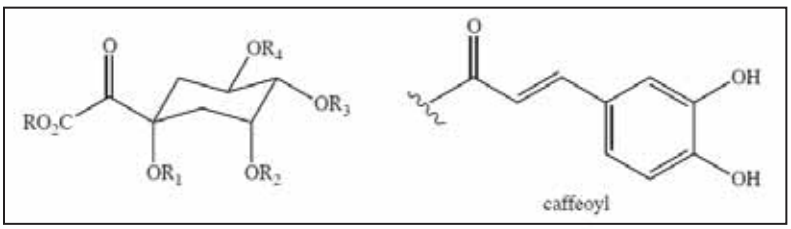
3. RESULTS AND DISCUSSION

3.1 Intestinal maltase inhibitors from *P. indica* leaves

P. indica leaves showed the potential intestinal maltase inhibition. The 50% aqueous methanol extract of *P. indica* leaves was partitioned between ethyl acetate (EtOAc) and water. The EtOAc fraction was subjected to silica gel column chromatography using chloroform-methanol gradient and the active fraction was further fractionated using ODS column chromatography. The active ODS fractions were further purified using preparative HPLC resulting in the isolation of five caffeoylquinic acid derivatives: 3,5-di-*O*-caffeoylquinic acid (**1**), 4,5-di-*O*-caffeoylquinic acid methyl ester (**2**), 3,4,5-tri-*O*-caffeoylquinic acid methyl ester (**3**), 1,3,4,5-tetra-*O*-caffeoylquinic acid (**4**), and 3,4,5-tri-*O*-caffeoylquinic acid (**5**).

The structure of all isolates (**1**–**5**) was determined using ESI mass and 1H- NMR spectra. The NMR pattern of all compounds showed characteristic signals of caffeoylquinic acids and their structures were finally confirmed by comparing with published spectral data. The structures of the isolated compounds are shown in

Figure 1. This finding is consistent with the hypoglycemic effect of this plant extract on streptozotocin-induced diabetic rats.[8]



3,5-Di-*O*-caffeoylquinic acid (**1**) : R = R_1= R_3= H, R_2 = R_4 = caffeoyl 4,5-Di-*O*-caffeoylquinic acid methyl ester (**2**) : R=Me, R_1=R_2=H, R_3=R_4=caffeoyl 3,4,5-Tri-*O*-caffeoylquinic acid methyl ester (**3**) : R=Me, R_1=H, R_2=R_3=R_4=caffeoyl 1,3,4,5-Tetra-*O*-caffeoylquinic acid (**4**) : R=H, R_1 =R_2=R_3=R_4=caffeoyl 3,4,5-Tri-*O*-caffeoylquinic acid (**5**) : R=R_1=H, R_2=R_3=R_4=caffeoyl

Figure 1. Intestinal maltase inhibitors isolated from *P. indica* leaves (1 – 5)

This is the first report for the identification of compounds **1**–**3** from *P. indica*, whereas compounds **4** and **5** were recently isolated as constituents for this plant.[27] Interestingly, the 3,5-isomer of dicaffeoylquinic acid was isolated as a free form (**1**), whereas the 4,5-isomer was isolated only as a methyl ester (**2**). This suggests that methyl esterification selectively occurs in plant tissues.

The isolated caffeoylquinic acid derivatives were compared for their rat intestinal maltase inhibitory activity (Table 1). Half maximal inhibitory concentration (IC_{50}) values were used as measures of the effectiveness of each compound to inhibit maltase function. On the basis of IC_{50} values, compound **3** had the highest inhibition level among the caffeoylquinic acid derivatives isolated from *P. indica* leaves, followed by **5**, **4**, **2**, and **1** respectively. Compounds **1** and **5** have been previously reported as maltase inhibitors.[28] However, compounds **2**, **3**, and **4** have not been evaluated for their intestinal maltase inhibitory activity.

Table 1. IC_{50} values for the inhibition of intestinal maltase by isolates of *P. indica* leaves (1–5) and acarbose

Compounds	IC_{50} (μM)	Yield (mg) *
3,5-di-*O*-caffeoylquinic acid (**1**)	1166	10
4,5-di-*O*-caffeoylquinic acid methyl ester (**2**)	208	75
3,4,5-tri-*O*-caffeoylquinic acid methyl ester (**3**)	2	42
1,3,4,5-tetra-*O*-caffeoylquinic acid (**4**)	13	41
3,4,5-tri-*O*-caffeoylquinic acid (**5**)	11	19
Acarbose	0.5	-

*Yield is calculated from 94 g leaves.

This suggests that, when comparing the inhibitory activity of compounds **1**, **4**, and **5**, increasing numbers of caffeoyl groups attached to quinic acid moiety enhanced maltase inhibitory activity. This is consistent with a previous study in which the caffeoyl group played an important role in intestinalα -glucosidase inhibitory activity.[28] Among the compounds with the same number of caffeoyl groups, compounds **2** and **5** had five-fold higher inhibitory activities than compounds **1** and **3**, respectively. This suggests that methyl esterification of the carboxylic group in quinic acid has an additional effect of enhancing maltase inhibitory activity. This is the first report for the importance of the methyl esterification ofα caffeoylquinic acids with respect for their -glucosidase inhibitory activity, although the mechanism still remains undetermined.

In the present study, increases in maltase inhibitory activity were due to both the number of caffeoyl groups attached to the quinic moiety and to the presence of methyl esterification on the carboxyl group. Considering the yields and IC50 values of the caffeoylquinic acid derivatives isolated, the inhibitory activity of P. indica leaves is likely to be due to compounds **3**, **4**, and **5**, although compounds 1 and **2** may also contribute to the activity. According to the intestinal maltase inhibitory effect of acarbose, IC_{50} value of compound **3** is 4-fold higher compared to acarbose.

Caffeoylquinic acid derivatives from this plant may be important medicinal substances, which may delay postprandial hyperglycemia. *P. indica* is an indigenous

plant that may potentially be used as a functional food or as a medicinal supplement for DM treatment and prevention. Further studies are needed to address the potential function of di-, tri-, and tetra -*O*-caffeoylquinic acid.

3.2 Intestinal maltase inhibitors from *C. sappan* wood

In this study, the constituents in *C. sappan* wood that are able to inhibit intestinal maltase were isolated and identified using enzyme assays and chromatographic techniques. The inhibitory activity was found in the ethyl acetate soluble fraction of the initial 50% methanol extract. Fractionation and purification was done using silica gel chromatography and preparative HPLC to yield four active compounds: brazilin (**6**), sappanchalcone (**7**), protosappanin B (**8**), and protosappanin C (**9**). The structures of the isolated compounds are shown in Figure 2. Brazilin (**6**) is a well-known homoisoflavone constituent of C. sappan[29], and sappanchalcone (**7**) is considered to be its biogenetic precursor.[30] Protosappanins B (**8**) and C (**9**) have also been identified.[25,26]

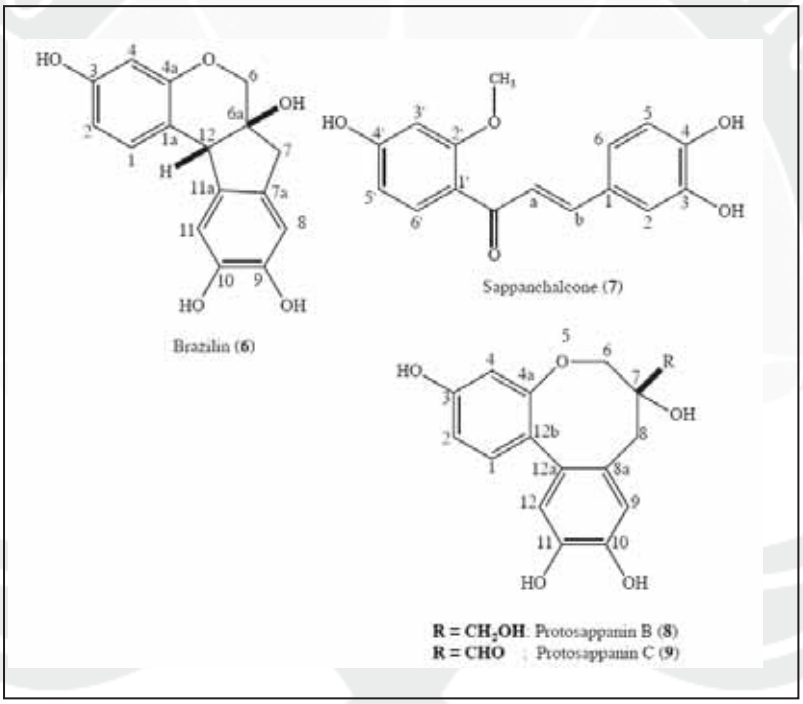


Figure 2. Intestinal maltase inhibitors isolated from *C. sappan* wood

In this study, the antidiabetic potential of the widely used traditional medicine, *C. sappan* wood was investigated, since this valuable herb has not previously been investigated for antidiabetic activity relating to inhibition of carbohydrate digestive enzymes, in particular, intestinal maltase.

Compounds **7** and **8** showed relatively higher maltase inhibitory activity than **6** and **9** (Table 2). This is the first report of **7** and **8** as intestinal maltase inhibitors obtained from *C. sappan* wood, although many homoisoflavonoid-related biological activities have been identified in this plant.[31] Therefore, the use of sappanchalcone (**7**) and protosappanin B (**8**) as intestinal maltase inhibitors could be investigated further in the future. Compound **9** has the same dibenz[b,d]oxocin structure as **8** and yet did

not show significant maltase inhibitory activity. The presence of a hydroxymethyl group on the C-7 position of **8** might result in a higher maltase inhibitory activity than the aldehyde group in **9**. Considering the yield of the isolates from *C. sappan* wood, the intestinal maltase inhibitory activity of the crude extract would be arising from **8** and partially contributed by **6, 7** and **9**.

This study is the first to report on the use of active compounds from the potentially antidiabetic Indonesian herb, *C. sappan* wood to lower glucose through the αinhibition -glucosidase, in particular intestinal maltase. The inhibitory activities of the isolates are relatively low compared to commercially available glucosidase inhibitors, such as acarbose; however, considering that *C. sappan* wood is a non-endangered plant that has been widely used throughout history, this research maybe useful for the development of functional foods, alternative medicines, and complementary therapies for diabetes prevention and management.

Table 2. IC_{50} values for the inhibition of intestinal maltase by isolates of *C. sappan* wood (6–9) and acarbose

Compounds	IC_{50} value (mM)	Yield* (mg)
Brazilin (**6**)	3.83	60
Sappanchalcone (**7**)	0.96	20
Protosappanin B (**8**)	0.81	1000
Protosappanin C (**9**)	2.59	240
Acarbose	5×10^{-4}	-

*From 200 g *C. sappan* wood.

4. CONCLUSIONS

This research reveals the potency of Indonesian α medicinal plants as - glucosidase inhibitors. *C. sappan* and *P. indica* are well known indigenous plants in Indonesia. Theseα findings revealed the active principles against -glucosidase inhibition. The isolated compounds should be investigated for their selectivity in binding the individual subunits of maltase-glucoamylase and sucrase-isomaltase, for the potential of modulating on glucose release could be optimized. Further investigation related to human safety, such as *in vivo*-experiment, should be done for developing the plant use as a functional food, alternative and complementary medicine for diabetes treatment and management.

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Mixture of Sambiloto (*Andrographis panniculata* Nees.) and Salam (*Syzygium polyanthum* (Wight.) Walp.) Extract to Improve GLUT4 and PPAR-γ Expression in Hyperglicemic Wistar Rats

*Wahyu Dewi Tamayanti[1] , Ferawati[1] , Iwan Sahrial Hamid[2], Elisabeth C. Widjajakusuma[1]
[1]Department of Clinical and Community Pharmacy, Faculty of Pharmacy, Widya Mandala Chatolic University, Surabaya, Indonesia
[2]Department of Pharmacology, Faculty of Veterinary, Airlangga University, Surabaya, Indonesia
dewffua@gmail.com

Abstract

The increased prevalence of diabetes mellitus stimulates the ongoing study of molecular mechanisms of sambiloto (*Andrographis panniculata* Nees.) and salam (*Syzygium polyanthum* (Wight) Walp.) in relieving the disorder. The molecular study was done by treating hyperglicemia Wistar rats with a combination of sambiloto (AP) and salam (SP) extract. After 14 days of treatment, the rats were sacrificed and the liver organs were collected. The organs were stored in a solution of 10% buffered formalin to be blocked in paraffin afterwards. Subsequent molecular test was performed to identify GLUT4 and PPARγ gene expression by immunohistochemistry method. This study showed that administration of a combination of sambiloto and salam extracts (6:1) in rats was able to increase the expression level of GLUT4 which resembled the expression level of GLUT4 after insulin administration. It was also found that the expression level of PPARγ was increased after rats were treated by combination of AP and SP extracts (6:1). It showed that the increased expression of PPARγ resembles the expression of PPARγ after metformin stimulation. Interestingly, the administration of AP:SP extract (6:1) combination showed a level of GLUT4 expression that was not in line with PPARγ expression.

1. INTRODUCTION

The prevalence of diabetes mellitus (DM) that remains increased stimulates the ongoing study of molecular mechanisms of sambiloto and salam in relieving the disorder. In hyperglycemic condition, ROS (reactive oxygen species) from mitochondria electron transport was occurred. If this condition persisted, cellular organelles may be damaged and enzymes activities may also be disturbed. Thus, increased lipid peroxydation and insulin resistance may ultimately be stimulated. Increased level of ROS can be diminished by induction of PPAR-γ coactivator, PGC-1α (PPAR Gamma Coactivator-1a), MnSOD (manganese superoxide dismutase), and activation of AMPK (adenosine monophosphate activated protein kinase). The mechanism of action is carried out by metformin and pioglitazone. The thiazolidinedione (TZD) is another oral antidiabetic agent that acts as Peroxisome Proliferator Activated Receptorγ (PPARγ) agonis to stimulate insulin sensitivity in cells (Braissant *et al*., 1996). Increase level of PPARγ may activate GLUT4, the gene

that plays role in regulating transport of glucose to the adipose tissue and skeletal muscle, as a response of increase insulin level in the blood (Watson *et al*., 2004). It was mentioned that both sambiloto and salam are effective in reducing blood glucose level (Widjajakusuma *et al.*, 2010). Therefore, this molecular study was conducted to explore the efficacy of a mixture of sambiloto and salam extract in increasing PPARγ and GLUT4 in hyperglycemic Wistar rats.

2. METHODS

2.1. Preparation of Extracts

Fresh Andrographis paniculata Nees. herbs (AP) and Syzygium polyanthum Wight. (Walp.) (SP) leaves that obtained from Pandaan, East Java were cleaned, cut, dryed, and water extracted (Kaneria *et al*., 2012). The water extracts was spray dried in order to obtain the dried extracts.

2.2. General Procedures

Healthy Wistar rats that were aged 2-3 months, weighted 200 – 250 g, blood glucose level of 65-100 mg/dl. The rats were randomly grouped into 8 groups of 6, namely: negative control group (K I); normal control group (K II); positive control group I (K III) – insulin; positive control group II (K IV) - metformin 9 mg/kg BW; treatment group I (K V) - AP extract; treatment group II (K VI) - SP extract; treatment group III - mixture of AP:SP extracts (6:1); treatment group IV (K VIII) – AP:SP mixture extract (2:1); treatment group V (K IX) – AP:SP mixture extract (1:2); treatment group VI (K X) – AP:SP mixture extract (1:6). Dose of extract was 200 mg/kg BW. Prior to the treatment, rats were administered by monohidrate alloxan 150 mg/kg BW through intraperitoneal route in order to reach the hyperglycemic condition (blood glucose level of 300-400 mg/dl). When hyperglycemic condition was achieved, mixture of *AP and SP* extracts was given for 14 days. After 14 days of treatment, the hyperglycemic rats were sacrificed and the liver was collected. The organs were stored in a solution of 10% buffered formalin to be blocked in paraffin afterwards.

2.3. Immunohistochemistry of GLUT 4 and PPARγ

The rats livers were paraffine blocked and sliced into 3-4 µm and incubated overnight 450 °C, then deparaffined with xylene. Subsequently, washed with PBS (phosphate buffer saline) and incubated in 3 % hydrogen peroxide (H_2O_2 in methanol). The antigen retrieval was conducted by incubating the slides in the citrate buffer pH 6. The 0,1% protease was added in 37 °C, digested with 100 µg/ml proteinase K in buffer (0,01 mol/l trish HCl pH 7,8 0,005 mol/l EDTA dan 0,5 % SDS) and incubated in mouse serum. Subsequently, the normal mouse serum was cleaned by primer antibody (1 : 50) of IgG1 anti GLUT 4 and PPARγ monoclonal antibody, in separate. Afterwards, incubation with secondary antibody IgG goat anti rat was conducted, streptavidin-peroxidase was dropped, and incubated in DAB for 5-15 minutes. Counterstain with hematoxylin eosin and rehidration were conducted with addition of absolute ethanol, 95% ethanol, 80% ethanol and xylol. The medium mounting was conducted lastly by adding gliserol gelatin prior to closing by deck glass.

3. RESULTS AND DISCUSSION

3.1. GLUT 4 Expression

This study showed that administration of AP and SP mixture extracts (6:1) in rats was able to increase the expression level of GLUT4 (figure 1 in brown) which resembled the expression level of GLUT4 after insulin administration (table 1). This study indicated that the mixture extracts were able to stimulate glucose transport to the cells by the high level of GLUT4 expression similar as insulin stimulation. This result is in line to the study of Widjayakusuma *et al.,* 2010 who reported the reduction of blood glucose level in rats after administrtion with mixture extracts of Ap and Sp.

Table 1. GLUT4 Expression (n=6) GLUT4 Expression in the liver of hyperglycemic Wistar Rats

	Groups	Means±SD
K I	Negative control	179±11
K II	Normal control	51±18
K III	Insulin	231±15
K IV	Metformin	54±20
K V	Ap (*Andrographis paniculata*)	141±23
K VI	Sp (*Syzygium polyanthum*)	151±23
K VII	Ap:Sp (6:1)	234±11
K VIII	Ap:Sp (2:1)	62±18
K IX	Ap:Sp (1:2)	70±15
K X	Ap:Sp (1:6)	43±18

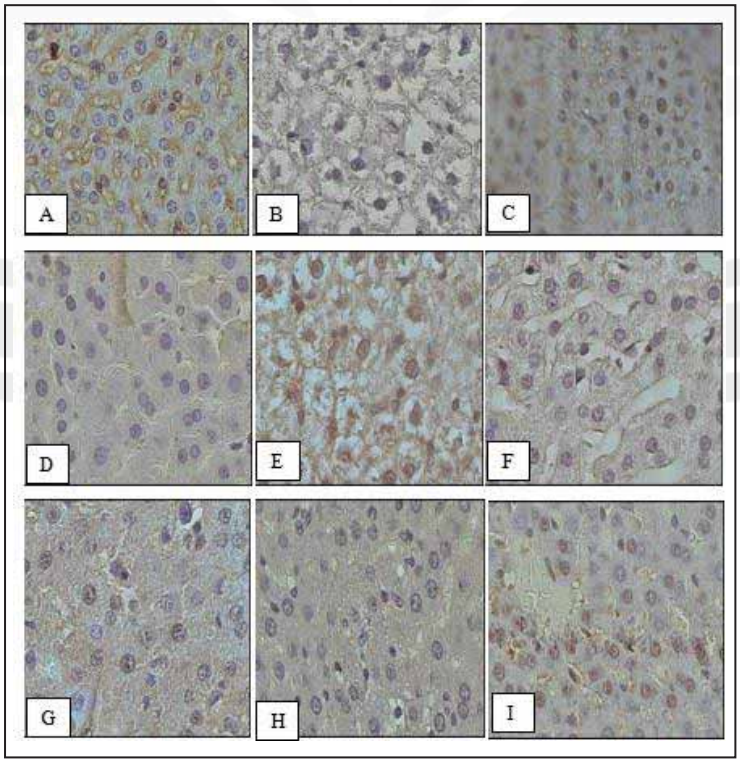

Figure 1. GLUT4 Expression in the liver of hyperglycemic Wistar Rats
(A) Negative control group **(B)** Normal group **(C)** Insulin group **(D)** Metformin group **(E)** Apgroup **(F)** Sp group **(G)** Ap:Sp=6:1 **(H)** Ap:Sp=2:1 **(I)** Ap:Sp=2:1

GLUT4 is a glucose transporter that was exspressed by adipose tissue, muscle, and liver cells (Fukumoto *et al.*, 1989). GLUT4 is activated and facilitates glucose transport to the adipose tissue and muscle when insulin hormone is released. It was proved that insulin was the main regulator of GLUT4 mRNA expression in adipose tissue (Garvey *et al.*, 1989). This study found that GLUT4 was expressed in relatively same level as insulin, thus it was predicted that active compound of AP is able to induce glucose transport by stimulating the activation of GLUT4. The expression of GLUT4 was also increase in high-fat-fructose-fed rats (Nugroho *et al*., 2011). Moreover, AP was also increase glucose cellular uptake in STZ-diabetics-induced rats by increasing the GLUT4 mRNA and protein level (Zhang *et al.*, 2009). Andrographolide, the active compound of AP also reported its ability to increase glucose uptake into the isolated ileus muscle of STZ-diabetic-induceds rat (Yu *et al*.,, 2008).

3.2. PPARγ expression

This study also showed the increased expression level of PPARγ after rats were treated by mixture of sambiloto and salam extracts (6:1). It showed that the increased expression of PPARγ resembles the expression of PPARγ after metformin stimulation (table 2).

Table 2. PPARγ Expression

Groups		Means±SD
K I	Negative control	164±11
K II	Normal control	169±9
K III	Insulin	183±8
K IV	Metformin	147±24
K V	Ap (*Andrographis paniculata*)	178±13
K VI	Sp (*Syzygium polyanthum*)	150±19
K VII	Ap:Sp (6:1)	141±12

Table 2 showed relative high expression of PPARγ that maybe indication of high reactivity of monoclonal antibody used in the immmunohistochemistry process. Moreover, this result maybe also an indication that PPARγ expression need to be studied in other organs or tissues such as adipose tissue, pancreas, or muscle tissue that are related to glucose transport. In figure 2, the PPARγ expression can be seen brown in colour.

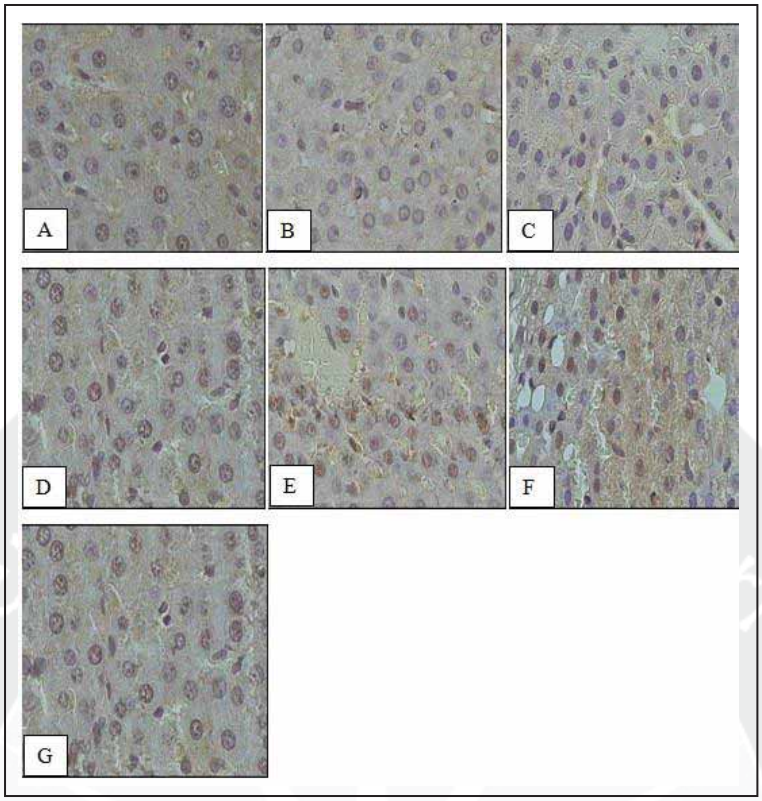

Figure 2. PPAR-γ Expression in the liver of hyperglycemic Wistar Rats
(A) Negative control group **(B)** Normal group **(C)** Insulin group **(D)** Metformin group **(E)** Ap group **(F)** Sp group **(G)** Ap:Sp=6:1

PPARγ expression expressed in adipose and muscle tissue. PPARγ promotes tcel differentiation adipose cells (Braissant *et al*., 1996). GLUT4 activation during adipogenesis is closely related to the role of PPARγ (Wu Z *et al.*, 1998). The role of PPARγ to GLUT4 expression was shown in a study of wild ginseng in reducing body weight (Ollah *et al.*, 2008). In the study, wild ginseng administration of dose 100 and 200 mg/kg for 4 weeks, revealed the lowering blood glucose level and increasing of PPARγ and GLUT4 expression, as well as insulin receptor in muscle and liver. Active compounds of SP was previously studied and revealed that campest-4-en-3-one, exhibited a significant protein tyrosine phosphatase 1B inhibitory (PTP1B) activity (Syaifudin *et al.*, 2012). PTP1B is an enzyme that found in insulin-targetting tissue (liver, muscle and adipose) that pays a role as a negative regulator in insulin signal transduction (Byon *et al.*, 1998).

Interestingly, the administration of AP and SP mixture extract (6:1) showed a level of GLUT4 expression that was not in line with PPAR-γ expression. The expressed PPAR-γ gene that were stimulated by the mixture extract maybe expressed in the different form as was previously studied, thus no relation with the level of GLUT4 was observed. These results explained that there maybe another gene expression than PPARγ was expressed and induced activation of GLUT4 following the administration of Ap and Sp mixture extract.

4. CONCLUSIONS

Mixture extracts of *Andrographis paniculata* Nees and *Syzygium polyanthum* Wight. (Walp.) able to increase GLUT4 expression in alloxanne-diabetic induced.

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PCR Detection of Early Mortality Syndrome in *Penaeus vannamei* and *Penaeus monodon* in the Philippines

Irma M. Dabu[1] , Mary Beth B. Maningas[1,2,3]

[1.] The Graduate School, University of Santo Tomas, España, Manila, Philippines
[2]Research Center for the Natural Sciences, University of Santo Tomas, España, Manila, Philippines
[3]Department of Biological Science, College of Science, University of Santo Tomas, España, Manila, Philippines
imdabu19@yahoo.com|marybethmaningas@yahoo.com | mbmaningas@mnl.ust.edu.ph

Abstract

A new, emerging disease is threatening the shrimp industry, a bacterial disease which exhibits a highly pathogenic plasmid, creating a deadly toxin that causes high mortality in shrimp. The disease has been identified as acute hepatopancreatic necrosis disease (AHPND) or commonly known as early mortality syndrome (EMS). To help in the efforts of reviving the shrimp industry, the study is focused on detecting *Vibrio parahaemolyticus* causing AHPND/EMS affecting *Penaeus vannamei*, (Pacific white shrimp) and *Penaeus monodon* (Black Tiger shrimp) in different locations around the Philippines. Microbiological methods and conventional Polymerase Chain Reaction (PCR) were applied to confirm the presence of AHPND/EMS. Results show that the prevalence of the pathogenic strain of *V.parahaemolyticus* from the shrimps collected were: 33% for Luzon, 21% for Visayas and 5% for Mindanao. Recognizing the significance of this newly emerging disease in the shrimp industry is very essential as it is the first step in identifying affected sites towards strategizing ways to combat the disease. Specific primers for the detection of the virulent strains of AHPND/EMS *V. parahaemolyticus* through PCR was utilized so that timely possible measures to prevent AHPND outbreaks can be developed.

1. INTRODUCTION

Early mortality syndrome (EMS) has been described as a newly found disease that caused mass mortality in shrimp industry in China (2009), Vietnam, (2010), Malaysia (2011) and Thailand (2012) (Flegel *et al.*, 2012) and recently, in Mexico (2014). It causes necrosis in the hepatopancreas of the infected shrimp, making them lethargic and anorexic. It appears within 30 days of stocking and affects both *P.vannamei and P. monodon*. (Lightner *et al.*, 2012). Due to other causes of early mortality, a more precise name which is AHPND or acute hepatopancreatic degenerative necrotic disease was used in 2013 to avoid confusion. The causative agent of the disease was a strain of *V.parahaemolyticus* (Tran *et al.,* 2013).

Recent studies claim that the cause of the pathogenicity is not present in all *V.parahaemolyticus* strains. According to a draft genome sequence presented by Kondo *et al.,* (2014) the pathogenic strains contain conserved sequences that have not been identified in existing *V.parahaemolyticus* strains. Consequently, these finding led Timwongger *et al.,* (2014) to generate primers that would target the

specific region of the plasmid resulting to the pathogenicity of *V.parahaemolyticus*.

In the Philippines, molecular-based detections like PCR is one of the procedures in shrimp stock disease management and prevention. This is used in tandem with microbiological techniques employing the use of a selective medium that allows the growth of the target pathogen such that effective management techniques could be implemented to prevent severe disease outbreaks and production losses (Caipang & Aguana, 2011).

Recent report by the Network of Aquaculture Centres in Asia-Pacific and Food and Agriculture Organization of the United Nations (2014), the Bureau of Fisheries and Aquatic Resources (BFAR) in cooperation with Dr. Donald V. Lightner revealed that the presence of AHPND/EMS in the country is suspected but not confirmed.

2. METHODS

2.1 Chemicals

V.parahaemolyticus was isolated from shrimp samples by homogenizing the hepatopancreas in 500 µL 0.8% natural sodium saline (NSS) solution. One loopful of the lysate was then streaked in a Thiosulfate Citrate Bile Salt (TCBS) agar and incubated at 28°C for 24 hours. Green/Blue-green colonies were picked and then subcultured on Nutrient agar (NA) for Gram staining and biochemical tests (IMViC) for confirmation of identity. Clones were also preserved by subculturing them in one-fourth strength Nutrient broth and stored. The PCR primers and the protocol used were adapted from Tinwongger *et al.,* (2014). The PCR products were analyzed with 1% agarose gels stained with ethidium bromide and visualized under UV transilluminator. The positive result produced a strong band at approximately 360 bp.

For histopathology, hepatopancreas samples were fixed with AFA Davidson's fixative, processed and stained with hematoxylin and eosin (H&E) using routine histological methods described by Lightner (1998).

2.2. Procedures

2.2.1 Shrimp Sampling Collection and Morphology Analysis

Biased sampling was employed in this study. Shrimps suspected of being afflicted with AHPND/EMS were observed to have an empty midgut and stomach with a pale hepatopancreas compared to that of healthy shrimps (Fig.2). This is in accordance with the descriptions of Tran *et al.,* (2013) on AHPND/EMS infected shrimps. *P.vannamei* and *P.monodon* were from fish ports and collected from shrimp farms in Bulacan. Pampanga, Bataan, Batangas, Cebu, Bohol, General Santos and Sarangani in the Philippines. Fifteen (15) shrimps per pond were collected for microbiological and PCR detection. Samples were stored in a cooler and transported to the Thomas Aquinas Research Complex of the University of Santo Tomas. Sampled shrimps were stored in a -80^{0}C freezer until the commencement of the experiment.

2.2.2 Isolation of *V. parahaemolyticus*

V.parahaemolyticus was isolated from shrimp samples by homogenizing the hepatopancreas in NSS solution. Upon incubation at 28°C for 24 hours, the streaked lysate of the hepatopancreas of shrimp samples that showed blue-green colonies in TCBS agar (Fig.1) and transparent colonies were observed in Nutrient Agar (NA)

plates upon further isolation.

Gram-negative staining produced a pink stain when viewed under light microscopy. These samples in the microbiological tests were then subjected to PCR analysis.

2.2.3 DNA Extraction and PCR

The bacteria grown in NA was used for DNA extraction. From the NA, a small colony was picked and suspended in 50ul of ultrapure water and heated for two minutes at 95°C, followed by centrifugation. The supernatant was then used as DNA tremplate for amplification. The PCR primers and the protocol used were adapted from Tinwongger *et al.,* (2014). The PCR conditions were as follows: two minutes at 95°C, followed by denaturation at 95°C for 30 sec, annealing at 56°C for 30sec, and final extension at 72°C for 30 cycles. The PCR products were analyzed with 1% agarose gels stained with ethidium bromide and visualized under UV transilluminator. The positive result produced a strong band at approx. 360 bp.

2.2.4 Histopathology

For histopathology, hepatopancreas samples were fixed with AFA Davidson's fixative, processed and stained with hematoxylin and eosin (H&E) using routine histological methods described by Lightner (1998). The histological sections were analyzed by light microscopy (10x, 40x in Nikon E 100) for AHPND/EMS lesions in the organ.

2.2.5 Data analysis

Prevalence of AHPND/EMS and *V.parahaemolyticus* in the shrimps collected from the selected sites was evaluated. Number of shrimps which were found positive for AHPND/EMS and *V. parahaemolyticus* were divided by the total number of shrimps collected multiplied by 100. The AHPND/EMS-positive samples showed a band at approximately 360 bp while the non-AHPND samples have no bands.

2.2.6 Statistical Analysis

In determining the percentage (%) of the frequency count distribution from the sample, the formula below was used:

$$\text{Percentage} = \frac{\text{frequency}}{\text{Number of samples}} \times 100$$

3. RESULTS AND DISCUSSION

Results upon PCR amplification indicate that bacteria isolated from suspected shrimps were positive for the disease-causing toxin of AHPND/EMS. The prevalence of the pathogenic strain of *V. parahaemolyticus* from the shrimps collected were: 33% for Luzon, 21% for Visayas and 5% for Mindanao. A band around the size of 360 bp, similar to the positive control, was amplified from *V. parahaemolyticus* strains isolated from the hepatopancreas of the shrimp. PCR products were sent to Macrogen, Korea and sequencing confirmed that the amplicons sent had more than 300 nucleotides similar to the published sequences of the EMS/AHPND-causing *V.*

parahaemolyticus (Figure 5A). Similarly, the nucleotide sequence produced exhibited 99% likeness to published sequences of the EMS/AHPND-causing *V. parahaemolyticus* (Figure 5B). Preserved samples of shrimp hepatopancreas for histology have shown the effects of AHPND/EMS indicated by gross morphologhical signs and tissue analysis. Results of histopathology studies are parallel to the initial study of Tran *et al.,* (2013). Moreover, the different phases of the infection were present in the sampled hepatopancreas used in the study beginning with the acute phase progressing towards the early terminal phase and terminal phase of the infection.

Degeneration and dysfunction of the tubule epithelial cells are shown as lesions in the hepatopancreas (Figure 6G). This early event causes the progressive dysfunction of the hepatopancreas. Sloughing of cells from the hepatopancreas tubule basement membrane into the tubule lumen are observed as the disease progresses. During the terminal phase of infection, the tubule epithelial cells degenerate, nuclei becomes hypertrophic and enlarged.

Figure 1. Colonies of *V. parahaemolyticus* grown on TCBS agar plates extracted from shrimp hepatopancreas

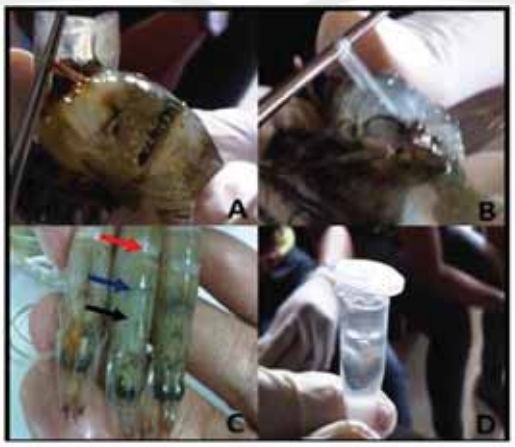

Figure 2. A. Normal midgut; B. Empty midgut; C. Gross Signs of EMS: Empty midgut (red), Pale hepatopancreas (blue) and Empty stomach (black). ; D. Hepatopancreas in NSS

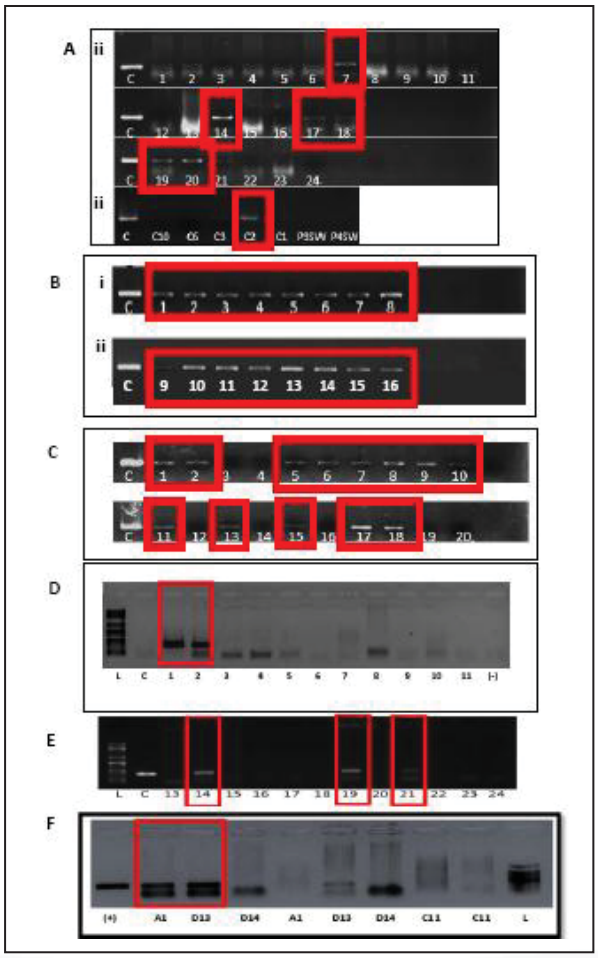

Figure 3. PCR Results. A. Bulacan Samples, i. *L. vannamei* EMS Positive results (red) and ii. C2 EMS positive (red). Postive control at 360 bp; 3B, Bataan Samples. i, *L. vannamei* EMS positive (red); 3ii. *P. monodon* EMS positive (red); C. Pampanga samples. *L. vannamei* EMS positive (red); D.Cebu samples *L. vannamei*; E. Bohol samples *L. vannamei*; F. General Santos samples *L. Vannamei*

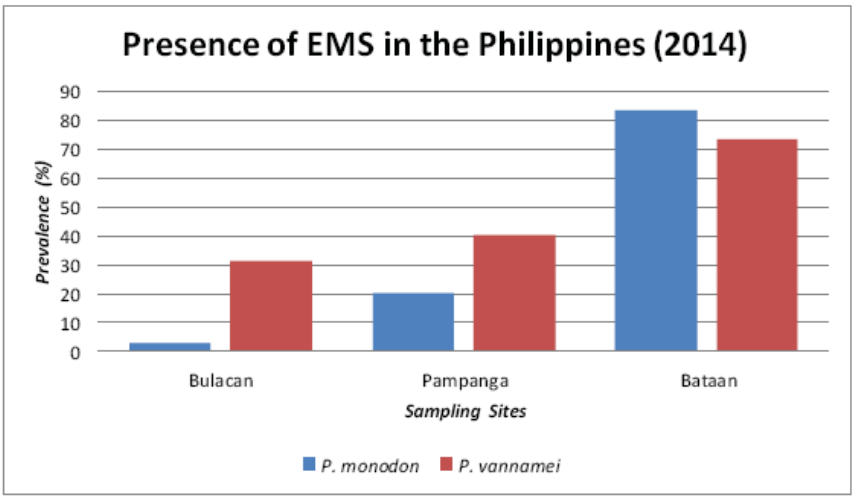

Figure 4A. Prevalence of EMS (2014) among selected sites in the Philippines. *P. monodon* and *L. vannamei* from randomly selected regions

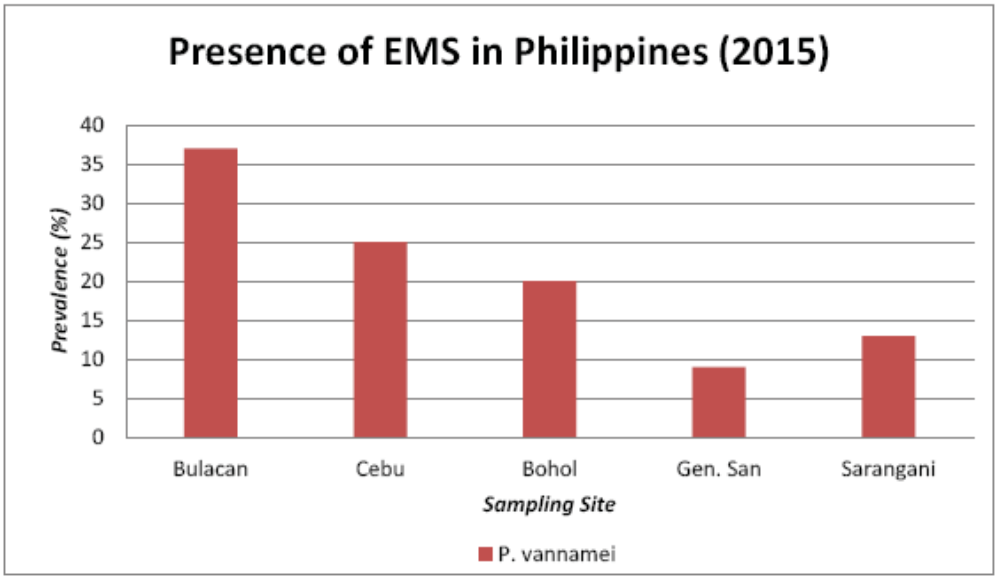

Figure 4B. Prevalence of EMS (2015) among selected sites in the Philippines. *P. vannamei* from randomly selected regions

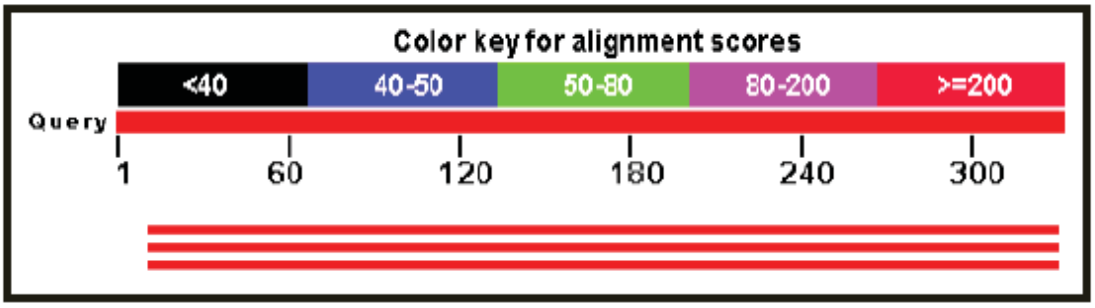

Figure 5A. EMS/AHPND-causing *V. parahaemolyticus* showing greater than 200 nucelotide sequence similarity to known pathogenic *V. parahaemolyticus* strains

Figure 5B. EMS/AHPND-causing *V. parahaemolyticus* showing 99% similarity to other published pathogenic *V. parahaemolyticus* strains.

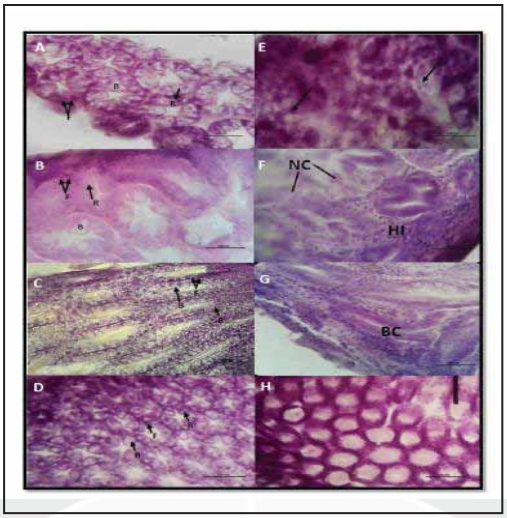

Figure 6. Histological sections from the sampled shrimps. A.-D. Normal hepatopancreas showing the preence of B- (normal secretory cell), F- (highly basophilic cells) & R-cells (fat storage). E. Acute phase with sloughing of hepatopancreas tubule epithelial cells (arrow); F. Early terminal phase; necrotic sloughing of HP tubule epithelial cell (NC) and the remnants of HP tubules are surrounded by hemocytic infiltration (HI); G. Terminal phase: Sloughing of cells are shown in the HP tubule lumens with significant bacterial colonization (BC). H. Hepatopancreas showing collapsed tubule epithelia. Scale bar: 100um

4. CONCLUSIONS

In conclusion, this study suggests that conventional PCR confirmed the presence of the pathogenic strain of *V.parahaemolyticus* causing AHPND/EMS in shrimps. Prevalence of the pathogenic strain of *V.parahaemolyticus* in shrimps collected from Luzon corresponded to 33% while 21% prevalence for Visayas and 5% for Mindanao. More investigation and research on the pathogenic mechanism and virulent strain of *V. parahaemolyticus* is needed. To our knowledge, this is the first record on the presence of AHPND/EMS in th Philipppines. This baseline information can be utilized in the formation of policies and management of Philippine shrimp aquaculture industry.

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Simple, Efficient and Inexpensive: Innovations to WSSV Diagnostics for The Shrimp Industry

*Mary Beth B. Maningas[1,2,3], Pocholo Mari T. Arabit[1,2], Sharlaine Joi Ann B. Orense[1,2],Joselito A. Tabardillo Jr.[2], Benedict A. Maralit[1], Erica M. Ocampo[4], Patrick Ellis Z. Go[4], Ricardo S. Balog[4], Christopher Marlowe A. Caipang[5]
[1]Graduate School, [2] Thomas Aquinas Research Complex, Research Center for the Natural Sciences, [3]Department of Biological Science, College of Science, [4]College of Engineering, University of Santo Tomas, España Boulevard, Manila 1008, Philippines
[5]School of applied science, Temasek Polytechnic, Singapore
mbmaningas@mnl.ust.edu.ph/ marybethmaningas@gmail.com

Abstract

The shrimp aquaculture industry is considered a major source of income and livelihood globally but diseases such as White spot syndrome virus (WSSV) causes 100% mortality resulting to devastating losses in the industry. The study aims to develop a low-cost WSSV diagnostic kit. Shrimp pleopods were used in the DNA isolation procedure. The DNA template generated from the kit's DNA isolation protocol was tested via PCR and LAMP for WSSV detection. In addition, the kit was compared to a commercially available DNA isolation kit in yielding results for PCR and LAMP. A comparison of each amplification assay's analytical sensitivity and specificity was performed to assess the kit's viability and efficiency as well as its limit of detection (LOD). Finally, field testing of the diagnostic kit was performed in selected shrimp farms comparing on-field LAMP assay with standard laboratory PCR assay. Utilizing the LAMP assay, a total of 87.5 % representative samples were tested positive for WSSV whereas conventional PCR only diagnosed 25% samples. The locally fabricated heat block machine used for the LAMP assay in field testing proved to be simpler and cheaper than the thermal cycler used in PCR. Adding convenience to field usage, LAMP products can be viewed using a simple and cheap portable black light with the addition of a non-toxic fluorescent DNA intercalating dye such as GelRed Nucleic acid stain.

1. INTRODUCTION

Aquaculture is one of the fastest growing sectors in the Philippines. In 2002, it was recorded that 1,338,175 tons of produce came from this sector, contributing a growth of 12% annually. The industry has been increasing production until 2013. A decrease of 0.15% in the fisheries sector was observed in 2014 (FAO, 2014). Shrimp aquaculture in the Philippines significantly contributes to the increase of aquaculture production and economic growth. The importance of this sector is undeniable, however, the shrimp industry has been declining due to occurrence of pathogenic infections. To date, common infections are of viral and bacterial origins. Outbreaks of diseases has been constantly reported and is still a problem of the industry. Hence, the rapid detection of the disease must be done in order to prevent loss in production.

Rapid advances in the field of molecular biology has been useful in terms of molecular detection of these diseases. Some of the conventional kits utilized in diagnosis are the IQ2000, IQplus (Tsai *et al*., 2014), Shrimple (Merican, 2004) and the conventional PCR method. These diagnostic kits are known for their high specificity yield and sensitivity, however these kits are very expensive. A comparable detection technique was developed in 2000 by Notomi *et al*., and is now one of the most popular detection technique in the aquaculture industry, the loop mediated isothermal amplification (LAMP) assay. Known for its capacity to yield highly specific and sensitive results, the assay additionally offers speed and cost-effectiveness (Biswas & Sakai, 2014). The technology has been utilized in the diagnosis of several pathogens known in aquaculture such as Edwardsiellatarda (Savan *et al*., 2004), red seabream virus (Caipang *et al*., 2004), koi herpes virus (Gunimaladevi *et al*., 2004), white spot syndrome virus (WSSV) (Kono *et al*., 2004), infectious hematopoietic necrosis virus (Gunimaladevi *et al*., 2005) and spring viremia of carp (Shivappa *et al*., 2008).

Despite these advancements, molecular detection of shrimp diseases remains to be inaccessible and is still a challenge for shrimp farmers. The use and adoption the LAMP kit would highly improve disease monitoring and farm management. Previous collaborative work (Caipang *et al*., 2012b, Maralit *et al*., 2012) have produced LAMP primers specific for the WSSV Philippine isolates. A previous study made by Nicolasora (2014) made use of the developed LAMP assay and locally fabricated heat block (UST, College of Engineering) in detecting both WSSV and Vibrio spp. in selected Philippine sites. Our research utilized cheap and readily available reagents as well as the locally fabricated heat block machine in the development of the WSSV diagnostic kit for accessible and affordable on-site diagnostics.

2. METHODS

2.1 Sampling sites

Five (5) sites were initially sampled for WSSV infection: Bataan, Bulacan, Cebu, Davao, General Santos from December 2014 to June 2015. Shrimp samples include Peneausmonodon and Peneausvannamei and range from juveniles to adults.

2.2 DNA Extraction

A simple DNA extraction procedure was developed based on the binding properties of DNA in silica particles and the use of low-cost reagents and materials. Pleopods from the samples were homogenized with the lysis and binding buffers. The DNA was then eluted using Ultrapure water. The DNA extraction was done on-site before the LAMP assay.

2.3 LAMP Assay

The LAMP assay was done according the protocol described by Nicolasoraet. al. in 2014 and used a heat block fabricated using locally-available and cheap materials. DNA extracts were added to the prepared LAMP mastermix and placed in 95C for two (2) minutes, then cooled down in ice for another two minutes before adding the Bst polymerase. The DNA was then amplified in 63C for 45 minutes. The results were then viewed by adding GelRed Nucleic Acid Stain illuminated under a black

light. The results were then verified by performing a separate LAMP and PCR assay using the same DNA sample.

3. RESULTS AND DISCUSSION

3.1 Analytical Sensitivity and Specificity

The analytical sensitivity and specificity of the LAMP-based diagnostic kit were compared with conventional PCR-based diagnosis. WSSV diagnostic procedures using the PCR technology have been widely practiced in screening of Philippine farms (Caipang and Aquana, 2010; Maralit *et al*., 2011; Magbanua *et al*., 2000) making it an ideal standard for comparison with the developed LAMP assay.

Tests on analytical sensitivity have shown that the developed LAMP reaction is 10 times more sensitive than the PCR reaction being able to amplify DNA even in 10-6 serial dilution whereas PCR was only able to amplify up to 10-5 serial dilution (Figure 1 – A,B,C,D). Similarly, visualization of LAMP products utilizing the fluorescent dye (SYBR) was achievable at 10-6 serial dilution adding to its applicability to field testing. The detection limit of the developed LAMP assay was 1.363pg of DNA. These results are congruent with the findings of project collaborators Caipang *et al*., (2012b) and Maralit *et al*., (2012) obtaining a more sensitive LAMP reaction in comparison with PCR. In the study of Caipang *et al*., (2012a) on MonodonBaculovirus (MBV), LAMP was also able to exhibit a significantly higher sensitivity than PCR. Additionally, previous studies from other countries agreed with the obtained LAMP sensitivity (Kono *et al*., 2004, Sun *et al*., 2006, Nimitphak *et al*., 2008). Finally, DNA extracted from the developed kit proved to be comparable with the commercial kit in terms of obtaining quality PCR and LAMP products (Figure 1 - A,C,E compared with B,D,F). Focusing on analytical specificity, the LAMP reaction for the developed kit (Figure 2 – 1B) is comparable with the commercial kit (Figure 2 – 2B), obtaining products specific for WSSV only. Similar results can be viewed using the added fluorescent dye (Figure 2 – 1, 2, 3C). Likewise, the PCR primers and the developed LAMP primers gave out the same specificity for WSSV (Figure 2-A and B).

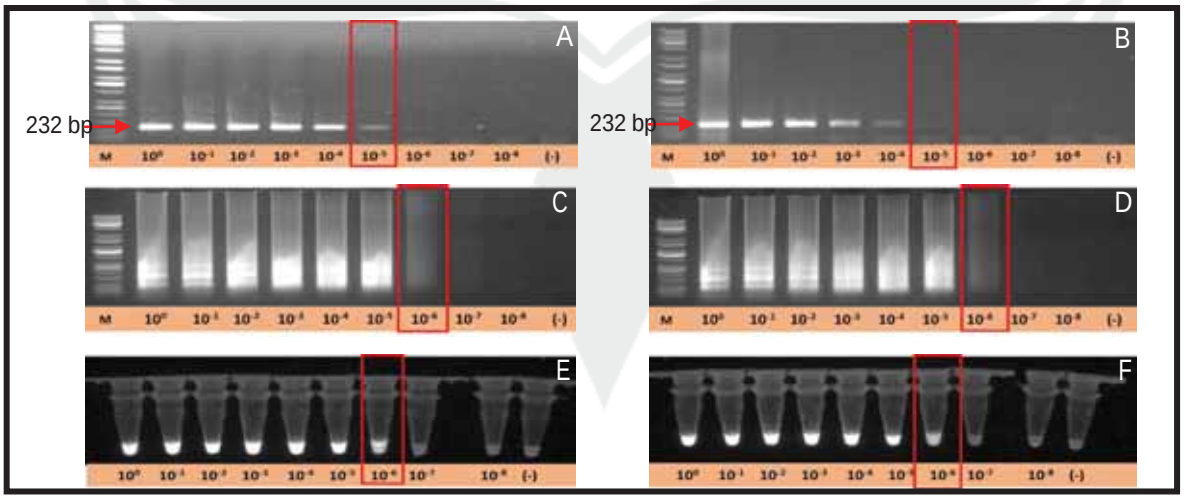

Figure 4. Analytical Sensitivity. A,C,E – DNA isolated using commercial kit; B,D,E – DNA isolated using developed kit. (A and B) - PCR, (C and D) – LAMP, (D and E) – LAMP visualized using SYBR dye.

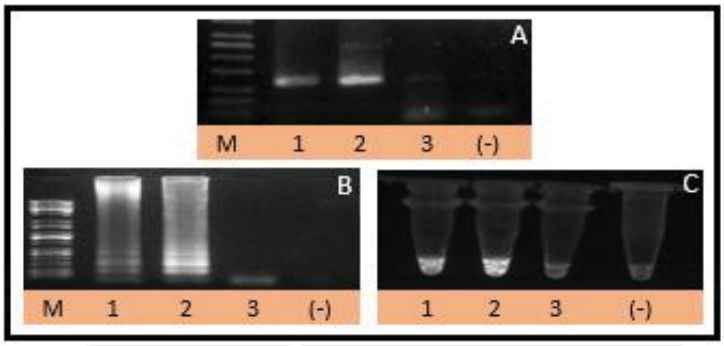

Figure 5. Analytical Specificity. 1– DNA isolated from developed kit, 2-DNA isolated from commercial kit, 3-Taura Syndrome Virus cDNA. (A)- PCR, (B)- LAMP, (C)- LAMP visualized using SYBR dye.

3.2 Site Monitoring and Field Performance

Collected shrimp samples from different sampling sites were tested for the presence of WSSV. Shrimp samples from Bulacan, Bataan and General Santos City were subjected to laboratory WSSV screening using the standard LAMP assay and developed WSSV diagnostic kit (Fig. 3, 4 and 5). Field diagnosis was supported by subsequent laboratory LAMP and PCR screening (Figure 3, 4 and 5).

Using the LAMP assay, 93% of the P. monodon samples from Bulacan (REGION 3) were tested positive for WSSV infection (Figure 3). The characteristic laddering pattern and the presence of smear are indicators of a positive LAMP reaction (Notomi *et al*., 2000). Collected P. vannamei samples from Bataan (REGION 3) (Figure 4) and General Santos City (REGION 12) (Figure 5) harboured WSSV infection with a total of 80% and 73% of the samples, respectively.

Field testing of the LAMP based diagnostic kit in Davao showed no presence of WSSV infection. However, laboratory test using PCR revealed than 20% of the samples were positive(Fig. 6A) whereas the developed LAMP assay diagnosed 50% of the samples were positive (Fig. 6B).

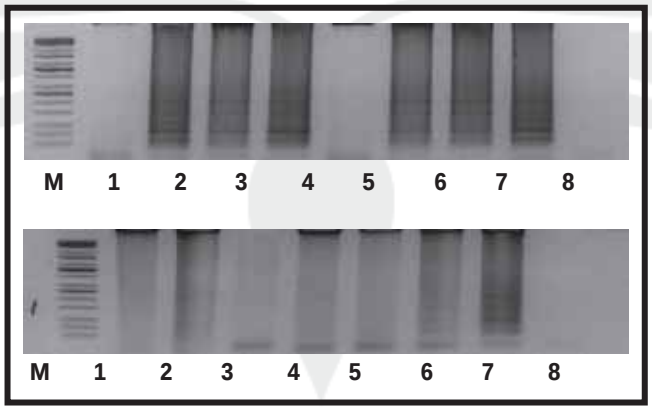

Figure 6. Bulacan area Penaeusmonodon screening for WSSV using LAMP assay. (M)-DNA ladder, Well 1 – negative for WSSV, Wells 2-15 – positive for WSSV, (N)- Negative control

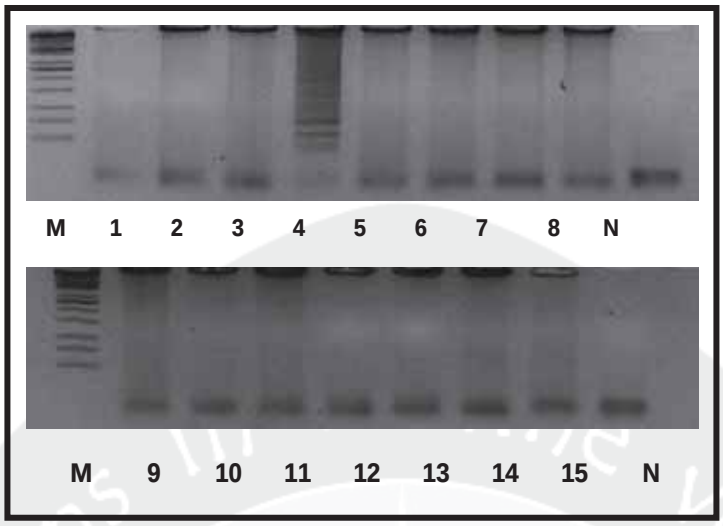

Figure 7. Bataan area Penaeusvannamei screening for WSSV using LAMP assay. (M)-DNA ladder, Well 1, 12 and 13 – negative for WSSV, Wells 2-11, 14 and 15 – positive for WSSV, (N)- negative control

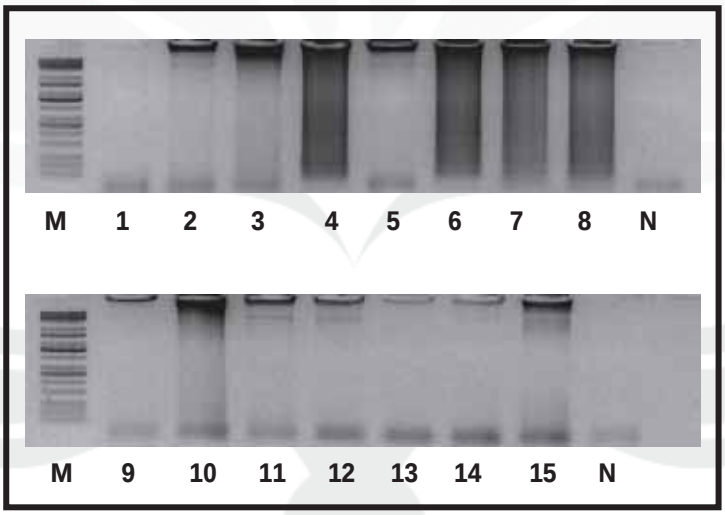

Figure 8. General Santos city area Penaeusvannamei screening for WSSV using LAMP assay. (M)-DNA ladder, Well 1, 9, 13 and 14 – negative for WSSV, Wells 2-8, 10-12 and 15 – positive for WSSV, (N)- negative control

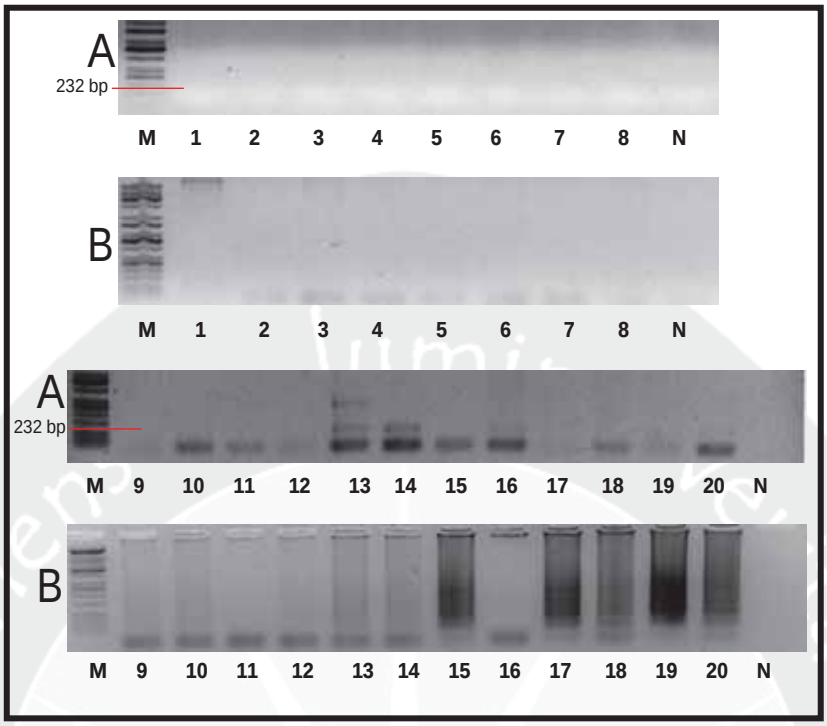

Figure 9.Davao city area Penaeusvannamei screening for WSSV using PCR (A) and LAMP (B) assay. (M)-DNA ladder, (N)- negative control, samples 1-8 negative for WSSV via PCR (A) and LAMP (B), samples 13,14, 16 and 20 positive for WSSV via PCR (A), samples 9, 10, 12-15, 17-20 positive for WSSV via LAMP (B) assay

These results showed that the greater analytical sensitivity of the LAMP assay over PCR (Figure 1) translates to its diagnostic performance, testing a larger percentage of the samples positive for WSSV in comparison to PCR. In addition, sample number 20 which tested positive via PCR and LAMP but the DNA source of the sample came from a non-shrimp species. The work of Hossain *et al*., (2004) and Flegel *et al*., (2006) supported the field test finding stating that various crustaceans including crabs can carry infection. Additionally, similar studies on crabs have also shown their role as carriers of WSSV infection contributing to the spread of disease (Corbel*et. al*., 2001; Flegel, 2001 and Huang *et al*., 2001).

Diagnostic kit testing in Cebu (Region 7) showed WSSV positive results for samples 1-4. Laboratory screening confirmed that 25% and 63% of the samples were positive for WSSV via PCR and LAMP assay, respectively (Figure 7). The high sensitivity of the developed LAMP assay allows it to detect WSSV even in low copy numbers often undetectable by PCR.

The most recent disease surveillance in Bataan (Region 3 - Central Luzon) revealed samples positive for WSSV via the on-field LAMP assay 90% (Figure 8-C). Laboratory confirmation of the results have shown that 0% (0/10) and 60% of the sample tested were positive for WSSV via conventional PCR and nested PCR respectively (Figure 9-A and 9-B). The diagnostic performance of the LAMP assay was comparatively more effective than PCR and nested PCR. The utilization of the

portable black light in the visualization of results allowed farmers to easily screen their farms. This fortifies the applicability of the diagnostic kit in field setting.

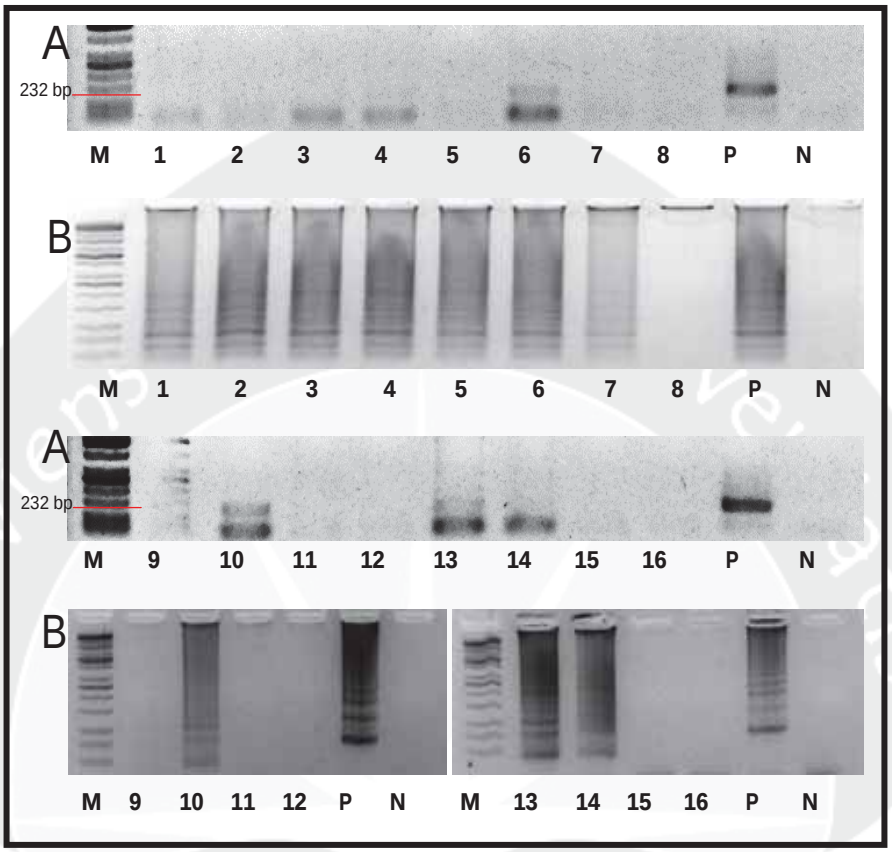

Figure 10. Cebu area Penaeusvannamei screening for WSSV using PCR (A) and LAMP (B) assay. (M)-DNA ladder, (P) – positive control, (N)- negative control, samples 4,6,10,13 positive for WSSV via PCR (A), samples 1-7, 10, 13,14 positive for WSSV via LAMP (B) assay.

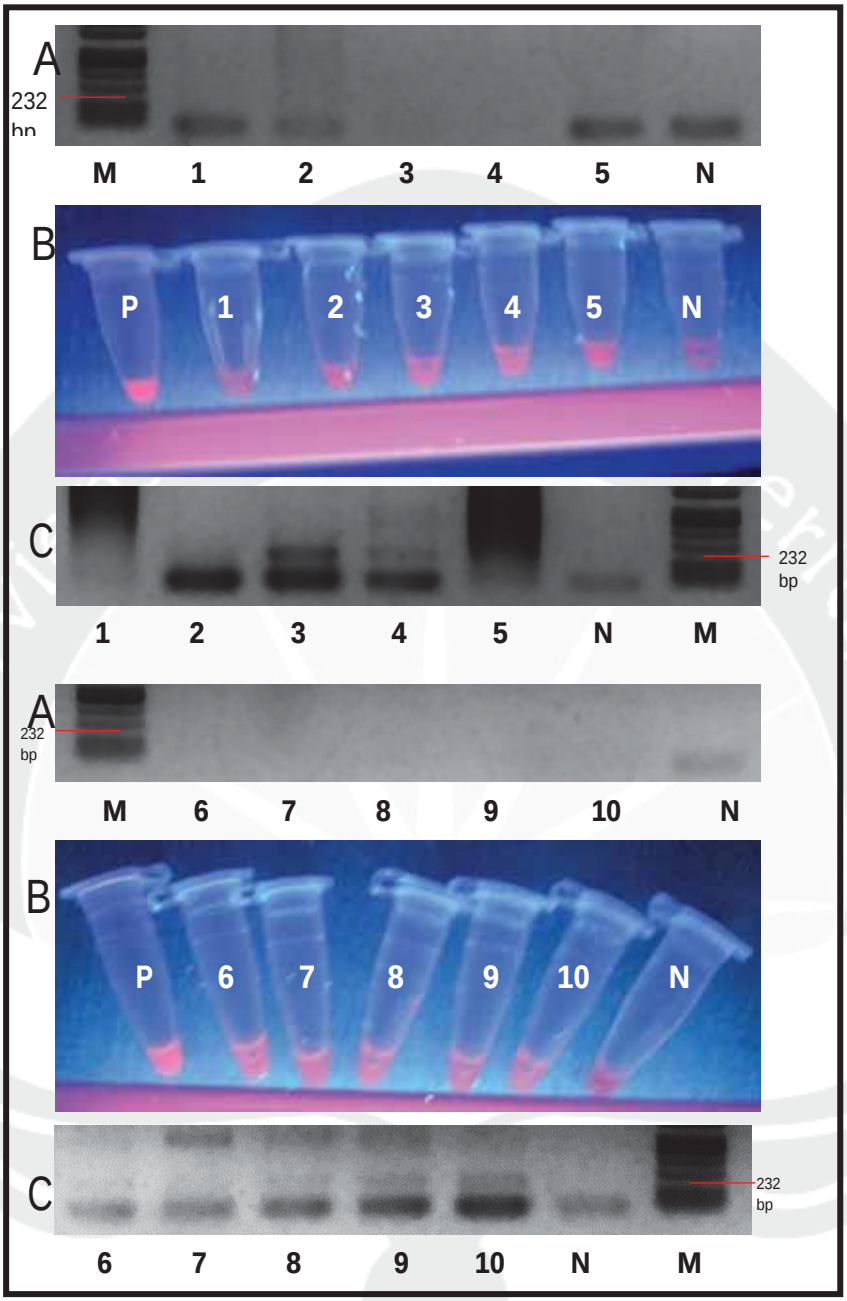

Figure 11. Bataan area Penaeusmonodon screening for WSSV using conventional PCR (A), LAMP (B) assay and nested PCR (C). (M)-DNA ladder, (P)- positive control, (N)- negative control, samples 1-10 diagnosed negative for WSSV via conventional PCR (A), samples 2-10 diagnosed positive for WSSV via LAMP (B) assay, samples 2,3,6 and 8-10 diagnosed positive for WSSV via nested PCR (C).

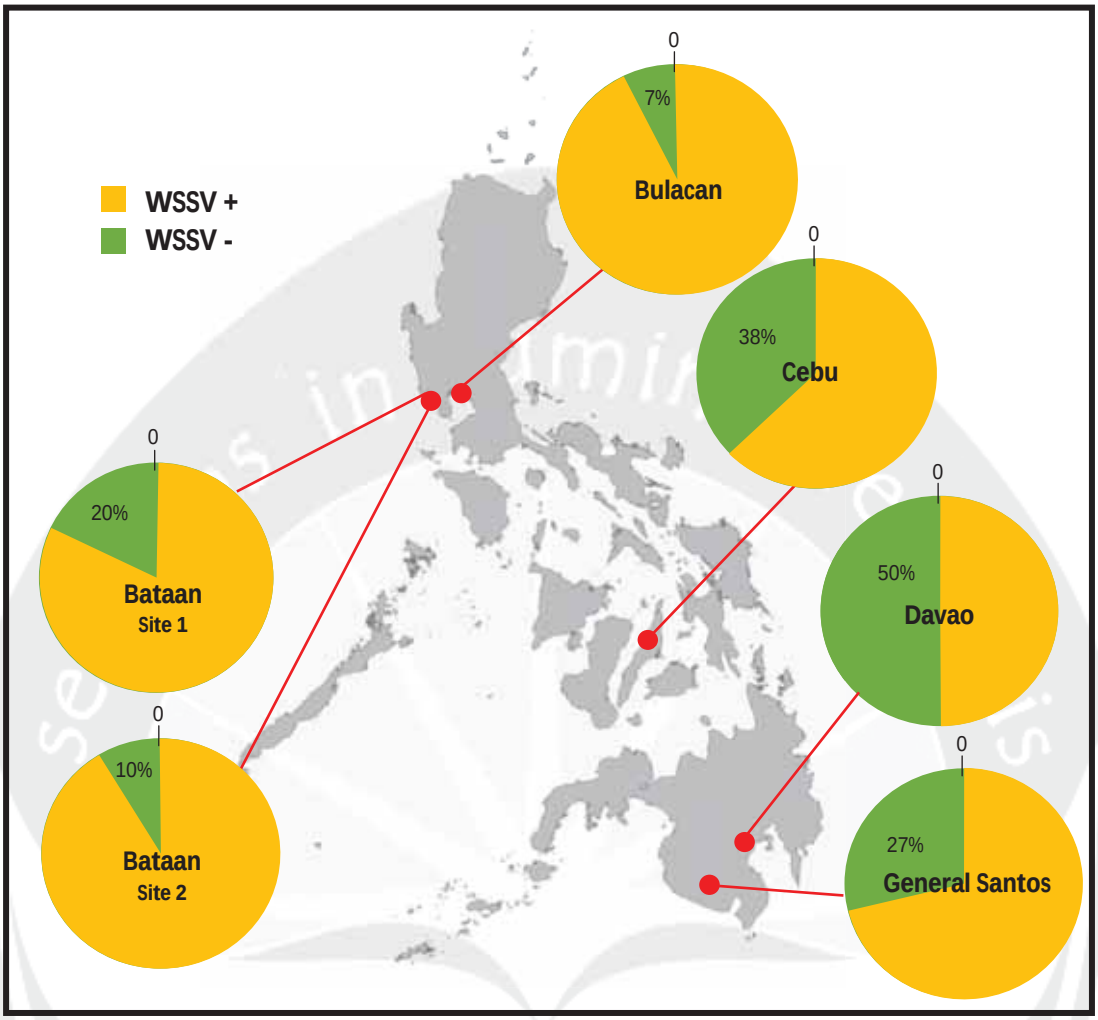

Figure 12. Prevalence of WSSV from six (6) seleted sites in the Philippines acquired using the developed LAMP assay.

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Molecular Aspects of Zinc Intake (Zn) and Selenium (Se) on Glycosylated hemoglobin (HbA1c) in patients with type 2 Diabetes Mellitus (DMT2)

Indranila KS[1], Judiono[2], Yuliati Widiastuti[3]
1) Departemen Patologi Klinik, FK UNDIP Semarang
2) Ketua PS D III Gizi, Jurusan Gizi Politeknik Kesehatan Bandung
3) Dosen Sekolah Tinggi Ilmu Kesehatan Immanuel Bandung
E-mail : nila_fkundip@yahoo.com

Abstract

Diabetes mellitus (DM) is a metabolic disease characterized by hyperglycemic. HbA1c is the result of the examination for glycemic control. Zinc and Selenium are metaloenzim factors, play a role in the mechanism and regulation of insulin synthesis. This study aims to explore the relationship between zinc and selenium with HbA1C in patients with type 2 diabetes mellitus. Cross sectional study to patients with type 2 diabetes mellitus. Samples numbered 108 people conducted at several hospitals in Bandung from years 2011 to 2013. The sample was done by purposive sampling. Zinc and Selenium are collected by SQFF. HbA1c is measured by the method of affinity chromatography. Data were analyzed by Fisher's Exact test and Spearman correlation ($p < 0.05$). The study showed that there was a significant relationship between Zinc, Selenium and HbA1c were significantly ($p < 0.001$). There is a relationship between Zinc and Selenium with HbA1c, so the management of diet with intake of Zinc and Selenium is needed in the regulation of patients with type 2 diabetes mellitus.

Keywords: Intake Zinc, intake Selenium, levels of HbA1c

1. INTRODUCTION

Diabetes Mellitus (DM) is one of the chronic degenerative diseases that the prevalence continues to increase by year to years.[1] DM is also a group of metabolic diseases by characteristies chronic hyperglycemia due to a defect in insulin secretion, insulin or both.[2] The type 2 diabetes pathogenesis based on impaired insulin secretion by pancreatic beta cells and impaired insulin action due to insensitivity (resistance) to insulin target tissues. Shaw showed, the worldwide prevalence of diabetes in the adult population aged 20-70 years was 6.4% in 2010, affected 287 million adults and is expected to increase to 7.7% and affected 439 million adults in 2030.[3] The prevalence of DM in Indonesia is expected to increase from 5.1% in 2000 to 6.3% in the year 2010.[4] Furthermore, Missmanagement could lead to complications and increased morbidity and mortality pasien.[5] Glycosylated hemoglobin or (HbA1c) is one of the laboratory tests for blood sugar control.[6] Persistent hyperglycemia causes glycosylation of the protein hemoglobin. It is estimated by the percentage of glycosylated hemoglobin glycation of hemoglobin (HbA1c), which are used clinically since 30 years ago to assess the degree of chronic hyperglycemia in patients with mild severe of DM.[7] Its value indicates the

average level of sugar in the period of 3 months; disglycemia helpful to know the characteristics of the study population because it is simple compared to oral glucose tolerance test (OGTT). In diabetes, one percent increase in HbA1c associated with 20-30% that it would lead to increase in mortality and morbidity of the cardiovascdiseasular.[7,8]

DM management is necessary to seek the way the blood sugar levels closely to normal. The main pillars is a medical nutrition therapy. Medical nutrition therapy, or better known as diet or meal arrangements for persons with DM is a very important factor in controlling blood sugar. [9] Dietary management generally still rarely pay attention to the availability and adequacy of the trace elements and bioactives food. Dieticians tend more priority to macro-nutrients such as carbohydrates, fats and proteins .Trace minerals are important for the body, specifically in patients with type 2 diabetes. Dietary management generally still rarely pay attention to the availability and adequacy of the trace element minerals and bioactive food.

Minerals such as Zinc and Selenium including types of trace minerals, in the body there is a small amount, but it has a play very vital role.[10] This mineral belongs to a group of minerals that works as an antioxidant metaloenzim which can prevent free radicals, increase insulin receptor sensitivity, thereby potentially preventing the degenerative disease.[11]

Zinc for example is an element essential for the synthesis, storage and secretion of insulin. It is a component of several enzymes. Zinc has also an important role in maintaining the balance function of multiple networks and have an important role in modulating system imun.[12] Body's ability to synthesize and secrete insulin is affected by zinc in the body, because it is involved in the mechanism of regulation and synthesis of insulin receptors.[13]

Selenium serves as part of a protein known as Selenoprotein. Selenoproterin plays a role as a defensive mechanism to oxidative stress, to regulation of thyroid hormone activity, and for the redox status of vitamin C and other molecules. However, note that the "therapeutic window" Se limited, and the adverse effects on health may occur due to excessive intake of Se (supra nutritional) or below the level required to toxicity.[14] Selenium acts as an antioxidant and contributes in regulating cell membrane integrity and lowering the risk of oxidative damage.[16] High-Se diet can stimulate the release of glucagon, promotes hyperglycemia, or can cause excess of gluthathion peroxidase-1 and other antioxidants Selenoprotein resulting in insulin resistance and obesitas.[15] This study aims to explore the relationship between zinc and selenium with $HbA1_C$ in patients with type 2 diabetes mellitus.

2. METHODS AND MATERIALS

This study is cross sectional study design, which was implemented in January 2011 to December 2013. The experiment was conducted at several hospitals in Bandung. Research was used all patients by type 2 diabetes who did endocrine outpatients clinic at Hospital in Bandung and incorporated Diabetes Association Members (Persadia). The samples was taken as many as 108 patients. They were obtained by purposive sampling with the following inclusion criterias: Patients with type 2 diabetes who have a history of results of HbA1c, age <65 years, did not have a blood disorder,

has been getting nutrition education, without the complications of the disease, not pregnant and was willing to be the subject of research by signing informed consent.

The eating habits questionnaire was colledted by Food Frequency Questionnaire (FFQ). This data were analyzed by Nutri survey. Nutrition intake of trace minerals such Zn, Se was collected by Food Frequency Questionnaire (FFQ). HbA1c was measured by affinity chromatography method. Processing and analysis the data was used by computer software with a significance level of $p < 0.05$ and 95% confidence level. Data were analyzed in univariate and bivariate format. The corelation between independent variables and the dependent variable was analyzed by non-parametric statistical analysis the Fisher Exact, Spearman correlation ($p < 0.05$). This study has approved by ethical clearance.

3. RESEARCH RESULT

3.1 Characteristics of Samples

The study sample characteristics include age, educational background, employment, and others in this study it is presented in Table 1.

Table 1. Distribution of the characteristics of the study sample by gender

Samples Characteristics	Category by Sex					
	Boy		Girl		Total	
	n	%	n	%	N	%
Age						
<50 years	5	11,6	7	12,7	12	11,1
≥50 years	38	88,4	48	87,3	96	88,9
Education level						
Elementary	8	18,6	52	80,0	60	55,5
Higher	35	81,4	13	20,0	48	44,5
Occupation						
Work	23	53,5	64	98,5	87	80,6
Unemploy	20	46,5	1	1,5	21	19,4
Family DM History						
Yes	25	58,1	23	35,9	48	44,4
No	18	41,9	42	64,6	60	55,6
Duration suffer DM						
<5 year	19	44,2	31	47,7	50	46,3
≥5 year	24	55,8	34	52,3	58	53,7
Medical therapy						
Yes	39	90,7	59	90,8	98	90,7
No	4	9,3	6	9,2	10	9,3
Exercise (Sport)						
Yes	15	34,9	11	90,8	26	24,1
No	28	65,1	54	9,2	82	75,9
Nutritional Status						
Normal	25	58,1	28	43,1	53	49,1
Malnutrition	18	41,9	37	49,2	55	50,9

3.2 Intake of Zinc, Selenium and levels of HbA1c samples

Based on finding, Zinc intake showed it achieved at a mean of 8.3 ± 2.62 mg, with a minimum intake value of 5.2 and a maximum of 18 mg. The recommended dietary allowance level (RDA) of less than 80% by 56 (61.5%). The intake of selenium showed on the average position of 74.62 ± 15.46 ug with a minimum intake value of 41 and a maximum of 104 ug that can be seen in Table 2. The findings suggest that most of the patients were in the intake of zinc is not ideal to meet the recommended nutrient required by RDA.

Table 2. Distribution of the intake of zinc, selenium intake and levels of HbA1c samples.

Variabel	Statistical analysis		
	χ± SD	Min	Max
Zinc Intake (mg)	8,3 ± 2,62	5,2	18
Baik (≥80% RDA)	35 (38,5%)		
Kurang (80%RDA)	56 (61,5%)		
Selenium Intake (µg)	74,62 ± 15,46	41	104
Good (≥80% RDA)	91(100%)		
Less (<80%RDA)	0(0%)		
HbA1c Level (%)	8,4 ± 2,17	5,7	15,4
Controlled (≤7%)	32(35,2%)		
Uncontrolled (> 7%)	59 (64,8%)		

Based on the study of HbA1c levels showed that it achieved at the mean of 8.4 ± 2, 17 percent which the achievement of a minimum value of 5.7 and a maximum of 15.4 percent. This showed that the majority of patients are at high HbA1c levels or uncontrolled regulation.

3.3 Relationship intake of zinc and selenium intake with levels of HbA1c

Based on the data analysis of the relationship intake zinc, selenium with HbA1c levels obtained the data as it is presented in Table 3 below.

Table 3. Relationship intake of zinc and selenium intake with levels of HbA1c

Variabel	n	r	P
Zinc intake and HbA1c level	108	-0,482	0,001*
Selenium intake and HbA1c level	108	-0,863	0,001*

**) Korelasi Spearman p<0,05*

Results of correlation analysis on samples of zinc intake patients with type 2 diabetes showed that zinc intake was significantly associated with HbA1c levels (r = -0.482, p <0.01). The present invention provided an indication of improvement zinc intake which meets the nutritional adequacy (RDA) can reduce HbA1c levels in patients with

type 2 diabetes. Selenium intake also showed significantly association with HbA1c levels (r = -0.863, p <0.05). Results of this analysis indicated that an increased intake of selenium in accordance with the level of adequacy of the advice showed a decrease of the levels of HbA1c.

3.4 Effect of intake of zinc, selenium on HbA1c

Based on regression analysis of the effect of the intake of zinc, selenium against HbA1c levels of data obtained as follows: Levels of HbA1c = 13.6 - 0.44 (zinc intake) - 3.03 (selenium intake), with coefficient R = 0.773 or 77.3% HbA1c is determined by the intake of zinc and selenium, rest influenced by other factors, namely obedience berdiit, drug consumption, exercise, heredity, habits and eating patterns (p <0.001).

4. DISCUSSION

This study has shown a significantly association between the intake of zinc with HbA1c levels, despite a weak negative relationship (r = -0.4). This study also showed a statistically significant association between the intake of Selenium with HbA1c levels (p <0.001). These results were consistent with research conducted by Jayawardena *et al*., 2012, that the better grades of zinc in the blood, then the individual will be in the regulatory status of DM were better, but lower when compared with the regulations on the individual non DM.[16] Song study, patients with type 2 diabetes who given pro-z (flour and zinc) for 3 months was able to reduce HbA1c levels in bermakna.[17]

Zinc (Zn) is an essential micronutrient that is needed for more than 300 different cellular processes, including DNA, protein synthesis, enzyme activity, and intracellular signaling. Require compartmentalization of cellular homeostasis Zn into intracellular organelles, which are closely regulated through the integration of transport mechanism.[18] Zinc works as an antioxidant to protect intracellular oxidation process produces free radicals which will also work as a synthesizer, storing and secreting a protective role insulin.[19] Zinc affect on damage pancreatic beta cells. Lack of zinc affects the beta cells of the pancreas in response to the call of the body to produce and secrete insulin, lowers insulin secretion and improve insulin resistance.[20] If the pancreas does not produce and secrete enough insulin in the body's glucose levels remain high, so that with continued high levels of glucose in the body of the regulation of blood sugar is not good.[21]

The role of zinc as an antioxidant is inhibition of ROS via the reduction of glucose toxicity by Zn. Zinc stimulates transcription of metallothionein. Metallothionein itself have antioxidant effects. Zinc provides protection against free radical attack immune mediators (immune-mediated free radical attack) to protect sulfhydryl groups (SH) against oxidation. Also participation in the inhibition of the production of free radicals (Haber Weiss cycle) to compete with the transition metal. Zinc contributes to stabilizing SH by protecting proteins from oxidation. It also reduces direct and radical $^{\cdot}O_2$ $.OH^-$, H_2O_2, and the level of xanthine oxidase, thereby improving mitochondrial function. This radical decline decrease lead to lipid peroxidation. Zinc also stimulates the activity of insulin promoter PDX-1, and inhibits the activity of xanthine oxidase, thus reducing lipid peroxidation. [22]

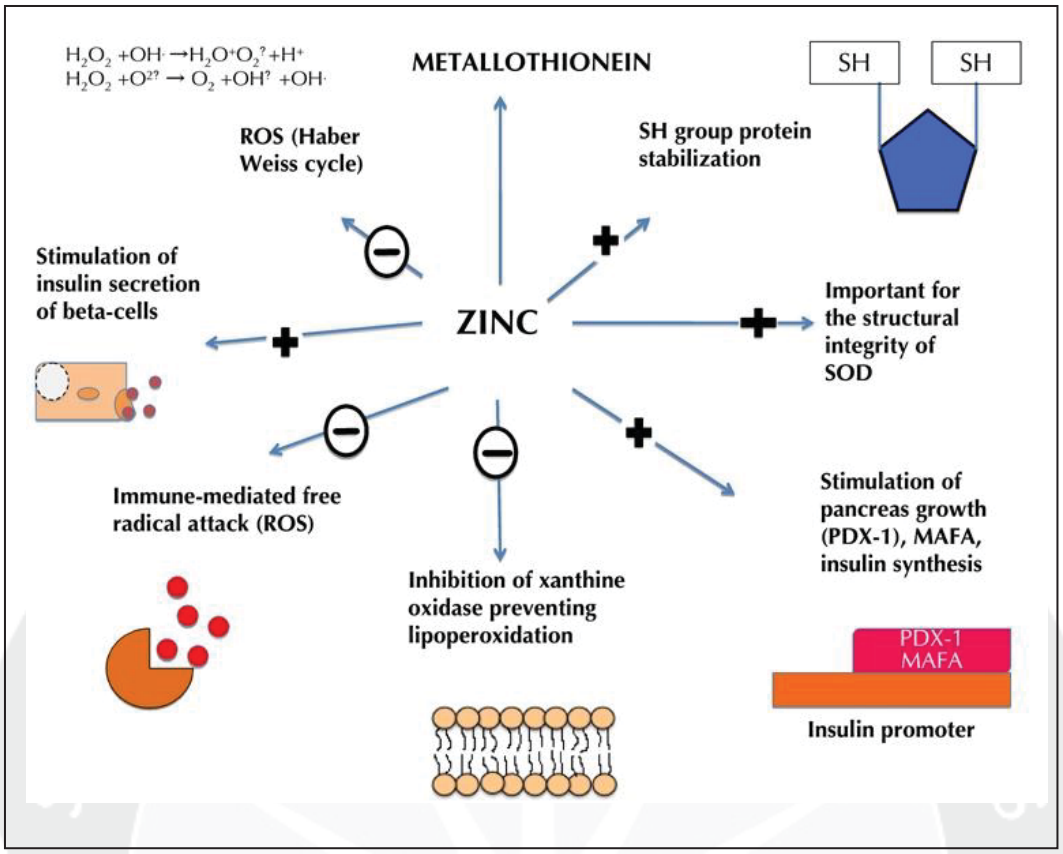

Figure 1. (Modified by [22]): The role of zinc as an antioxidant; +: Stimulation pathways.; -: Inhibition track

Research on intake of selenium (Se) in patients with diabetes, previous findings indicated that contrary to the possibility of a relationship between the level of control of diabetes and changes in the levels of this mineral. Se intake in this study was measured and the relationship between the intake and the metabolic control of diabetes, as determined by glycosylated hemoglobin (HbA1c). A negative correlation between the intake of Se and HbA1c was found. Some studies show lower serum selenium levels in the diabetic group compared with the non diabetes.[23] Subjects of research data showed that selenium plays a role in regulation of beta cell-specific target genes and potentially push the overall improvement in the function of the island Langerhans.[24] On the other hand, has shown that high levels of selenium are associated with the prevalence of diabetes.

In addition due to the intake of zinc and selenium that already meet adequacy, HbA1c levels were also influenced by other factors, including the use of pharmacological therapy. The results showed that nearly all of the samples (90%) using the pharmacologic therapy. Oral hypoglycemic drugs and injection drug given to patients with diabetes mellitus can reduce HbA1c between 05 to 3.5% .25

5. CONCLUSIONS AND RECOMMENDATIONS

This study showed an association between the intake of zinc and selenium with HbA1c levels in patients with type 2 diabetes mellitus (T2DM). It is suggested that the management of diet in patients with type 2 diabetes that it needed to pay attention to

the intake of zinc and Se in sufficiency recommended with respect to obtain a controlled HbA1c levels. Futher research It is needed to be examined gluthathion peroxidase-1 and selenoproteins other antioxidants such as Copper (cu) which resulted in insulin resistance and obesity.

6. ACKNOWLEDGEMENT

The author would like to thank Danone Indonesia Foundation, Goiter Indonesia Laboratory (GAKI) Diponegoro University of Semarang, The Integrated Laboratory Centre of The Bandung Health Polytechnic, MOH R.I which has provided research grants, and all samples that have been willing to be a subject of research, and all those who helped to the completion of the study.

7. AUTHOR CONTRIBUTIONS

All authors participated in data collection, participated in the study design, statistical analysis and preparation of the manuscript. All authors gave final approval for publication. There is no conflect interest with any company in this research.

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Sorghum (*Sorghum bicolor* L. Moench) Leaves Bioethanol Production

Birgitta Narindri[1], Muhammad Nur Cahyanto[2], Ria Millati[2]
[1]Faculty of Biotechnology Universitas Atma Jaya Yogyakarta
[2]Food Science and Technology, Faculty of Agricultural Technology
Universitas Gadjah Mada Yogyakarta
birgitta_narindri@mail.uajy.ac.id

Abstract

The aim of this research was to analyze the potency of sorghum leaves as biomass in ethanol production. Sorghum leaves will be used as a substrate in an enzymatic hydrolysis process and the reduction sugar that produced will be used as substrate in fermentation. Hydrolysis was conducted without pretreatment. Sorghum leaves were degraded into 30 mesh powder before used as biomass in enzymatic hydrolysis using Celluclast (novozyme) 90FPU/g dry weight. Reduction sugar concentration and Cellulose Conversion Ratio (CCR) will be used to analyze degradability of sorghum leaves cellulose at enzymatic hydrolysis. Reduction sugar in hydrolysate as the result of enzymatic hydrolysis will be fermented by *Saccharomyces cerevisiae* FNCC 3012 to produce ethanol. Initial *S. cerevisiae* concentration was 10^6 CFU/ml and added 10% of hydrolysate volume. Obseravation carried out on reduction sugar and ethanol concentration during fermentation. The results presented that some cellulose cannot be enzymatically hydrolysed directly because of the low value of CCR. Highest reduction sugar concentration after enzymatic hydrolysis was 3.18 mg/ml. Highest level of ethanol concentration was 0,23%(v/v).

Keywords: bioethanol, sorghum leaves, enzymatic hydrolysis, ethanol fermentation

1. INTRODUCTION

The scarcity issue and environmental problems as the result of the fuel usage have become the concern of the world community, including Indonesia. Ethanol has attracted attention as an alternative energy since the oil crisis in the world (Shen *et al.,* 2012). Ethanol can be produced from the fermentation process using wide variety of materials such as agricultural materials containing lignocelluloses, or materials that contain saccharin (Goshadrou *et al.*, 2011).

Popular types of agricultural crops cultivated in Indonesia are cereal crops such as rice, corn, glutinous rice, and sorghum. Parts of plants such as corn stalks, wheat straw, rice straw, rice husk, and baggase are residue and byproducts which can be used as raw materials for production of bioethanol (Saha and Michael, 2006).

The agricultural wastes contain cellulose which can be hydrolyzed into sugars to be used as a substrate in the fermentation process. Hydrolysis can be done using various methods such as physical method through evaporation, chemical method using acid and or enzymatic method. *Saccharomyces cerevisiae*, which can produce ethanol in large quantities and has a high tolerance for alcohol, as well as easy

application to industrial scale, is commonly used in industrial fermentation of glucose into ethanol (Elevry and Surya, 2006).

This present research used sorghum leaves which will be enzymatically hydrolyzed using cellulose enzymes to convert the cellulose into simple sugar. The result of the enzymatic hydrolysis will be used as a substrate in ethanol fermentation process using *S. cerevisiae*. Analysis of sugar level reduction and ethanol produced were used to measure the efficiency of fermentation.

2. MATERIALS AND METHODS

2.1 Characteristic Analysis

Water content analysis was conducted using AOAC (2005) method. Two gr sample were added into measured test bowl before dried at 100-105°C until the weight was constant. The weight was measured and counted using the formula below.

$$\text{Water Content} = \frac{(finalweight + emptybowl) - (startingweight + emptybowl)}{startingweight} \times 100\%$$

Lignocellulosic in sorghum leaves was conducted using Chesson (1981) method. Some stages of lignocellulosic degradation were conducted using hot water and acid to analyze the amount of water soluble content, cellulose, hemicelluloses and lignin.

2.2 Sorghum Leaves Enzymatic Hydrolysis

Sorghum leaves from numbu putih varieties were taken from Banguntapan, Yogyakarta. Sorghum leaves were collected and degraded to 30 mesh powder. This research was also used PASC (Phosporic Acid Swollen Cellulose) as control in enzymatic hydrolysis. PASC was made by mixing cellulose powder and phosphoric acid for 1hour in 4°C, then diluted with water and mixed for 1 hour in 4°C. The dilution then washed using aquadest and $NaHCO_3$ 1% (w/v) until pH 7. PASC was saved in citrate buffer pH 4,8 before used (Walseth, TAPPI, 1952).

Cellulase activity was measured using Ghose (1987). 1 mL of 0,05 citrate buffer (pH4.8) pre warmed into 40°C and added to the test tube containing 1 whatman filter paper strip (1x6cm). 0,5 ml of cellulase of Celluclast ® 1,5L (Novozyme) added into the test tube then transferred into waterbath 50°C for 1 hour. The mixture then analyzed using DNS method (AOAC, 2005) to measure the reduction sugar. The result was stated in FPU (Filter Paper Unit). One unit was amount of enzyme to release 1 µmol glucose in 1 minute at 50°C.

Enzymatic hydrolysis was modified according to the previously published method of Musatto *et al.,* (2008). 90FPU/substrate dry weight was added in 50 nM Sodium citrate buffer (pH 4,8) and supplemented by 0.02%(w/v) sodium azide then mixed with 2% (w/v) sorghum leaves. Mixing was incubated at 50°C for 72 hours and sampling was done on 0,12,24,36,48,60,72 hours. Reduction sugar in sample was analyzed using DNS (AOAC, 2005) method after sentrifuged with 4000rpm for 10 minute.

%CCR (Cellulose Conversion Ratio) was analyzed using formula below

%CCR = (mg reduction sugar/mg cellulose) x 0,9 x 100%

Reduction sugar weight can be obtained from reduction sugar analysis result and mg cellulose can be obtained from characteristic analysis result.

2.3 Ethanol Fermentation

Ethanol fermentation was done using Cao *et al.,* (2012) modified method. *S.cerevisiae* was prepared by moving from slide agar to PGY (Peptone Glucose Yeast) media containing 7,5g/L Peptone; 4,5g/L yeast extract; 20g/L glucose. One ose of *S.cerevisiae* moved into 100mL of PGY broth media before incubated at 30^{o}C for 12 hours. Hydrolysate was sterilized in 121^{o}C for 30 minute before used. *S.cerevisiae* was added 10% (v/v) in hydrolysate then incubated at 30^{o}C for 36 hours and sampling was done on 0, 4, 8, 12, 24, 36 hours. All steps in this method were done under sterile condition. Reduction sugar was analyzed using DNS method and ethanol was measured using Gas Chromatography.

3. RESULT AND DISCUSSION

3.1 Sorghum Leaves Characteristic

Water content, Water Soluble Compound (WSC), and lignocellulosic composition of sorghum leaves were presented in Table 1.

Table 1. Sorghum leaves Characteristic

Composition		Amount
Water content (%)		10.02
WSC (%db)		20.99
Lignocellulose (%db)	Hemicellulose	32.93
	Cellulose	26.89
	Lignin	17.80

Sorghum leaves were in powder shape and affected the water binding capacity. Powder shape could bind moisture easily and made the water content higher. WSC (Water Soluble Content) was contained some components that soluble into water such as sugar, minerals, phenol, and others. The value of WSC was depended on the structure and composition of the source.

Hemicelluloses was the highest content of lignocellulose composition of sorghum leaves. Hemicelluloses can act as inhibitor in enzymatic hydrolysis by limiting the contact between enzyme and cellulose. Sorghum leaves contained hemicelluloses between 26,4 – 38,5 % (Murray, 2008).

3.2 Sorghum Leaves Enzymatic Hydrolysis

Cellulase was added into the mixture 90FPU/g biomass. Reduction sugar concentration during enzymatic hydrolysis was shown in Figure 1.

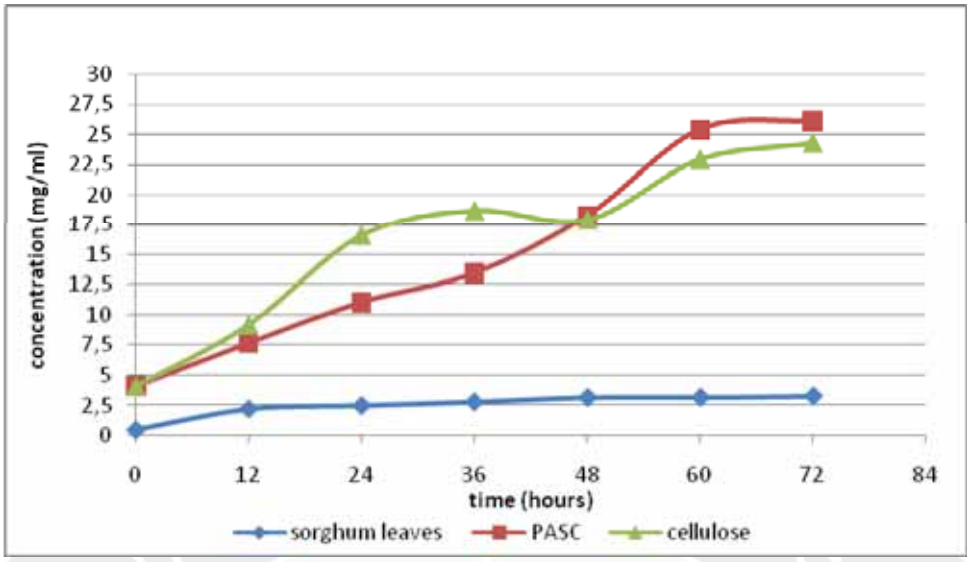

Figure 1. Reduction Sugar Concentration During Enzymatic Hydrolysis

In enzymatic hydrolysis, PASC and cellulose were used as control. Highest reduction sugar concentration from PASC and cellulose enzymatic hydrolysis were 26,15mg/ml and 24,26 mg/ml. PASC produced higher reduction sugar concentration because of the amorphous structure that made it easier to hydrolyzed.

Reduction sugar was increased during enzymatic hydrolysis. Cellulase actively converted cellulose into reduction sugar in 24-48 hours. At 60-72 hours there were no significan increasing of reduction sugar. It can be occurred because the amount of cellulose as the cellulase substrate has been decreased. Limited number of cellulose was decreased the chance of contact between cellulase and substrate. Highest production of reduction sugar was 3,18 mg/ml.

This result shown products from enzymatic hydrolysis can be used as an enzyme activity inhibitor. Cellulase activity inhibition can be done in enzymatic hydrolysis by sugar especially cellubiose and glucose (Taherzadeh and Karimi, 2007).

Lignocelluloses composition was caused different glucose production. In lignocelluloses biomass, cellulose was bound physically to hemicelluloses and chemically to lignin. Lignin and hemicellulose can inhibit cellulase access to cellulose. The inhibition and the decrease of hydrolysis efficiency will produce lower sugar (Mussato *et al.,* 2008).

3.3 Cellulose Enzymatic Hydrolysis Efficiency

Cellulose enzymatic hydrolysis was stated in % CCR (Cellulose Conversion Ratio). % CCR value from Sorghum leaves was 55,058 %. The result was meant that only 55 % cellulose converted into glucose. Enzymatic hydrolysis in this research was conducted without pretreatment. There were still hemicelluloses and lignin content that can inhibit cellulase to reach cellulose and produce low % CCR values.

% CCR can be affected by pretreatment severity. Pretreatment increased the surface area of the raw material samples, which leads to sufficient contact between cellulase and substrate. Thus, reduction sugar released increased (Lu, 2012)

The digestibility of cellulose presented in lignocellulosic biomass was hindered by many physicochemical, structural, and compositional factors. In the conversion of lignocellulosic biomass to fuel, the biomass needs to be treated so that the cellulose in the plant fibers was exposed. Pretreatment used various techniques, including ammonia fiber explosion, chemical treatment, biological treatment, and steam explosion to alter the structure of cellulosic biomass in order to make cellulose more accessible (Kumar, 2009).

Recalcitrance of lignocellulosic biomass was attributed to numerous factors: low substrate accessibility, high degree of polymerization (DP) of cellulose, presence of lignin and hemicellulose, high crystallinity, particle size, and porosity. Most of these factors were correlated with substrate accessibility, which was suggested to be the most important substrate parameter impacting hydrolysis rate. Ball milling and cellulose dissolution in cellulose solvents followed by regeneration in anti-solvents can greatly increased cellulose accessibility before enzymatic hydrolysis (Sathisuksanoh, 2011).

3.4 Ethanol Fermentation

S. cerevisiae was added 10% (v/v) with concentration $1x10^6$ CFU/ml before incubated at 30°C for 36 hours. Reduction sugar concentration and ethanol changing during fermentation shown in Figure 2.

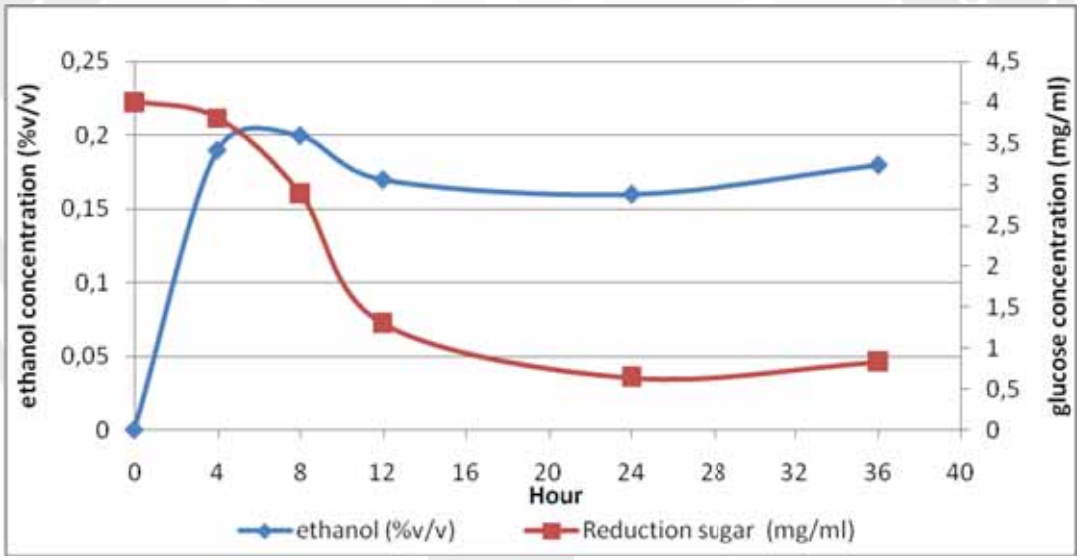

Figure 2. Reduction Sugar Concentration during Fermentation

Based on picture 2 above, on 12 first hour reduction sugar decreasing was balanced by increasing of ethanol concentration. It was declared that reduction sugar converted into ethanol by *S. cerevisiae* trough fermentation. There were some sugar cannot be converted by *S. cerevisiae.*

Reduction sugar concentration was decreased during fermentation. Highest sugar reduction showed at first 12 hours and there were no significant changing on 24 and 36 hours. The first 12 hours were log phase for *S. cerevisiae*. *S. cerevisiae* consumed sugar in a big amount for growth and ethanol production. Final reduction sugar concentration in hydrolysate was almost zero. Initial reduction sugar

concentration from hydrolysate was low and cannot fulfill the growth of *S. cerevisiae*. Optimum *S. cerevisiae* growth needed 10% reduction sugar concentration (Afriyanti, 2014).

Highest ethanol concentration was produced during 12 first hour. Similar ethanol concentration result was found on 24 and 36 hour. On 12 first hour, *S. cervisiae* entered log phase and produced ethanol actively. On the next observation hour, *S. cerevisiae* entered stationary phase.

4. CONCLUSION

The use of untreated sorghum leaves as biomass in ethanol production produced low cellulose conversion into reduction sugar. The hemicelluloses and lignin contained in sorghum leaves can act as inhibitor in enzymatic hydrolysis. % CCR produced was 55,058% with the highest reduction sugar was 3,18 mg/ml. In fermentation phase, highest ethanol production was 0,23 % (v/v). Pretreatment was needed to breakdown the lignocelluloses binding and increase the contact between cellulose and cellulase.

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Dilation of The Brain Ventricles Due to Infection of *Toxoplasma Gondii*

Lucia Tri Suwanti[1,3,*] , Mufasirin[13] , Hani Plumeriastuti[2]
[1]Department of Parasitology, Faculty of Veterinary Medicine, Universitas Airlangga
[2]Department of Pathology, Faculty of Veterinary Medicine , Universitas Airlangga
[3]Researcher of Institute of Tropical Desease , Universitas Airlangga
Surabaya, Indonesia
email: tswant@gmail.com

1. INTRODUCTION

Toxoplasma gondii is an obligate intracellular parasite that infects warm-blooded vertebrates. Toxoplasma gondii infection acquired by pregnant women during gestation can lead to cause defects in the offspring, with clinical sign hydrocephalus or microcephalus, intracerebral calcification, and chorioretinitis (Dubey, 2008). Hydrocephalus is a pathologic disorder such as cerebral ventricel enlargement which is due to the flow of cerebrospinal fluid is disrupted (Felderhoff-Mueser *et al*., 2001). This study aims to determine whether the *Toxoplasma gondii* causes dilation of the ventricles of the brain of mice infected congenitally.

2. METHOD

Twenty pregnant mice aged 11.5 days were divided into 2 groups. Mice in the treatment group (T) were infected with *T. gondii* and the others as control group (c) were not infected. Infectious dose was 10 takizoit each mouse intraperitoneally. The control group was injected only 100 mL of physiologic saline. All mice maintained until delivery. Puppies were sacrificed and them brain were removed and fixed in 10% buffered formalin to prepare histology slides with HE staining to observe ventricular dilation (as a marker hydrocephalus).

3. RESULTS AND DISCUSSION

Representative frames from the histopathology of brain are in Figure, weighted ventricles showed significant differences between the uninfected (C) and infected (T) mice. Ventricles of uninfected mice were narrower than ventricles of uninfected mice. It means that *T. gondii* infection caused ventricular dilatation. Quantitation of the differences in ventricular size more than fivefold. This results differs with Hermes *et al.*, (2008), they showed that just moderate ventricular dilatation in infected mice. They were observed the ventricular dilatation in MRIs of chronically infected mice and they suggested that it was caused by neuronal cell loss or damage. Dilataion of venticles were associated with development of hydrocephalus in congenital toxoplasmosis.

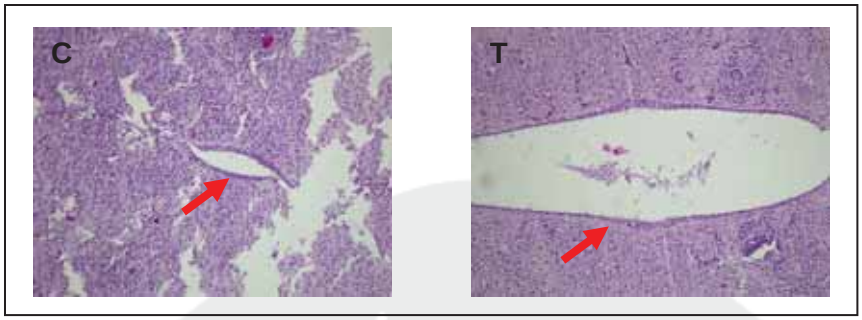

Figure 1. Brain Ventricle (C. Uninfected, T. Infected)

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Effect of Paclobutrazol on Growth and Saponin Content of Binahong (*Anredera cordifolia* (Ten.) Steenis)

Rosiana Dwi Wahyuni[1] and Kumala Dewi[2]
[1] Alumni of Faculty of Biology, Universitas Gadjah Mada, Jl. Teknika Selatan, Yogyakarta.
[2] Faculty of Biology, Universitas Gadjah Mada, Jl. Teknika Selatan, Yogyakarta 55281
Corresponding author: kumala.dewi@ugm.ac.id

Abstract

Binahong (*Anredera cordifolia* (Ten.) Steenis) is well known as medicinal plant. It contains saponins that act as antioxidants, anti bacterial or anti fungal. This research was aimed to evaluate the effect of paclobutrazol on growth and saponin content of binahong. Bulbs of binahong were selected, planted in a plastic bucket containing a mixture of soil and compost (3 : 1 = v:v) and watered twice a week. After two months, paclobutrazol of 0 ppm, 100 ppm, 150 ppm, 200 ppm or 250 ppm were applied by spraying to the whole plant. For each concentration 5 plants were used as a replicates. All plants were grown for another two months and then growth parameters were observed namely plant height, leaf number, stomata density, length and width of stem's epidermis cell, chlorophyll content as well as saponin content. The result showed that paclobutrazol significantly reduced plant height and leaf number, however paclobutrazol increased chlorophyll content, stomata density, width of stem's epidermis cells and saponin content in binahong.

Keywords: Binahong (*Anredera cordifolia* (Ten.) Steenis), paclobutrazol, saponin

1. INTRODUCTION

Andredera cordifolia is one of herbal medicine plants and it belongs to family of basellaceae. This plant originated from South America but it spreads worldwide. In Indonesia this plant is called "Binahong" and it has been reported that this plant produced secondary metabolites such as alkaloids, flavonoids, polyphenols, flavonoid and mono polysaccharide including L-arabinose, D-galaktose, L-rhamnose (Rachmawati, 2008; Khunaifi, 2010). *Andredera cordifolia* grows as a climbing succulent plants and it has been widely cultivated as an ornamental vine in tropical regions (Astuti *et al*., 2011). The growth of *Andredera cordifolia* is relatively rapid and it is also difficult to limit the spreading of this plant as it can twine up trellises, fences, or rock walls easily. Often this plant is considered as weed, thus it is important to limit the growth of *Adredera cordifolia* so that it can be cultivated in a small area. The rapid growth of a certain plant normally is determined by cell elongation which is controlled by hormones such as brassinosteroids and gibberellin. It has been reported that rice mutants deficient in either brassinosteroids or gibberellin display a dwarf phenotype (Tong *et al*., 2014).

Paclobutrazol is a growth retardant that act through inhibition in gibberellin biosynthesis (Rademacher, 2000). As the level of endogenous gibberellin decreased, the cell elongation will also reduced and then it caused the plant become semidwarf

or dwarf following paclobutrazol application. Nazarudin *et al*., (2007), reported that application of paclobutrazol of 1.25, 2.50 and 3.75g/L inhibited plant height and leaf expansion of *Syzygium campanulatum*. In *Zinnia*, paclobutrazol of 10, 20 and 30 mg/L reduced plant height and length of lateral branch, however, it increased fresh and dry weight of aerial parts and roots, lateral branch number as well as flower number (Asgarian *et al*., 2013). Beside reducing growth, paclobutrazol aplication may increased secondary metabolite content. It has been reported that total phenol was increased considerably in *Ocimum sanctum* plants treated with paclobutrazol compared to control (Gopi *et al*., 2009). According to a review reported by Haralampidis *et al*., (2002), saponins are plant secondary metabolites that have surfactant properties and give stable, soap-like foams in aqueous solution. Saponins represent a group of structurally diverse molecules that consists of glycosylated steroids, steroidal alkaloids and triterpenoids. In various plants, saponins often occur as complex mixtures. The composition and content of saponin may vary markedly depending on the genetic background of the plant material, the tissue type, the age and physiological state of the plant and environmental factors. Astuti *et al*., (2011) reported that leaves, stems and tubers of "Binahong" plant contain saponins triterpenoid and steroid. The effect of paclobutrazol on saponin content in "Binahong" was also evaluated in this experiment.

2. METHODS

2.1 Chemicals

Chemicals used in this experiments were paclobutrazol, acetone and ethanol. Paclobutrazol (GoBEST 250 SC, PT Petrokimia Kayaku, Gresik) was obtained from a fertilizer shop in Gresik, East Java. Acetone was used for chlorophyll analysis , whereas ethanol 96% was used to make leaf extract of "Binahong" for saponin determination.

2.2 Procedures

This experiment was conducted by randomized block design. Aerial tubers of *Adredera cordifolia* having girth of 2 cm were selected and planted in a plastic bucket containing a mixture of soil and compost (3 :1 = v:v). Tubers were watered regularly, and after tubers were sprouted (week 2) paclobutrazol of 0 ppm (control), 100 ppm, 150 ppm, 200 ppm or 250 ppm were sprayed to the plants. Five replicates were made for each treatments and all plants were grown for another three weeks. Parameters observed were plant height, leaf number, chlorophyll content, stomatal index, epidermis cell length and saponin content. Chlorophyll content was determined spectrophotometrically according to Harborne (1987). Leaf sample of 0.1g was grinded using a mortar and pestle and poured with 10 ml of 80% acetone. Leaf extract was then filtered through a Whatman No 3 filter paper. The clear green leaf extract was then put in a small tube and its absorbance was measured using spectrophotometer (Spectronic D-2000) at α of 663 nM and 645 nM. Saponin content was determined according to method explained in Yachya (2012). Leaf was sampled and dried under sunlight. Dried leaf was then grinded and 40 mg of dried leaf powder was extracted in 4 mL of 96% ethanol. Extract was then placed in a waterbath of 80^{o}C for 45 minutes and and dried until the volume left was 0.2 mL. Extract was diluted with 10 mL aquadest and absorbance of extract was measured using

spectrophotometer (Spectronic D-21) at α of 365 nM (Stahl, 1985). Saponin content in leaf extract was determined using a standard Saponin (Merck). Data were analyzed by One Way Anova and Duncan Multiple Range Test with a significant level of 5%.

3. RESULTS AND DISCUSSION

The effect of paclobutrazol on plant height and leaf number is presented in Fig. 1.

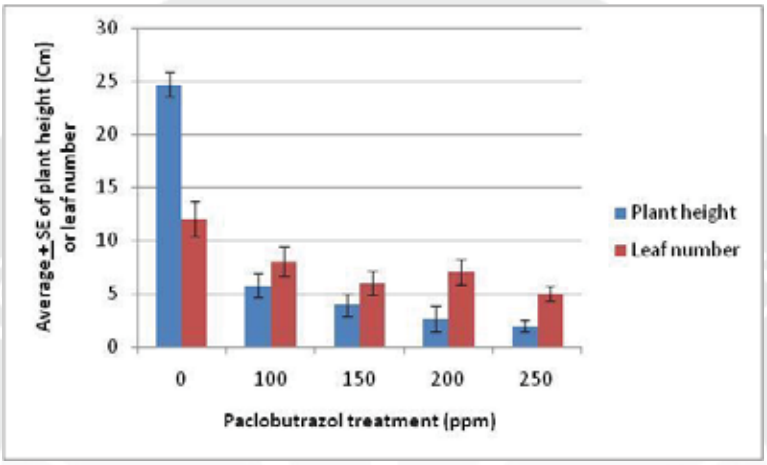

Figure 1. Effect of paclobutrazol on average internode length and leaf number of *Andredera cordifolia* (n = 5)

From Fig. 1 it can be seen that paclobutrazol decreased the average plant height significantly. It has been known that paclobutrazol inhibit the activity of enzymes that converting the *ent*-kaurene to *ent*-kaurenoic acid (Berova and Slatev, 2000). Both *ent*-kaurene and *ent*-kaurenoic acid are gibberellin precursors. As the biosynthesis of gibberellin precursors was inhibited by paclobutrazol, so the level of endogenous gibberellin in paclobutrazol treated plant is predicted to be low and it leads to an inhibition in cell elongation and reduction in plant growth. The leaf of *Andredera cordifolia* grows alternately along its stem, so as the plant height was reduced by paclobutrazol, the internode length was also shorter in plants treated with paclobutrazol compared to control (Fig. 2). The leaf number consequently was also less in plants treated with paclobutrazol compared to control (Fig. 1). Mansuroglu *et al*., (2009), reported that application of paclobutrazol in *Consolida orientalis* also reduced plant height and internode length so the plants become sturdy. However, plants treated with paclobutrazol had darker green leaves and deeper violet flowers than that of control plants. In other ornamental plants, paclobutrazol has been widely applied to make a dwarf plants, to accelerate flowering as well as to enhance flower colour, such as in ornamental kale (*Brassica oleracea* var. acephala) (Mello *et al*., 2012).

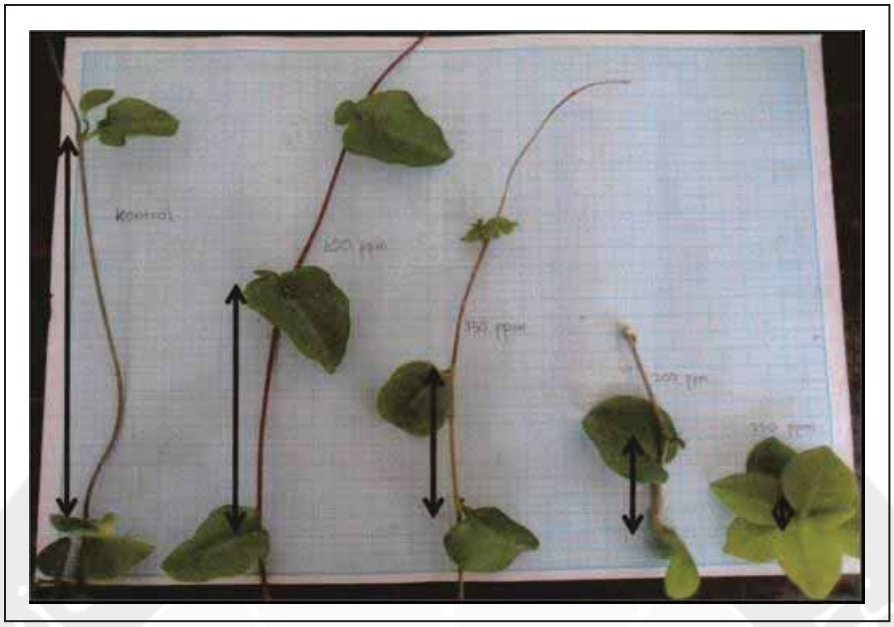

Figure 2. Effect of paclobutrazol on internode length of *Andredera cordifolia*

The effect of paclobutrazol on chlorophyll content is presented in Fig. 3. It was found that chlorophyll-a and total chlorophyll content increased in "Binahong" plants treated with paclobutrazol compared to those of control, whereas chlorophyll-b relatively similar either on "Binahong" plants treated with paclobutrazol or control.

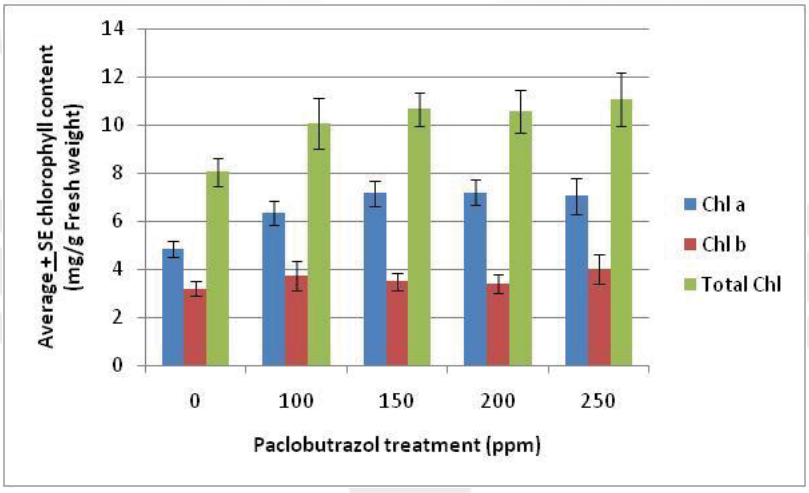

Figure 3. Effect of paclobutrazol on chlorophyll content of *Andredera cordifolia* (n=5)

The effect of paclobutrazol on chlorophyll content may differ amongst species. Nazarudin *et al*., (2006), reported that in *Syzygium campanulatum* Korth, chlorophyll content did not altered by paclobutrazol treatment. However, in *Camelina sativa* L. Crantz, chlorophyll content increased by paclobutrazol. As the leaves were also photosynthetically active for longer period, paclobutrazol treatment of 100 mg/L

increased seed yield and oil content in *Camelina sativa* (Kumar *et al*., 2012). It has also been reported that paclobutrazol reduced plant height and leaf area but increased chlorophyll content and number of flower bud in potting *Hibiscus rosa-sinensis* L (Nazarudin, 2012). The higher chlorophyll content in paclobutrazol treated "Binahong" may be related to the influence of triazole on endogenous cytokinin levels. Tsegaw *et al*., (2005), proposed that could stimulate cytokinin synthesis and it leads to chlooroplast differentiation and chlorophyll biosynthesis as well as prevention of chlorophyll degradation. in potted *Tabernaemontana coronaria* Stapf, it has been reported that paclobutrazol increased cytokinin content (Youssef *et al*., 2013). In addition, increasing chlorophyll content in paclobutrazol treated plants could also be due to an increase in phytol production which is essential part of chlorophyll molecule. Phytol is synthesized in the same pathway with gibberelin. As paclobutrazol treatment blocked gibberelin synthesis in early pathway, this caused gibberellin precursor namely geranylgeranyl-diphosphat is metabolized to phytol synthesis pathway (Chaney, 2005).

It has been known that paclobutrazol affect photosynthesis. However, the mechanism by which paclobutrazol increased or decreased photosynthesis rate is not clearly explained. In peach (*Prunus persica* (L.) Batsch) leaves treated with paclobutrazol, stomata density increased linearly with the doses, on the other hand, length of stomata also decreased linearly (Blanco *et al*., 1997). In "Binahong' treated with paclobutrazol, similar finding was found in which stomata density in adaxial suface of leaf increased but length of stomata decreased as the level of paclobutrazol increased (Fig.4)

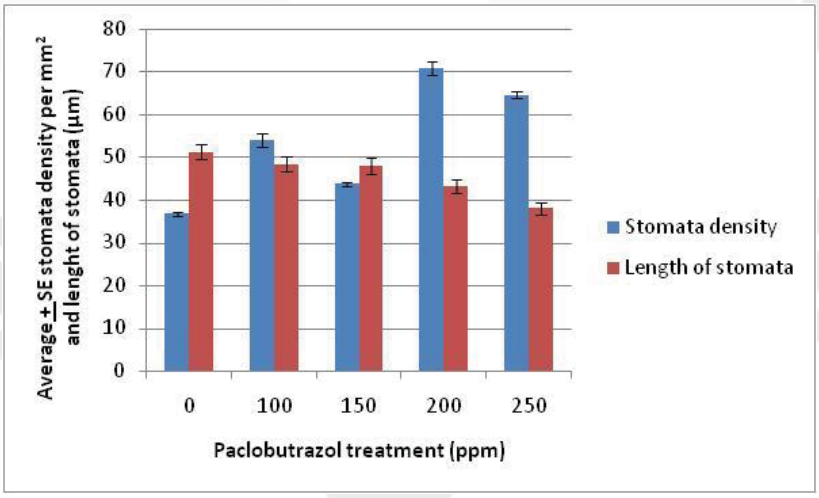

Figure 4. Effect of paclobutrazol on stomata density and length of stomata on adaxial leaf surface of *Andredera cordifolia* (n = 15)

The density or size of stomata may vary over the surface of leaf, but according to Yim *et al*., (1977), increased stomatal density are commonly found in plants treated by growth retardant that showed reduction of the leaf area. Stomatal density could indirectly influence photosynthesis as it may influence the leaf capacity in absorbing CO_2. As the chlorophyll content in paclobutrazol treated plants also increased, this could increased phootosynthesis and photoassimilate production. It has been

reported that paclobutrazol could also enhance plant resistance to stress condition (Tari, 2003). This could possibly due to the ability of paclobutrazol in altering stomatal size and density which may also affecting transpiration rate. This assumption warrants further investigation.

The effect of paclobutrazol on lenght and width of stem's epidermis cell have been evaluated and it was found that epidermis cell of "Binahong" plants treated with paclobutrazol were shorter compared to control, on the other hand the width of epidermis cells were greater on "Binahong" plants treated with paclobutrazol compared to control (Fig. 5). It is known that the direction of cell expansion is determined by the orientation of cortical microtubules (Lloyd, 2012), whereas specific pattern of cell division and cell expansion determine plant's morphogenesis.

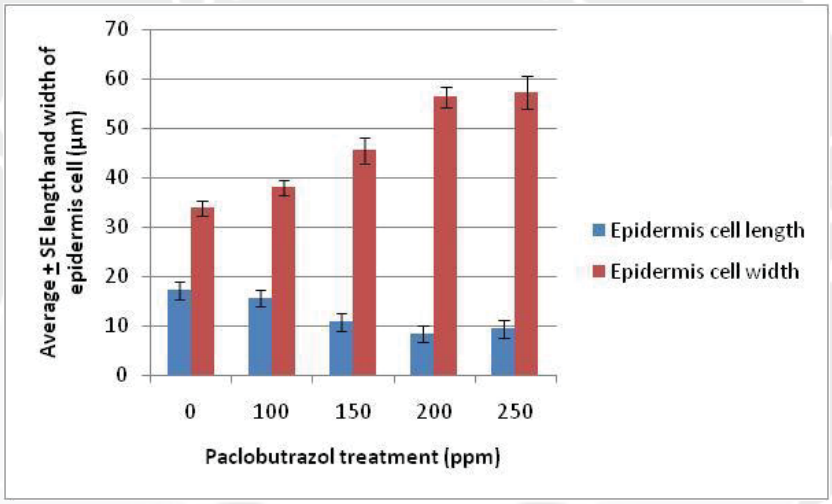

Figure 5. Effect of paclobutrazol on length and width of stem's epidermis cell of *Andredera cordifolia* (n=15)

Baluška *et al*., (1993), reported that in maize mutant having deficiency in gibberellin biosynthesis as well as in wild ttype maize treated with paclobutrazol, roots apical cells become wider and shorter and it was due to cortical cells in these regions were impaired in their ability to develop highly ordered transversal arrays of cortical microtubules. The normal arrangement of cortical microtubules and the polarity of cell growth characteristic of roots were able to be re-established by gibberellin application. In addition, Locassio *et al*., (2013), reported that in Arabidopsis plant treated with paclobutrazol, most of α-tubulin dissociated from β-tubulin and appear in its monomeric form. It was suggested that microtubule orientation is regulated indirectly through physical interaction between DELLA proteins and the prefoldin complex, a cochaperone required for tubulin folding. In the presence of gibberellin, DELLA proteins are degraded and the prefoldin complex stays functional in the cytoplasm, but in the absence of gibberellin, the prfoldin complex is localized to the nucleus, which severely compromise α/β tubulin heterodimer availability, and affecting microtubule organization.

The effect of paclobutrazol on saponins content in leaves of "Binahong' is presented in Fig. 6.

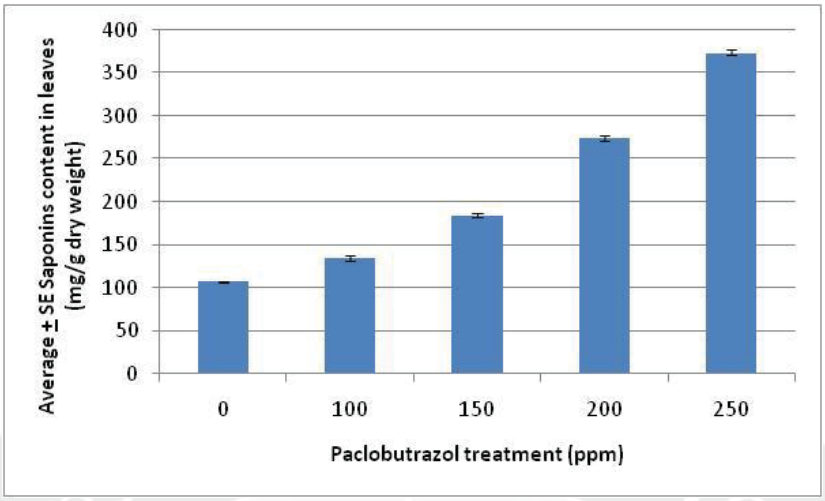

Figure 6. Effect of paclobutrazol on saponins content in leaves of *Acrider cordifolia* (n=3)

From Fig. 6 it can be seen that the higher the level of paclobutrazol applied, the level of saponins in leaves of "Binahong" increased accordingly. It has been reported that triterpenoid saponins are synthesised via the isoprenoid pathway by cyclization of 2,3-oxidosqualene to give primarily oleanane (bamyrin) or dammarane triterpenoid skeletons. The triterpenoid backbone then undergoes various modifications (oxidation, substitution and glycosylation), mediated by cytochrome P450-dependent monooxygenases, glycosyltransferases and other enzymes (Haralampidis *et al*., 2002). According to Rademacher (2000), saponins biosynthesis is also catalyzed by cholesterole 26-hydroxylase which is a cytochrome P450 dependent monooxygenase. As there are many isozymes of cytochrome P450 dependent monooxygenase, it could be that paclobutrazol inhibits ent-kaurene oxidase that leads to an inhibition of gibberellin biosynthesis but as a compensation it possibly increased the activity of 26-hydroxycholesterol thus saponins content also increased in paclobutrazol treated "Binahong". The mechanisms by which saponins content increased by paclobutrazol treatment warrant further examination.

4. CONCLUSIONS

Based of the results and discussion, it can be inferred that paclobutrazol application reduced growth of "Binahong". Paclobutrazol of 200 ppm made "Binahong" plants become dwarf, whereas application of higher dose of paclobutrazol (250 ppm) caused severe dwarf plant. It is suggested that cultivation of "Binahong" in a small area can be conducted by creating dwarf plants of through paclobutrazol application. The internode length of "Binahong" treated with paclobutrazol decreased and it was due to the reduced length in stem's epidermis cells. However, the total chlorophyll content and saponins content increased in "Binahong" plants treated with paclobutrazol. An increased saponins content in "Binahong" leaves through paclobutrazol application probably could improve the value of "Binahong" as a herbal medicinal plant.

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Preproduction Chitin Deasetilase from Fisheries Waste

Rischa Jayanty[1], Maharani Pertiwi Koentjoro[2], and Endry Nugroho Prasetyo[1]
[1]Biotechnology Laboratory, Department of Biology, Institut Teknologi Sepuluh Nopember, Gedung H 1st floor, Kampus ITS Keputih Sukolilo Surabaya, 60111 Indonesia
[2]Laboratory of Environmental Microbiology, Department of Biological and Environmental Science, Faculty of Agriculture - Shizuoka University & Structural Biology Research Center, Inter-University Research Institute Corporation - High Energy Accelerator Research Organization (KEK), Tsukuba-Ibaraki, Japan
*Corresponding author email address: maharanipertiwi@yahoo.com

Abstract

Chitin Chitin is abundant, easily obtained, and is the second renewable natural polymer after cellulose which has limited benefits. However the product of chitin deacetylase, chitosan, has many benefits and applications. So far, chemical methods which had been used to produce chitosan require much energy, non eco-friendly, and the results are less uniform. Chitinolytic microbes which capable producing chitin deacetylase can be the alternative solution. Chitin deacetylase can produce chitosan with better quality since the enzyme is selective and not damaging the structure of chitosan chains. Fisheries waste contains chitin polymer is potential for bacteria that can produce chitin deacetylase. The aim of this study is to explore chitin deacetylase-producing bacteria from fisheries waste. The pure colonies bacteria were identified by biochemical character according to Bergey's Manual of Determinative Bacteriology. Isolates of bacteria that had been degrading chitin was screened by observing the of the clear zone chitin selective agar media. Then chitin deacetylase activity was tested by Tokuyasu's method. This study was able to isolate 13 strains of bacteria from fisheries waste. Four of the bacterial strains designated as *Bacillus* B3, *Bacillus* B5, *Bacillus* B6 and *Bacillus* B14 showed clear zone when grown in chitin medium. Highest chitin deacetylase activity was shown by *Bacillus* B3 which was 360.37 U/mL.

Keywords: *Bacillus,* Bacteria, Chitin, Chitin deacetylase, Chitosan.

1. INTRODUCTION

Chitin is a linear heterogeneous polysaccharide of N-acetyl-D-glucosamine and D-glucosamine (Ravi, 2000; Synowiecki and Al-Khateeb, 2003; Tharanathan and Kittur, 2003; Dutta *et al*., 2004; Kurita, 2006; Pillai *et al*., 2009; Domard, 2011). Chitin existence is abundant, easily obtained, and is the second renewable natural polymer after cellulose (Sandford, 1989 ; Ravi, 2000; Patil *et al.,* 2000).Chitin is a biopolymer found in invertebrates, insects, marine diatoms, algae, fungi and yeast (Synowiecki and Al-Khateeb, 2003).

Chitin is an insoluble material and limited benefits in the (Muzzarelli, 1996; Somashekar and Joseph 1996; Shahidi *et al.,* 1999; Tsigos *et al.,* 2000). Results deacetylation of chitin is called chitosan have numerous applications in foods, pharmaceuticals and biotechnological products, cosmetics, textiles, in waste water

treatment and in agriculture (Shahidi *et al.*, 1999;. Gryndler *et al*., 2003; Kato *et al.,* 2003; Senel and McClure, 2004; De Jin *et al.,* 2005;. Kim & Mendis, 2006; Gortari and Hours, 2008; El Hadrami *et al*., 2010; Park and Kim, 2010; Portes *et al.,* 2009; Ramírez *et al*., 2010;. Rangel-Mendez *et al*., 2010; Limam *et al.,* 2011; Muzzarelli *et al.,* 2012; Raja *et al.,* 2012).

Chitosan has been produced by deacetylation of chitin using chemical method (Tsigos and Bouriotis 1995). Chemically conversion of chitin into chitosan has many disadvantages including consumes a lot of energy and produce chitosan has a diverse molecular weight and has are less uniform physical and chemical properties, so it is difficult to apply (Kaur *et al*., 2012: Sugita *et al.*, 2009). Enzymatic methods in the conversion of chitin into chitosan produce better quality, enzymes that selectively and do not damage the structure of the chitosan chains (Khanafari *et al.,* 2008).

Chitin deacetylase is an enzyme produced by the yeast, fungi (mold), bacteria and also in some insect species (Kashyap *et al.,* 2014; Vincy *et al.,* 2014). Fisheries waste is the remains of bones, spines, scales, crustacean shells, shells of invertebrates which are a source of chitin polymer (Arbia *et al.,* 2011). The existence of chitin in the fisheries waste allows high population of bacteria that degrade chitin, therefore exploration of bacteria producing chitin deacetylase in fisheries waste is needed. So in this study performed a selection of chitin deacetylase bacteria that could potentially change the chitin into chitosan.

2. METHODS

2.1 Isolation, Characterization and Identification Bacteria

Sampling was taken in a composite of five different points on the fisheries waste. Fishery samples were selected based on visual observation which showed the condition has degraded. Bacteria isolated from Kenjeran fisheries waste samples, using serial dilution method and spread plate method in chitin medium consists of : yeast extract 10 gr, $(NH_4)_2SO_4$ 4 gr, KH_2-PO_4 0.15 gr, 0.1 gr of chitin, agar 20 gr, distilled water 1 L. Bacteria were incubated 24-48 hours.

Bacterial colonies were selected based on differences in character and pigmentation by macroscopic observation. Selection result of bacterial colonies was purified used streak plate method. Cell shape observed microscopically with simple methylene blue staining and Gram staining to confirm the isolates purity. Isolates that have cell shape and the same stain results is pure isolates. Pure isolates were further characterized by biochemical tests : endospores staining, acid-fast staining, catalase test, oxidase test, needs oxygen test, carbohydrates fermentation, and Triple Sugar Iron Agar (TSIA). Identification of bacteria carried to the genus level. Bacteria identified by Bergey's Manual of Determinative Bacteriology.

2.2 Screening of Chitin Degradation Activity

Isolates of hitinolytic enzyme-producing bacteria was observed clear zone around the colony. The bacteria have pure inoculated into selective medium with the composition: chitin 10 gr, $NaNO_3$ 2 gr, K_2HPO_4 1 gr, KH_2PO_4 1 gr, $MgSO_4$ 0.5 gr, P and N 0.5 gr, agar 20 gr, distilled water 1 L. Bacteria then incubated at room temperature for 3-5 days. Isolates were formed clear zone then tested for chitin deacetylase activity.

2.3 Crude Enzym Preparation

Chitin deacetylase enzyme obtained by liquid fermentation with chitin source substrate added to the fermentation medium. Chitin source in the fermentation medium is derived from commercial chitin. The composition of the fermentation medium is chitin 0.5 gr, yeast extract 0.2 gr, ammonium sulphate 0.2 gr, KH2PO4 0.1 gr, tryptone 0.1 gr, MgSO4.7H2O 0.01gr in 1 liter of distilled water.

2.3 Chitin Deacetylase Activity Test

Chitin deacetylase activity was measured using a modified method Tokuyasu *et al*., (1996) with a standard curve of glucosamine that has the equation y = ax + b. y is the value of the concentration of glucosamine. x is the value of the absorbance of glucosamine. Digestion solution consisting of 3 ml of the enzyme, 8 mg chitin and 1 ml of buffer were incubated for 30 minutes at a temperature of 50ºC, then the enzyme activity was terminated by the addition of acetic acid 33% as much as 200µl. For control, the addition of the enzyme conducted shortly after the addition of acetic acid.

After digestion, the concentration of glucosamine residue formed from deamination reaction is calculated based on the oxidation using $NaNO_2$, following the spectrophotometric method using HCl indole according to Dische and Borenfreund (1950) which has been modified as follows: digestion solution 200 mL was added 200 mL of acetic acid 33% and 200 mL of 5% $NaNO_2$. Vortex solution and left for 10 minutes at room temperature. Added 500 mL of 0.1 mM ascorbic acid and shaken for 30 minutes at room temperature. Then added 800 mL of 5% HCl and 80 mL of indole 1% in ethanol. The reaction mixture simmer in boiling water for 5 minutes to form a red color. The solution was cooled then added 800 mL of absolute ethanol and vortex. Glucosamine concentrations known formed by reaction of reddish brown color that occurs and measured at λ 492 nm. Chitin deacetylase activity was measured in units. One unit of enzyme activity is expressed as the amount of enzyme that produces 1 mM glucosamine residue per minute. The standard used was the concentration of glucosamine at 1 mg / 1mL.

3. RESULTS AND DISCUSSION

3.1 Isolation, Characterization and Identification Bacteria

Isolation of bacteria producing chitin deacetylase by diluting the sample, then inoculated with spread method in chitin agar medium. Bacterial colonies was calculated by total plate count method (TPC) in colony forming units (CFU) (Table 1). The following data and observations made in the method of total plate count (TPC).

Table 1. The Results of Bacterial Colonies

The Number of Colonies			The Number of Bacteria (CFU/mL)
10^{-2}	10^{-4}	10^{-6}	2,5 x 10^{6}
TMTC	25	TLTC	

TMTC : too much to count
TLTC : too little to count

Data from Table 1 shows that The number of bacterial colonies obtained in this isolation was 2.5 x 106. Based on the shape and color of colonies colonies obtained 13 pure bacterial isolates (Table 2).

Table 2. Observations Morphology Colonies of Bacteria

Isolates Code	Form	Margin	Elevation	Colour
B1	Irregular	Undulate	Umbonate	White milk
B2	Circular	Undulate	Flat	White transparent
B3	Circular	Entire	Convex	Turbid white
B4	Circular	Undulate	Raised	White
B5	Circular	Undulate	Raised	White
B6	Circular	Undulate	Raised	White yellowish
B7	Circular	Entire	Convex	Yellow
B8	Circular	Entire	Convex	Yellow
B9	Circular	Entire	Raised	White yellowish
B10	Circular	Undulate	Umbonate	White milk
B11	Circular	Undulate	Umbonate	White milk
B12	Irregular	Undulate	Raised	White milk
B13	Circular	Entire	Convex	White yellowish

Pure isolates were further characterized by biochemical tests : endospore staining, acid-fast staining, catalase test, oxidase test, needs oxygen test, carbohydrates fermentation, and Triple Sugar Iron Agar (TSIA) (Table 3).

Table 3. Observations Biochemical Characterization of Bacteria

Isolates Code	Shape Cell	Gram	Endospore	Acid-fast	oxidase	Catalase	F. Glucose	F. Lactose	F. Mannitol	Motillity	H_2S	Needs Oxygen Test	Genus
B1	Bacilli	+	+	-	+	+	+	-	+	+	-	Aerob	*Bacillus*
B2	Bacilli	+	+	-	+	+	+	-	+	+	-	Aerob	*Bacillus*
B3	Bacilli	+	+	-	-	+	+	-	-	+	-	Aerob	*Bacillus*
B4	Bacilli	+	-	-	-	+	+	-	-	+	-	Aerob	*Corynebacterium*
B5	Bacilli	+	+	-	-	+	+	-	-	+	-	Aerob	*Bacillus*
B6	Bacilli	+	+	-	-	+	+	-	-	+	-	Aerob	*Bacillus*
B7	Cocci	+			-	+	+	+	+	-	-	Aerob	*Staphylococcus*
B8	Cocci	+			-	+	+	+	-	-	-	Aerob	*Staphylococcus*
B9	Bacilli	+	-	-	-	+	+	+	+	-	-	Aerob	*Corynebacterium*
B10	Cocci	-			+	+	+	+	+	-	-	Aerob	*Neisseria*
B11	Cocci	-			+	-	+	-	-	-	-	Aerob	*Neisseria*
B12	Bacilli	+	+	-	-	+	+	+	+	+	-	Aerob	*Bacillus*
B13	Cocci	+			-	+	+	-	-	-	-	Aerob	*Micrococcus*

3.2 Screening of Chitin Degradation Activity

Chitin degradation screening to determine the ability of the isolated bacterial isolates to degrade chitin. Isolates were grown in a medium that chitin as carbon source, suspected isolates produce enzymes chitinolytic will produce a clear zone around the colony (Figure 1) (Kaur *et al.,* 2012). Clear zone is formed due to the termination of bond β -1, 4 homopolymer of N-acetylglucosamine in chitin by enzyme chitinolytic into monomer N-acetylglucosamine. The magnitude of the resulting clear zone is directly proportional to the amount of monomer N-acetylglucosamine from chitin hydrolysis process (Susi, 2002; Muharni, 2009).

Thirteen isolates that were isolated, having grown in medium chitin agar and incubated for 5 days, there are four isolates were able to produce a clear zone that *Bacillus* B3, *Bacillus* B5, *Bacillus* B6 and *Bacillus* B12. The resulting clear zone punctuated by Congo Red (0.1%) staining and washed with 1M NaCl solution.

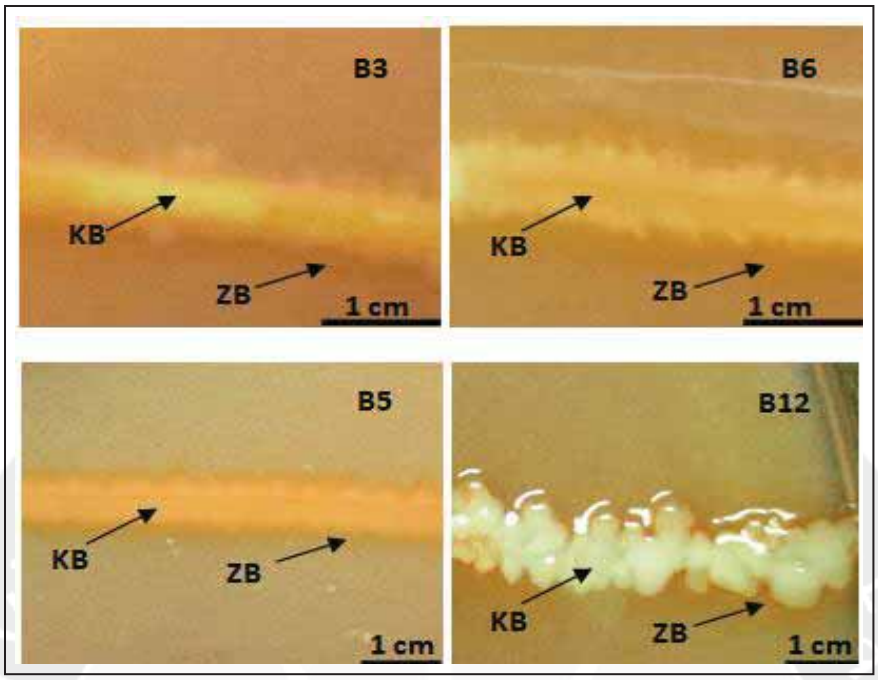

Figure 1. Clear zone by chitin degradation, KB : bacterial colonies, ZB: clear zone

3.3 Confirmation of bacterial strains infection on *K. alvarezii*

After the screening of chitin degradation, isolates that produce a clear zone is then performed chitin deacetylase activity assay as a confirmatory test that isolates the clear zone capable of producing chitin deacetylase. Crude enzyme chitin deacetylase obtained by liquid fermentation substrate chitin in the fermentation medium. Generally, enzymes that degrade extracellular chitin produced. This enzyme is released by the cell and are on the medium, then centrifuge. Given the enzyme is easily degraded the crude extract enzyme centrifugation process is carried out at low temperature (4°C) (Natsir, 2002). Chitin deacetylase activity was measured using a standard curve of glucosamine by the equation y = 832.64x + 16 515, and the value of R2 = 0.9938. Results of chitin deacetylase activity in each isolate can be observed in Figure 2.

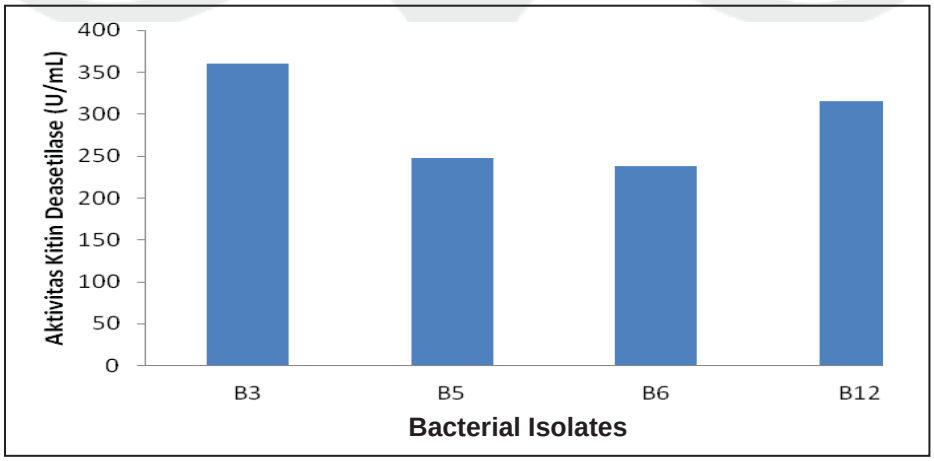

Figure 2. Results of Chitin deacetylase activity

The highest activity of chitin deacetylase results produced by *Bacillus* B3 is equal to 360.37 U / mL. The lowest chitin deacetylase activity produced by *Bacillus* B6 was 237.47 U / mL. *Bacillus* B5 have chitin deacetylase activity was 247.80 U / mL and *Bacillus* B12 was 315.41 U / mL. Chitin deacetylase is an enzyme that catalyzes the hydrolysis of chemical reactions Acetamido group-N-acetyl-D-glucosamine in chitin. The value of chitin deacetylase activity of each isolate the 2.5-hexose value anhydride stained indole reaction results chitin substrate that CN bond and peptide bonds in linear amide hydrolyzed by the enzyme chitin deacetylase (Natsir, 2002; Kashyap *et al.,* 2014).

4. CONCLUSIONS

Fisheries waste bacterial that can be isolated and potentially produce chitin deacetylase is *Bacillus* B3, *Bacillus* B5, *Bacillus* B6 and *Bacillus* B12. The highest chitin deacetylase activity produced by *Bacillus* B3 was 360.37 U/mL.

5. ACKNOWLEDGEMENT

The authors are grateful for the academic grant provided by Biomaterial and Enzyme Technology Research Team (2014/2015) supervised by Dr. techn. Endry Nugroho Prasetyo, M.T. in which the study was conducted. Special thanks are also given to father Imam Suwono and mother Sutini for the motivation, support and prayers; to Nur Shabrina, S.Si. for proof-reading the manuscript; to family of Scylla serrata 2011 and Biomaterial and Enzyme Research Team (2014/2015) for the motivation, support and assistance during the study.

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Growth Responses of Kencur (*Kaempferia galanga* L.) with Addition of IBA and BAP in *In Vitro* Propagation

Muji Rahayu[1], Bambang Pujiasmanto[1], Samanhudi[1]*, Ahmad Yunus[1], Dian Rahmawati[2]
[1]Faculty of Agriculture, University of Sebelas Maret, Surakarta, Indonesia
[2]Student of Faculty of Agriculture, University of Sebelas Maret, Surakarta, Indonesia
*Email: samanhudi@ymail.com

Abstract

Kencur (*Kaempferia galanga* L.) is one of the important medicinal plant because the properties that can cure several diseases such as ekspetoransia, diuretics, and stimulant. Cultivation with conventional breeding can't meet the demand of kencur seed in the market, so *in vitro* cultivation can be used as an alternative to supply rapid and uniformly of kencur seeds. Explants were taken from the rhizome tip and cultured on MS medium combined with IBA (*Indole Butiric Acid*) and BAP (*Benzyl Adenine Purin*) at concentration of 0 ppm, 1 ppm, 2 ppm, 3 ppm, and 4 ppm. The major variables is the number of micro-shoot on the explant. The highest frequency induction of micro-shoots cultured on MS suplemented with 0 ppm of IBA and 3 ppm of BAP. Mostly micro-shoot rooting at the 12 days after planting, and the fastest rooting at 7 days after planting cultured on MS medium suplemented with 2 ppm of IBA and 4 ppm of BAP.

Keywords: Kencur, *Kaempferia galanga*, IBA, BAP, in vitro

1. INTRODUCTION

Kaempferia galanga is one of medicinal plants belonging to the family Zingiberacea. *Kaempferia galanga* also known as a important medicinal plant that can cure various diseases, because of his advantages as ekspetoransia, diuretics, and stimulansia. In Indonesia *K. galanga* also treat for coughs, stomach ulcers, swelling, vomiting, tetanus, pain, headache, menstrual smooth and influenza (Yan Nie *et al*., 2012).

The high demand of *K. galanga* especially from medicine factory encourage the farmers to be able to provide *K.galanga* in a large quantities. *K. galanga* crop cultivation has been done by using rhizomes have some drawbacks such as low productivity, susceptible to pests and diseases and costly (Rahman 2005). Tissue culture became one of the alternatives for *K. galanga* propagation with uniformly and high result. The advantages of seeds suppliying through tissue culture are obtained superior plant material in large quantities and uniform, and can be obtained sterile culture (mother stock) so it can be used as material for further propagation (Bieber 2008 cit. Endang 2011). In the use of tissue culture propagation plant growth regulator (PGR) is very important, which is to regulate organogenesis and morphogenesis in the formation of buds, roots and callus (Bieber 2011). *K. galanga* tissue culture supplemented with auxin (IBA) and cytokinins (BAP) on Murashgie and Skoog media. The present study was conducted to obtain a combination of the concentration of auxin (IBA) and cytokinin (BAP) which is use on *K. galanga* tissue culture.

2. MATERIAL AND METHODS

2.1 Collection of explants and surface sterilization

Rhizome was execised aseptically from in vitro grown cultures originally established from field. The rhizome explants were excised and cleaned thoroughly under continuously fushing of running tap water for 30 minutes to remove all the soil. Then washed with solution of antiseptic (sunlight) 10 % for 3 hours, and washed with aquades 2 times. The explants were then immersed with Dithane (fungicide) for 4 hours then antibioyic (streptomicyn 2 %) for 1 hours. Then washed with aquades 2 times. Surface sterilization was done either with sodium hypoclorite (20%) with drop of tween 80. The last should be whased 2 times with sterile destilated water under laminar air flow chamber.

2.2 Culture condition

After surface sterilization, the explants were trimmed to appropriate sizes and inoculated on MS medium for shoot multiplication. To finding out the best results for shooting and rooting, the medium was supplemented with different concentrations of IBA either alone or IBA with different concentrations of IBA and BAP. The regenerated micro shoots were placed on half-strength MS medium supplemented singly with various concentrations of IBA for rooting.

The pH of the medium was adjusted to 5.8-6.3 before adding agar and the medium was autoclaved for 45 minute with 1.5 kg/cm^2 under pressure. Each explant was cultured in a culture bottle containing 15ml of sterilized semisolid medium. Each experiment was repeated three times per treatment were taken into account. All the data were analyzed by using descriptive analyze.

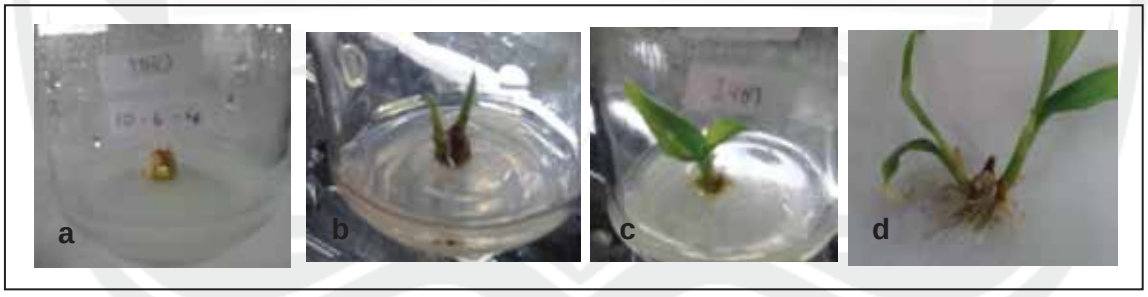

Figure 1. a-d : Plantlet regeneration from rhizome bud of *Kaempferia galanga* under in vitro condition

a) Shoot bud initiation from rhizome bud explants on MS media with IBA 0 ppm after nine days cultured
b) Development of multiple shoot and root initiation on MS media with IBA 4 ppm and BAP 2 ppm after 30 days cultured
c) Elongation of shoot on MS media with IBA 4 ppm and BAP 1 ppm after 43 days cultured
d) Harvesting of plantlet after 48 days cultured on MS containing IBA 2 ppm and BAP 4 ppm

3. RESULT AND DISCUSSION

3.1 Shoot Induction

Shoots development in tissue culture method influenced by the composition of PGR in the media. Chirangini (2004) said that the rhizomes of *K. galanga* and *K. rotunda*

can induce shoots when cultured on media Murashgie and Skoog (MS) combined by PGR.

The explants were cultured will be develop form roots, shoots and leaves. Shoot induction influenced by the use of PGR are usually from cytokines, a type cytokines used in this study is BAP (Benzyl Adenine Purin). Faster appeared bud then faster produced material for plant propagation. The following table is presented median and mode shoots induction with different concentrations of IBA and BAP.

Table 1.2. Mode and median of shoot number with combination of IBA and BAP

IBA	BAP															Median	Mode
	0 ppm			1 ppm			2 ppm			3 ppm			4 ppm				
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3		
0 ppm	18	18	10	6	11	-	6	6	-	11	15	-	6	6	-	10	6
1 ppm	7	5	13	13	5	5	11	-	-	10	6	-	6	6	-	6	5
2 ppm	22	6	-	5	-	-	10	7	11	-	-	-	5	-	-	7	5
3 ppm	7	-	-	18	5	-	6	-	-	12	-	-	10	14	-	10	-
4 ppm	41	10	-	12	-	-	6	10	9	14	-	-	-	-	-	10	10
Median		10			6			8			11.5			6			
Mode		18			5			6			-			6			

The time required to shoot induction ranged from 5 to 41 days of culture. Most of micro shooting induction appeared on 5 and 6 days of culture were cultured on MS treatment with IBA 0 ppm, 1 ppm and 2 ppm. While on treatment of BAP 1 ppm shoot induction have appeared on 5 HST. Combination of IBA and BAP in the media proved to stimulate shoot induction on explants, as repoted by Lestari (2011) that the combination of cytokinin and auxin can stimulate morphogenesis in bud formation.

In Table 1.2 shows that the range of number of shoots between one to five for each explants. The highest number of shoots was observed in IBA 0 ppm and BAP 3 ppm, in addition to the treatment I3B0 and I4B3 also able to induce3 of shoots, being other treatments can induce only 1-2 buds. BAP is a plant growth regulator of cytokinin that stimulates cytokinesis (division) of cells so that the administration of cytokinin can induce the formation of buds more. Results of research conducted by Chelantavur (2015) showed that the maximum shoot formation was observed on MS medium supplemented with BAP 3 mg / l and NAA 0.5 mg / l. On the formation and growth of shoots BAP has a very important role. BAP also induce shoots on some types of plants such as *Ipomoea sepiaria* and *Amorphopalus muelleri*. Optimum growth number of shoots occurred at 3 ppm concentration of IBA IBA concentration even further additions decreased the number of shoots.

Tabel 1.3. Median and mode root proliferation on MS media with combination concentration of IBA and BAP

IBA (ppm)	BAP (ppm)															Median	Modus
	0 ppm			1 ppm			2 ppm			3 ppm			4 ppm				
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3		
0 ppm	29	39	~	~	12	~	~	~	~	21	~	~	~	~	~	25	-
1 ppm	~	~	26	~	12	~	~	~	~	22	31	~	12	~	~	22	12
2 ppm	53	~	~	~	~	~	14	~	~	~	~	~	7	~	~	14	-
3 ppm	~	~	~	25	~	~	34	~	~	13	~	~	~	40	~	29.5	-
4 ppm	45	~	~	14	~	~	~	~	20	~	~	~	~	~	~	20	-
Median		39			13			20			21.5			12			
Modus		-			12			-			-			-			

Of all the explants were successfully induce shoots, not all managed to induce root, only about 43% of the overall managed to induce bud also develop to rooting induction. Table 1.3 shows that while emerging roots ranged from 7 to 53 days after cultured, but most of the shoots appear on the 12 days after cultured. Research conducted by Thingbaijam (2014) on culture *Zinggiber officinale* showed that the roots begin to form at the age of 21 days. When he appeared the fastest roots occurred in the treatment IBA 2 ppm and BAP 4 ppm. Root induction on explants affected by the presence of auxin were added, in terms of auxin physiological effect on cell development, phototropism, apical dominance, root growth (root initiation), Parthenocarpy, abscission, callus formation and respiration (Purnomo, 2010). Wiesman (1989) states that indol butirat acid (IBA) is more commonly used to stimulate rooting than NAA or other auxin. IBA is active, even if quickly metabolism become IBA-aspartate and at least be one conjugates with other peptides.

4. CONCLUSION

The best shoot proliferation present on MS media containing 1 ppm IBA and 1 ppm BAP. The highest frequency of multiple shoot initiations shown at BAP 3 ppm. The faster root initiation reported on MS media supplemented with 2 ppm IBA and 4 ppm BAP.

5. ACKNOWLEDGMENT

The author would like to thank the Ministry of Research, Technology and Higher Education that has funded this research through the National Innovation System Research Incentive (Insentif Riset SINas) for fiscal year 2015.

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Bacterial Diversity on Red Macroalgae *Kappaphycus alvarezii* Infected by Ice-Ice Disease

Nur Shabrina[1], Qintan Istighfarin Atmaja[1], Isdiantoni[2], Maharani Pertiwi Koentjoro[3*], and Endry Nugroho Prasetyo
[1]Biotechnology Laboratory, Department of Biology, Institut Teknologi Sepuluh Nopember, Gedung H 1st floor, Kampus ITS Keputih Sukolilo Surabaya, 60111 Indonesia
[2]Agrobusiness Department, Faculty of Agriculture, Universitas Wiraraja Jalan Raya Sumenep-Pamekasan Km. 5 Patean, Sumenep, 69451, Indonesia
[3]Laboratory of Environmental Microbiology, Department of Biological and Environmental Science, Faculty of Agriculture - Shizuoka University & Structural Biology Research Center, Inter-University Research Institute Corporation - High Energy Accelerator Research Organization (KEK), Tsukuba-Ibaraki, Japan
*Corresponding author email address: maharanipertiwi@yahoo.com

Abstract

Commercial red seaweed *Kappaphycus alvarezii* has been vastly cultivated in Indonesia for its carrageenan content. Yet in recent years, carrageenan quality product of the seaweed has been declining because of ice-ice disease. Ice-ice softens the seaweed texture, turns the color to white and leads to fragmentation. Unfavorable ecological conditions in the cultivation area are known to promote the disease. However, the role of occasional pathogenic bacteria as the lead factor in the disease development has been demonstrated. Management strategy to prevent and treat the disease is necessary by analyzing the bacterial diversity promoting the ice-ice disease. The objective of the current study is to explore the bacterial diversity on *K. alvarezii* infected by ice-ice disease. Confirmation of the bacterial isolates on *K. alvarezii* infected by ice-ice disease was conducted qualitatively through assessment of bacterial ability to hydrolyze carrageenan in a modified semi-refined carrageenan peptone medium. The current study was able to isolate 15 bacteria on *K. alvarezii* infected by ice-ice disease designated as I-1, I-2, I-3, I-4, I-5, I-6, I-7, I-8, I-9, I-10, I-11, I-12, I-13, I-14, and I-15. All of the isolates showed good growth in the modified medium, indicating that all of them are bacteria on *K. alvarezii* infected by ice-ice disease.

Keywords: bacteria, biodiversity, carrageenan, ice-ice disease, Kappaphycus alvarezii

1. INTRODUCTION

Kappaphycus alvarezii or Cottonii seaweed is one type of seaweed species of high economic value and thus massively cultivated in Indonesia (Prasetyowati *et al*., 2008; Kementerian Kelautan dan Perikanan, 2010). This is because *K. alvarezii* is a perpetual source for the hydrocolloid known as carrageenan which is of versatile usage in food industry as a gelling agent, thickener and stabilizer (Van de Velde *et al*., 2002; Campo *et al*., 2009; Mustapha *et al.*, 2011).

However, in recent years, Indonesian seaweed farmers have suffered from great economic loss because of the decline in carrageenan quality and yield as a result of ice-ice disease occurrence (Mendoza *et al.*, 2002; Widiastuti, 2009). Data from Development Service Unit Tri Merta Segara, Bali, Indonesia (cultivation coordination unit) show that harvest loss caused by ice-ice disease has declined seaweed production from 500 tons in 2007 to 200 tons in 2009. Consequently, Indonesian seaweed export has decreased by 97.8% in 2007 (Widiastuti, 2009).

Ice-ice disease symptoms began from yellowing of thalli to gradual whitening and softening of the tissues, resulting in necrosis (tissue death) (Largo, 2000; Fresco, 2012). The disease is triggered by unfavorable ecological conditions in cultivation area (high or low temperature, salinity and light intensity, insufficient nutrients, etc.) which serve as predisposing factors (Largo *et al*., 1995a). The stressed seaweeds become more susceptible to bacterial attack, mainly by certain opportunistic pathogens (Tisera dan Naguit, 2009; Largo, 2002). Identified bacteria causative of ice-ice disease are *Vibrio* and *Cytophaga-Flavobacterium* groups (Largo, 1995b). In addition, fungi of species *Aspergillus ochraceus, A. terreus* and *Phoma* sp. are also known to induce ice-ice disease (Solis *et al*., 2010).

Preventive management strategy and treatment against the disease needs to be carried out in order to increase the quality of seaweed through detection and identification of the disease causal. Study on bacterial diversity by morphological and biochemical characterization of the bacteria is one of the methods to detect and identify bacteria on diseased organisms. Since the first report on ice-ice disease initially made by Uyenco *et al*., (1977, 1981), ice-ice disease studies are still scarce particularly on bacterial diversity on *K. alvarezii* infected by ice-ice disease. By thoroughly understanding the diversity of pathogens on *K. alvarezii* infected by ice-ice disease, effective and efficient methods to address the disease can be devised (Spratt, 2004). Hence, this study explores the bacterial diversity on *K. alvarezii* infected by ice-ice disease through phenotypic characterization.

2. METHODS

2.1. Samples and parameters of physical and chemical cultivation area

The seaweed cultivation area in Palasa Village, Poteran island, Madura, Indonesia (S: 07°04'08.3" dan E: 114°01'49.7") was visited on January 25, 2015. Fresh materials of *K. alvarezii* with diseased thalli were obtained during this date by aseptic method. About 3 g of wet *K. alvarezii* materials were placed in sterile bottles containing autoclaved seawater. Seawater in the vicinity of ice-ice disease occurrence was also obtained aseptically for detection of total bacterial coliform. Wet materials and seawater were brought in chilled condition to Microbiology Laboratory of Institut Teknologi Sepuluh Nopember (ITS) (Surabaya, Indonesia). Physical and chemical seawater data obtained include temperature, pH, ocean current, salinity, and water clarity.

2.2. Isolation and characterization of bacteria

About 1 g of whitened thalli of *K. alvarezii* was homogenized in 9 mL of sterile seawater. A dilution series of up to 1:1000 were then prepared for each sample and 0.1 mL aliquots were then spread-plated on Alkaline Peptone Agar (APA) with salinity

of 30 ‰. Numbers of colonies were counted after incubation at room temperature (28-30^{o}C) for 24-48 hours. They are presented in this study as colony forming units per gram (CFU g^{-1}) of wet *K. alvarezii* sample. Differentiable colonies were randomly selected for purification and subsequent characterization. Bacterial characterization conducted include: colonial morphology and pigmentation, cell morphology, oxygen requirement in broth medium thioglycollate, catalase test with 3% hydrogen peroxide, and oxidase test with tetra-methyl-*p*-phenylene-diamine dihydrochloride solution, carbohydrate fermentation for glucose and Triple Sugar Iron Agar (TSIA). The strains were also tested for growth in certain sugars, mannose and xylose, of which red algae are known to produce (Lechat *et al.*, 1997).

2.3. Confirmation of bacterial strains infection on *K. alvarezii*

Selected bacterial strains were tested to grow in modified semi-refined carrageenan peptone medium. The medium was prepared with the composition of 10 g peptone, 15 g carrageenan, and 1 L seawater of 30 ‰. Positive control for the bacterial growth test was *Vibrio alginolyticus* from Balai Besar Perikanan Budidaya Air Payau (BBPBAP) collection, Jepara, while the negative control was *Escherichia coli* from Microbiology Laboratory collection of ITS. The bacteria were incubated at room temperature (28-30^{o}C) for 24-48 hours.

3. RESULTS AND DISCUSSION

3.1. Correlation of ecological factors and ice-ice disease

Ice-ice disease occurring on *K. alvarezii* cultivation site in Palasa village showed general symptoms of whitening and softening of thalli (Doty dan Alvarez, 1975; Trono, 1993; Tisera dan Naguit, 2009). Ice-ice disease is caused by two factors: abiotic factor of extreme ecological conditions and biotic factor of microbial pathogens attack (Largo, 2000). Extreme abiotic factors cause seaweeds to become less resilient and may predispose the seaweeds to pathogens attack (Jailani *et al.*, 2011). Table 1 presents physical and chemical seawater parameters in Palasa village.

Table 1. Parameters of physical and chemical seawater in Palasa village

Physical and chemical parameters	Measurement results
Temperature	28-30^{o}C
pH	8-8,5
Salinity	30-31‰
Ocean current	4-8,3 cm/s
Water clarity	100%

Data from Table 1 shows that the ecological factors in the cultivation site of Palasa village were categorized as fair for the optimum growth of seaweed with temperature, pH, salinity and water clarity of 27-30^{o}C, 6.5-8.5, 30-37 ‰, and 100% respectively (Setiyanto *et al.,* 2008; Aslan, 2005; Samsuari, 2006; Soenardjo, 2003). In contrast, ocean current in the cultivation site wasn't as good which was 4-8,3 cm/s. Ocean

current for the optimum growth of seaweed has to be in between 20-40 cm/s (Winarno, 1996). Ocean current serves as a platform for nutrients circulation and also functions to keep the water temperature constant (Nazam, 2004). Weak ocean current may subdued nutrients circulation and result in the immunosuppressed seaweeds, predisposing them to pathogens attack causative of ice-ice disease (Largo, 2000).

The presence of pathogens as a lead factor in the ice-ice disease manifestation was analyzed through detection of total coliform using MPN analysis. Although bacterial coliforms are generally not harmful themselves, they indicate the possible presence of pathogenic (disease-causing) microorganisms that also live in human and animal digestive systems (WHO, 1997; EPA). Result from MPN analysis showed that the presence of coliforms weren't harmful since it was below the maximum number of total coliform allowed in marine cultivation site (290 MPN/100 mL). However, bacteria causative of ice-ice disease could still attack immunosuppressed seaweed because they are opportunistic pathogens (Parker, 1978; Largo *et al.,*1995b dan 2000; Egan *et al.*, 2014).

3.2. Isolation and characterization of bacteria

Bacterial colonies count obtained from *K. alvarezii* infected by ice-ice disease ranged from $6x10^2$ to $1,2x10^5$ CFU g^{-1} of wet *K. alvarezii* sample. This result was below bacterial count reported by Largo *et al.,* (1995b) which ranged from $2,4x10^6$ to $1,5x10^7$ CFU g^{-1} of wet *K. alvarezii* sample. A total of 15 isolates designated as I-1 to I-15 were purified and characterized. The characteristics of the isolates are presented in Table 2. Circular and irregular colonies were predominant colonial morphology characteristic with pigmentation of white, translucent and milky white. Gram negative bacteria predominated with a total of 11 isolates.

Table 2. Characteristics of isolates from *K. alvarezii* infected by ice-ice disease

Code	Colonial morphology	Pigmentation	Cell morphology	Gram reaction	Endospore[a]	Catalase test	Oxidase test	Oxygen requirement[b]	Glucose	Mannose	Xylose
I-1	Irregular	Translucent	Coccus	-	nd	+	+	AF	+	+	+
I-2	Circular	White	Coccus	-	nd	+	+	AF	+	+	+
I-3	Circular	Milky white	Coccus	-	nd	+	+	AF	+	+	+
I-4	Circular	Sparkling white	Coccus	+	-	+	-	OA	+	+	+
I-5	Circular	Yellowish white	Coccus	-	-	+	+	AF	+	+	+
I-6	Punctiform	Translucent	Rod	+	-	+	+	OA	+	+	+
I-7	Circular	Milky white	Coccus	-	nd	+	+	OA	+	+	+
I-8	Irregular	White	Coccus	-	nd	+	+	OA	+	+	+
I-9	Punctiform	White	Coccus	+	-	+	+	MA	+	+	-
I-10	Irregular	Translucent	Coccus	+	-	+	+	OA	+	+	+
I-11	Punctiform	Milky white	Coccus	-	Nd	+	+	OA	+	+	+
I-12	Irregular	Yellow	Coccus	-	nd	+	-	OA	+	-	-
I-13	Punctiform	White	Rod	-	-	+	+	OA	+	+	+
I-14	Irregular	Yellow	Coccus	-	nd	+	+	AF	+	+	+
I-15	Punctiform	Translucent	Coccus	-	nd	+	+	AF	+	+	+

[a] nd – *not determined*.
[b] AF – aerobic facultative; OA – obligate aerobe; MA – microaerophillic.

3.3. Confirmation of bacterial strains infection on *K. alvarezii*

All of the isolates showed positive growth in semi-refined carrageenan medium after incubation of 24 hours. The same result was shown by the positive control, *V. alginolyticus*. On the other hand, *E. coli* as the negative control showed negative growth (Fig. 1). This result confirmed preliminarily that the isolates were the causative agents of ice-ice disease. This was because carrageenan is the dominant polymer to constitute the cell walls and extracellular matrices of *K. alvarezii* and represent 39% of the algae dry weight (Lechat *et al.*, 1997; Bixler, 1996). Microbes on *K. alvarezii* infected by ice-ice disease initiated their infection by penetrating the cell wall and lysing the epidermis cells rich in chloroplasts, leading to whitening of the tissues (Largo, 2000; Denny and Gaines, 2007; Solis *et al.*, 2010). Furthermore, Goecke *et al.,* (2010) stated that enzymes, such as agarases, alginases, cellulases, and carrageenases, that break down the heterogeneous polysaccharides of macroalgal cell walls can potentially act as primary virulent factors. Hence, theoretically, microbes which are able to grow on *K. alvarezii* infected by ice-ice disease have to produce hydrolytic enzymes (carrageenases) against carrageenan (Largo, 2000).

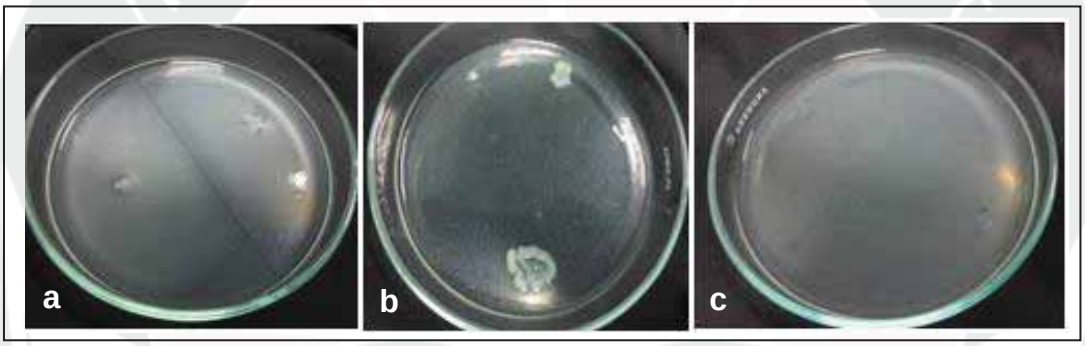

Fig. 1. Bacterial growth in semi-refined carrageenan medium. A) Isolate I-7; B) *V. alginolyticus*; C) *E. coli*

4. CONCLUSIONS

Bacterial diversity exploration on *K. alvarezii* infected by ice-ice disease through phenotypic approach was necessary to determine causative agents of ice-ice disease. Hence this study concluded that all of the isolates designated as I-1, I-2, I-3, I-4, I-5, I-6, I-7, I-8, I-9, I-10, I-11, I-12, I-13, I-14, and I-15 are isolates on *K. alvarezii* infected by ice-ice disease. Further study of infecting the isolates on healthy thalli of *K. alvarezii* following Koch postulates needs to be carried out. Phylogenetic analyses are also necessary to determine the evolutionary relationships between the isolates and known species with similar genotypic characteristics. Through understanding the disease causal accurately, preventive management strategy and treatment are expected to be formulated.

5. ACKNOWLEDGEMENT

The authors are grateful to the *Deutscher Akademischer Austauschdienst* (*DAAD*) / *German* Academic Exchange Service through Sustainable Island Development Initiatives (SIDI) program for the academic grant under which this study was conducted. She also thanks Mr. Adwi (local person in Palasa village, Poteran island,

Madura, Indonesia) for the on-field assistance. Special thanks are also given to Biomaterial and Enzyme Technology Research Team (2014/2015) for assistance and support, especially Risanda Martalina and Febrian Mayang Arumingtyas, for providing space and facilities in Surabaya, Indonesia during the study.

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Characteristics of Tilapia (*Oreochromis niloticus*) Fillet Chips on Different Formulation of Flour Dough during Storage

Nunuk Siti Rahayu
Study Program Agricultural Products Technology, Faculty of Agricultural Technology
Universitas Widya Dharma Klaten Central Java, Indonesia
nsitirahayu@yahoo.com; E-mail : info@unwidha.ac.id.

Abstract

Klaten district (Central Jawa, Indonesia) through the Local Government launched a farming of tilapia fish in the potential area known as Minapolitan region Kalungharjo (covering areas: Karanganom, Tulung and Polanharjo), and it is continued by the assistance of the processed tilapia craftsmen for diversification of processed products, namely processing of tilapia meat into tilapia fillets chips wrapped with flour. To help the chips artisans, there has been done preliminary research (Susi Hartati, 2014), with the objective: to know the chemical properties and consumer acceptance of tilapia fillets chips, fried in dough flour mixture: rice flour and tapioca in a variety of formulations, namely $R_{100}T_0$, $R_{90}T_{10}$, $R_{80}T_{20}$ and $R_{70}T_{30}$. For more complete information about the quality of chips for storage, then there is a continued research on tilapia fillets chips wrapped with flour mixture: rice flour and tapioca with formulations: $R_{100}T_0$, $R_{80}T_{20}$ and $R_{70}T_{30}$ during storage 3, 6 and 9 weeks, with the aim to find out chemical and organoleptic characteristics of the tilapia fillet chips that are made with different formulation of rice flour and tapioca dough during storage at the room temperature. Besides, it is to know the storability of tilapia fillets chips produced by tilapia fillet chips craftsmen in Daleman Village, Tulung District of Klaten Regency, Central java, Indonesia. Tilapia fillet chips that are wrapped in flour dough with various formulations are wrapped again in plastic of polyethilen 0.08 mm and stored for 3, 6 and 9 weeks at room temperature. Further chemical analysis: water content (AOAC, 1995), protein content (method Micro kjedahl) as well as the occurrence of rancidity (TBA levels Tarladgis method), and organoleptic analysis through sensory test: color, flavor, crispness (Scorring Test method), the overall favorite (method Hedonic Test).The result of research analysis of water content of all samples during storage still meet SNI standards, except for $R_{100}T_0W_9$, a slightly higher (3, 1%) compared to the standard (max. 3%), protein content of all samples meet SNI standards (minimum 6%), and TBA levels indicate that there are two samples: $R_{80}T_{20}W_9$ and $R_{70}T_{30}W_9$ have levels above the threshold (max. 2 mg / kg), ie 2, 11 mg / kg and 2.279 mg / kg. Organoleptic analysis resulted in: diminishing the amount of rice flour in the dough, color chips dwindle browned level towards a yellowish color; scores the highest sense of the average obtained on the sample $R_{80}T_{20}$ (3.8; savory, not rancid); crispness showed no difference in all samples, as well as the highest scores obtained on the sample a stored $R_{80}T_{20}$ 3 weeks (5.8; really like). From the study it can be concluded that the chips fillets of tilapia which have best chemical and organoleptic properties is made with formulations $R_{80}T_{20}W_3$, and the second best is

$R_{100}T_0W_3$. Its storability is up to 9 weeks from the starting point of the chemical and organoleptic deterioration.

Keywords: Characteristics, chips, fillet of tilapia, storage

1. INTRODUCTION

The future of freshwater fish product development should always be done based on the prediction of sea food source that have a possibility on the decreasing number of product because of the overfishing (Cecep Risnandar, in http://alamtani.com/ikan-air-tawat.html). To support the national fishery cultivation that has targeted the product development about 353% (from 5.25 million ton become 16.9 million ton), so in Klaten Regency by the local government, planned to cultivate the tilapia fish in the potential region that has been determined as the Minapolitan Kalungharjo area (because included 3 subdistricts region: Karanganom, Tulung and Polanharjo). Based on the data from 2011, cultivation freshwater fishery production in Klaten Regency, especially tilapia fish is 8.610, 4 tons, in 2012 is targeted until 11.100 tons and will be increased in the next years (Klaten Agriculture Department, 2010). Beside increasing tilapia fish production, Klaten Government also accompanying to the tilapia fish refined producers to get diversification refined product, beside being fried, baked or made to curry, be it processing tilapia fish become tilapia fish fillet chips that is wrapped with flour.

Early research about the tilapia fish refined product has been done with the tilapia fish filleT chips as the result that is produced by the tilapia refined producer in Daleman Village, Tulung Subdictrict, Klaten Regency by Susi Hartati (2014), to know chemical characters and consumer acceptance of the tilapia fish fillet chips that is fried and is wrapped by the mixing flour that consist of rice flour and tapioca flour in various comparison formulation, there are $R_{100}T_0$, $R_{90}T_{10}$, $R_{80}T_{20}$ and $R_{70}T_{30}$. Research result show another chemical character: water dose, protein and fat dose, also is known that the mixing flour usage as the chips wrapped (without saving) and as the favorite of the panels is the formulation rice flour : tapioca flour =100 : 0 but there is little differences with the 90 : 10 formulation. While the research about how long the saving time of the tilapia fish fillet chips in Daleman Village has not been done, so about that research should be done by the producer so they can predict chips production time. The fact show that period of the product being saved in seller etalage will affect the product quality.

The goal of this research is to know the chemical characteristic and tilapia fish fillet chips organoleptic that is made with different formulation rice flour and tapioca flour in a room temperature, also to know the storability of the tilapia fish fillet chips that is produced by the producer in Daleman village, Tulung Subdistrict, Klaten regency. With this research, I hope that can help the producer of tilapia fish fillet chips to know the storability of the product so it facilitate them to know the time production.

2. METHODS

2.1. Materials and tools

The sample that is used is 9 kg fresh tilapia fish fillet, 3 kg "Rose Brand" rice flour, 1 kg tapioca flour, 5 kg cooking oil, 500 ml water, some seasoning: 135 gram

coriander, 1012.5 gram garlic, 270 gram of salt which is bought in Klaten Market. The material to chemical analysis consist of aqua's, H_2SO_4 (Merck), Na_2SO_4, NaOH-$Na_2S_2O_3$ (Merck) solution, H_3BO_3, HCl, HgO (Merck), red methyl indicator, TBA reagent. Polyethylene plastic bag size 0. 08 mm to pack the chips that is fried. The research tools are stove, pan, implant, dustpan, scales, spoons, bowls, basins and filter. The tools that is used to do chemical analysis are porcelain bowls, desiccator, oven, pumpkin destruction, distillation equipment, erlenmeyer, extraction tool, waring blender, spectrophotometers, micro Kjeldahl unit and Analytical scales.

2.2. Experimental Design

The experimental design that being used in this research is Factorial Complete Randomized Design (RAL Factorial) that consist of 2 factors, first factor is formulation of rice flour and tapioca flour with 3 level: $R_{100}T_0$ (rice flour 100%, tapioka flour 0%), $R_{80}T_{20}$ (rice flour 80%, tapioca flour 20%) dan $R_{70}T_{30}$ (rice flour 70%, tapioca flour 30%). Second factor is the storage time that consists of 3 levels: W3 (3 weeks storage time), W6 (6 weeks storage time) and W9 (9 weeks storage time). Each treatment is repeated three times. The data result is analyzed with Analysis of Variance (ANOVA), if there is differences, it will be continued with Duncan's Multiple Range Test (DMRT) in 5% and 1% standard of quality (Gomez and Gomez, 1995). This analysis is done with computer with SPSS method.

2.3. Reserch Step

2.3.1 Fish Fillet Preparation

Big size tilapia fish is cut in head area and discard the entrails, weed the shell, wash in running water so will get a headless fish pieces without entrails, free of dirt, blood and mucus. The next step is slice the fish from behind the rear fins gill until behind the head and go to front slice in front area in order ribs to head where the slicer cut the bone thorn. Then, slice from the head area to the tail with knife and make the angle on the ribs and aligned with knife, after that take off the fillet from the rib immediately. Reverse the fish and do the same thing of the slice process. The next step is prepare the season and mix the flour based on the formulation. Therefore, the raw material formulation of tilapia fish fillet chips is in Table 1 .

Table 1. Raw Material Formulation of Tilapia Fish Fillet Chips Making

Material	Treatments		
	$R_{100}T_0$	$R_{80}T_{20}$	$R_{70}T_{30}$
Tilapia Fish	1000 gr	1000 gr	1000 gr
Rice Flour	650 gr	520 gr	455 gr
Tapioca Flour	0 gr	130 gr	195 gr
Water	500 ml	500 ml	500 ml
Salt	10 gr	10 gr	10 gr
Garlic	37,5 gr	37,5 gr	37,5 gr
Conander	5 gr	5 gr	5 gr

Source: Susi Hartati (2014)

2.3.2 Tilapia Fish Fillet Chips Making

Tilapia fish fillet is flavoured with the grind seasoning (salt, garlic and coriander) in ± 10 minutes so the fish will be flavoured. Then, pour it into the mixture of rice flour and tapioca flour fit with the formulation with being inverted. Next, fried in hot oil temperature of about 185 ° C-190 ° C for 15 minutes, drained, cooled further chips, then, packed in 0.08 mm polyethylene packaging plastic, the packaging is sealed using a sealer, and labeled according to treatment and stored at room temperature according to treatment retention time (3 weeks, 6 weeks, 9 weeks), then performe chemical analysis and organoleptic analysis after the chips are stored. The research sample can be seen in Figure 1.

Figure 1. Chips Tilapia Fish Fillet

2.3.3 Sample Analysis

Samples in the form of chips fillets of tilapia are stored according to the treatment performed chemical analysis that includes: water content method Thermogravimetri (AOAC, 1995), protein content method Micro Kjedahl (AOAC, 1995; Slamet Sudamandji *et al*., 2007) and levels of TBA (thiobarbituric acid, Tarladgis methods, to detect rancidity in products) (Anton Apriantono *et al.*, 1989) and organoleptic analysis including color, flavor and crispness (scoring test method) and the liking overall Hedonic Test. There are five color scores and its criteria: 1. Dark brown, 2. Brown, 3. Yellow brown, 4. Yellowish, 5. White Yellowish. There are also five flavor scores and its criteria: 1. Not savory, rancid; 2. Less savory, slightly rancid; 3. Slightly savory, slightly rancid; 4. Savory, not rancid; 5. Very savory, not rancid. There are also five crispness scores and criteria: 1. Not crispy (soggy), 2. Less crisp (rather sluggish), 3. Slight crisp, 4. Crisp , 5. Very crisp. Next, there are seven liking scores and its criteria: 1. Do not like at all, 2. Dislikes, 3. Do not like it much, 4. Rather like 5. Like, 6. Like it very much.

3. RESULTS AND DISCUSSION

3.1. Chemical Properties of Tilapia Fish Fillet Chips

Tilapia Fish Fillet Chips which is made with three formulation ($R_{100}T_{0}$,, $R_{80}T_{20}$, $R_{70}T_{30}$), each of it packed tightly in plastic polyethylene 0.08 mm. After storage 3, 6 and 9 weeks, in each storage, each chips in each formulation, were analyzed about water content, protein content and the level of rancidity with TBA test.

3.1.1. Water Content of Tilapia Fish Fillet Chips

The average water content of Tilapia Fish Fillet Chips, ranged from 1. 64 – 3.10%. The value of average water content of Tilapia Fish Fillet Chips is shown in Figure 2. Water content analysis result show that the diminishing proportion of rice flour (increasing of tapioca proportion) will cause the increasing average water content, but in $R_{80}T_{20}$ and $R_{70}T_{30}$ although water content value slightly increase, but there are no big differences between it *(P≤0.01)*. It happens because the proportion difference is just 10%. It also happens between $R_{100}T_0$ and $R_{80}T_{20}$, although the difference only 20%, there is slightly increasing water content (P≤0.01). It happens because the water content of the raw material, rice flour proportion is 12% (Directorate of Nutrition, Indonesian Republic Health Department, 1989), is equal with tapioca proportion, that is 12%, so the decreasing amount of one of the flour in the dough chips do not make a real contribution to the change in water content.

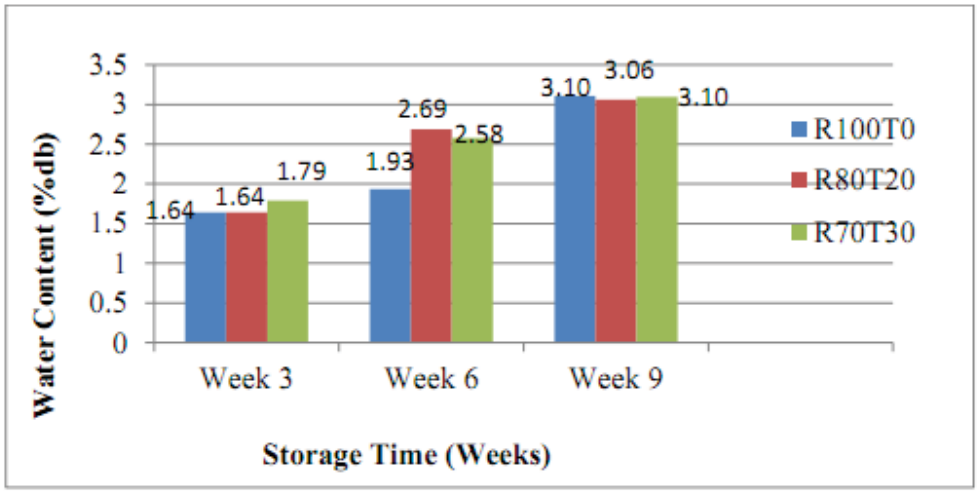

Figure 2. Water Content of Tilapia Fish Fillet Chips in various formulations for storage

The longer the storage time of chips, from 3.6 and 9 weeks, further increase the water content. The average value of water content based on storage time in succession: 1.604; 2.39; and 3.087. During storage, the increasing water content of the chips which are packaged in 0.08 mm polyethylene plastic bags, by Eko Nur Cahyo Ratna Dewi and Ibrahim (2008, in http://eprints.undip.ac.id 190821EKO_Nur.pdf.), who researching red tilapia fillet beef jerky and stored in Vacuum (vacuum) containers compared to the polyethylene plastic bag , storage for 30 days. Water content of beef jerky that stored in plastic bag increase significantly from 16. 280 b.b (0 days) to 18.760 b.b (30 days), while the water content of beef jerky that stored in Vacuum containers, water content decreases from 15.960 b.b (0days) to 13.970 b.b (30days). The increasing water content of the product that stored in plastic bag during storage because when the product is inserted in plastic bag, the plastic bag still contained the air, so even though it is sealed, the air in te bag wil be absorbed into the product. In addition, the packaging is still able to be influenced from the outside air humidity Quality requirements of

Tilapia Fish Fillet Chips that wrapped with different flour dough and being fried, are not in the Indonesian National Standard (SNI), then as a comparison, used quality requirements chips fried tempeh with rice flour pads, with SNI 01-2602-1992. In the Indonesian National Standard (SNI), quality requirement of maximum water content of tempeh chips is 3% (SNI Refined Product of THP in http://a289431serbaserbidajal.blogspot.com/2012/05/sni-produk-olahan-tph.html;

Based on the assumption, so all of the formulation of the dough with the mixing flour between rice flour and tapioca flour $R_{100}T_0$, $R_{80}T_{20}$ dan $R_{70}T_{30}$) which is used to wrap the fillet of tilapia fry chips for storage time of 3.6 and 9 weeks, the water content is still in the SNI specified maximum limit, so it means thaT it fulfills the quality requirements, unless the treatment $R_{100}T_0W_9$ with the 3. 1068 of water content, so it is slightly above the maximum limit of SNI standard.

3.1.2. Protein Content of Tilapia Fish Fillet Chips

Protein content of Tilapia Fish Fillet Chips with different formulation treatment of the flour dough during storage shown in Figure 3. The differences formulation between rice flour with tapioca flour which is used to wrap tilapia fish fillet chips during storage give the real impact (P≤0.01) to the differences of protein content. The number of tilapia fish fillet which is used in the making of the chips in each formulation is the same that is equal to 1000 grams. So, the protein of tilapia fish fillet do not impact to the changing of protein content.

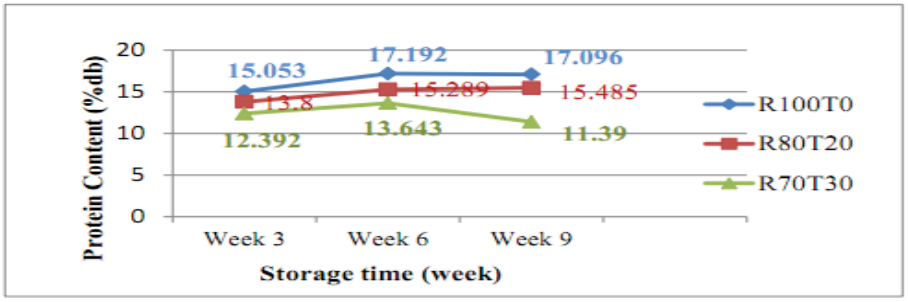

Figure 3. Protein Content of Tilapia Fish Fillet Chips in Various Formulation during Storage

It is possible that the protein content changing caused by the changing of the amount of rice flour that is formulated to tapioca. Protein content of rice flour is 7% in each 100 grams material (Directorate of Nutrition, Indonesian RepublicHealth Department, 1989), while the protein content of tapioca flour is 0.5% in each 100 grams material (Iis Istanti, 2005, in http://repository.ipb.ac.id/bitstream/handle/123456789/11518/C05iis.pdf?sequencehttp://repository.ipb.ac.id/bitstream/handle/123456789/11518/C05iis.pdf?sequence=1 thus the diminishing amount of flour used in the dough formulation will reduce the levels of the protein content. There is a fluctuation of protein content average value of the tilapia fish fillet chips that stored during 3, 6, and 9 weeks. It happens because the differences amount of tilapia fish fillet in each formulation chips dough which is used as sample to analyze the protein content.

Quality requirement of tilapia fish chips fillet assumed with Indonesia National Standard (SNI) for fish crackers. It is mentioned in SNI 01-2713-1999, that the minimum protein content of fish crackers is 5%. (Index SNI Produk Perikanan in http://www.bkipm.kkp.go.id/bkipm/sni/index/PRODUK%20PERIKANAN;NSAI(BSN),1999). In figure 3, it can be seen that all treatments in different formulation flour dough that used to warp the tilapia fish fillet chips that stored in 3.6 and 9 weeks fulfill the quality requirement that is described by SNI because the average protein content of all the above specified limits.

3.1.3. Rancidity Test through TBA levels of Tilapia Fish Fillet Chips

One factor determining the quality of food containing oils or fats include the emergence of a number Thiobarbituric acid (TBA). TBA number will show the rancidity level. Smell rancid mainly due to the formation of aldehyde or ketone in the stored food. In TBA, rancidity occurs in fat because of the emergence of detectable aldehydes as malonaldehyde (Slamet Sudarmadji *et al.*, 1996), as Malonaldehyde is the result of lipid oxidation (Anton Apriyantono *et al.*, 1989). Analysis result of TBA test of Tilapia Fish Fillet Chips during the storage shown in Figure 4.

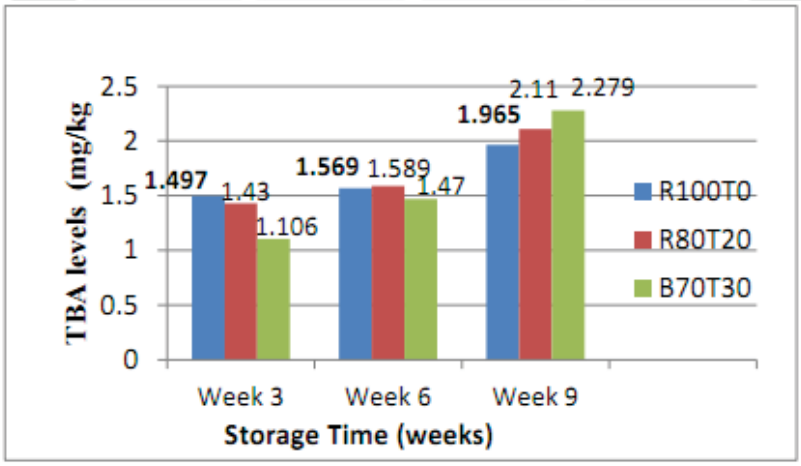

Figure 4. TBA levels of Tilapia Fish Fillet Chips in Different Formulation During Storage

Average TBA interaction number between rice and tapioca flour formulation and storage time ranging from 1.106 - 2.279 mg / kg malonaldehyde. In figure 4, the different formulation mixture of rice flour and tapioca flour as the dough pads of tilapia fish fillet chips, has no effect on the TBA numbers, and the average value of the levels of TBA in the formulation: $R_{100}T_0$, $R_{80}T_{20}$ and $R_{70}T_{30}$ row is 1.677; 1.71 and 1.621 mg / kg of malonaldehyde. But with the increasing storage time from 3.6 and 9 weeks will increase TBA levels, a row of 1.344; 1.545 and 2.12 mg/kg of malonaldehyde. Watts (1962) in Febrina (2012) said that limits value of TBA in foods is 1-2 mg / kg (Siti Nur Sayang and Abustam, 20013 in http://repository.unhas.ac.id/.../ARTIKEL%20SYG.doc?...2). Based on the TBA levels, it means that chips product began to show rancidity in storage at least 9 weeks. In this research, chips storage is done in polyethylene plastic bag and sealed using a sealer, to delay rancidity up to 9 weeks. Research of Eko Nurcahyo

Dewi and Ratna Ibrahim (2008, in http://eprints.undip.ac.id190821EKO_Nur.pdf) in tilapia fish beef jerky is packed in Vacuum container compared with polyethylene plastic bag for storage of 30 days, the polyethylene plastic is not able to prevent the growth of aerobic bacteria, means that packaging is not able resist oxidation. This is evidenced by the increasing number of total bacteria of 4.784 log colony / gram (at 0 day) to 5.195 log colony / gram after stored for 30 days. This shows that the plastic bag that still contain oxygen in the bag, so that allow the oxidation of fat or oil in the product.

Fatty meat fish contains 17-21% saturated fatty acids and 79-83% unsaturated fatty acids. The high content of unsaturated fatty acids cause the fishery products easily oxidized (Rahayu, *et al.*, 1992 in Evi Susilowati, 2005. http://repository.ipb.ac.id bitstream.....16500...A02esul....pd.). The big oxidation process mainly happen during material processing (filleting, seasoning until frying in high temperature) until before the product is packed. The frying step in this research took place at a high temperature (185°C-190°C) for ± 15 minutes. This process is one of the processes that trigger rancidity, especially it happens on food containing high unsaturated fatty acids. (Mudjajanto, 1991 in Evi Susilowati, 2005. http://repository.ipb.ac.id bitstream.....16500...A02esul....pd). The air oxygen in contact with tilapia fish fillet chips during frying, and the heat will attack the double bond in unsaturated fatty acids to form peroxides. The peroxides leads to fat or oil oxidation to produce the aldehyde (Ketaren, 1986) in the form of malonaldehyde which can be detected by TBA numbers (Slamet Sudharmaji, 1996). Rancidity process continues in plastic bag, because the plastic bag that do not vacuum still allows contain oxygen in the packaging.

3.2. Organoleptic Characteristic (Sensory Characteristic) of Tilapia Fish Fillet Chips During Storage

Organoleptic analysis in tilapia fish fillet chips that stored during 3.6 and 9 weeks is done by sensory test that consist of color, flavor, crispness and favorite.

3.2.1. Analysis Result of Color Test of Tilapia Fish Fillet Chips

The average score of color test of tilapia fish fillet chips during storage between 3.25– 4.35 with criteria: tawny – yellowish. Results of variance analysis of colors presented in Table 2.

Table 2. Average Analysis of Color Chips Fillet of Tilapia During Storage

Storage Time (weeks)	Flour Mixture			Average
	$R_{100}T_0$	$R_{80}T_{20}$	$R_{70}T_{30}$	
3	3,3 b	3,55 ab	4,35 a	3,743 B
6	3,4 b	3,5 b	4,05 ab	3,65 B
9	3,25 b	3,35 b	3,95 b	3,52 B
Average	3,32 B	3,47 B	4,12 AB	

Note: numbers followed by different letters indicate very significantly different (P≤0.01)

In Table 2, even though in variance analysis has pointed out a big difference, but color test score give average score that almost the same. The color changing show its differences in formulation $R_{100}T_0$ with $R_{70}T_{30}$ and $R_{80}T_{20}$ with $R_{70}T_{30}$. It shows that the diminishing amount of rice flour (increasing amount of tapioca flour) in the formulation of wrap chips dough, will decrease brownish color of the product and produce towards yellowish color. The biggest thing that made the changing color process is caused by the contribution of the protein in the chips material. The biggest rice flour formulation in the dough is 100% (tapioca 0%) with the treatment $R_{100}T_0$, the product color will leads to brownish. It is caused by protein content of the rice flour is bigger 7% than tapioca 0.5%. The bigger protein content upon heating is because of frying makes the Maillard reaction happen. This reaction occurs due to the heating process of chips wrapped in carbohydrate dough containing carbonyl groups of reducing sugars with amino (amine) primer of the protein material (Mauron, 1981; Winarno, 2004). Consequently, if the rice flour amount is reduced and tapioca flour is increases, the color of the product toward yellowish.

3.2.2. Analysis Result of Flavor Test of Tilapia Fish Fillet Chips

The average score of flavor test of tilapia fish fillet chips ranged from 3.55 – 4.1 (slightly savory, slightly rancid - savory, not rancid), more seen in Table 3.

Table 3. Average Analysis of Flavor Chips Fillet of Tilapia during Storage

Storage Time (Weeks)	Flour Mixture			The Average
	$R_{100}T_0$	$R_{80}T_{20}$	$R_{70}T_{30}$	
3	3,7 ab	4,1 a	3,9 a	3,9 A
6	3,6 ab	4 a	3,5 ab	3,7 AB
9	3,5 ab	3,35 ab	2,9 b	3,25 AB
The Average	3,6 AB	3,8 A	3,43 AB	

Note: numbers followed by different letters indicate very significantly different (P≤0.01)

Table 3 show that the flour dough formulation pointed out that the average flavor score has a little difference, but the highest average flavor is given by the panelist in the formulation $R_{80}T_{20}$ with score 3.8 (savory, not rancid). While the longer stage time (3.6 and 9 weeks), the average flavor score decreases in sequence 3.9 (savory, not rancid); 3, 7 (between rather savory, little rancid to savory, not rancid) and 3.25 (slightly savory, slightly rancid). The emergence of a little taste of rancid in storage up to 9 weeks in accordance with the rise of the TBA numbers above the threshold value (above 1-2 mg / kg), amounting to 2.279 mg/ kg. It can be assumed that the storage chips fillets of tilapia to 9 weeks (± 2 months, 7 days), panelists could still accept the crisps taste because this time as the onset of rancidity.

3.2.3. Crispness Test of Tilapia Fish Fillet Chips

The average crispness score in all treatments is ranged from 3.55 – 4.1 (slightly crispy – crispy), as presented in Table 4.

Table 4. Average Analysis of Crispness Chips Fillet of Tilapia during Storage

Storage Time (Weeks)	Flour Mixture			The Average
	$R_{100}T_0$	$R_{80}T_{20}$	$R_{70}T_{30}$	
3	4,1	3,95	3,8	3,95
6	4,1	3,95	3,9	3,98
9	3,55	3,75	3,55	3,62
The Average	3,92	3,88	3,75	

In Table 4, the average all treatments have score between 3.55 – 4.1 and show that all treatments of the formulation of the mixture dough of the rice flour and the tapioca flour and stored during 3.6 and 9 weeks doesn't show a big differences in P≤0.01 and P≤0.05. The differences increasing score of crispness is just a little differences. It is also supported that the water content of the data analysis, the chips were stored at 3.6 and 9 weeks - the average value of the water level is 1.604; 2.39 and 3.087%; so that they meet the quality tempeh chips requirements as the comparison is a maximum of 3%. It seems that increasing water levels are still very low, the panelists have not been able to distinguish the change of crispness.

3.2.4. Favor Levels of Tilapia Fish Fillet Chips

The average favor score tilapia fish fillet chips in all treatment ranged between 3.55-5.8 (rather like – like it very much) and shown in Table 5.

Table 5. Average Analysis of Favor Chips Fillet of Tilapia during Storage

Storage Time (weeks)	Flour Mixture			The Average
	$R_{100}T_0$	$R_{80}T_{20}$	$R_{70}T_{30}$	
3	5,1 c	5,8 a	5,2 b	5,37 B
6	4,65 d	4,9 c	3,95 e	4,483 D
9	3,95 e	4,4 d	3,55 e	3, 95 E
The Average	4,53 D	5,03 C	4,217 DE	

Note: numbers followed by different letters indicate very significantly different (P≤0.01)

In Table 5 show that the increasing storage time from 3.6 and 9 weeks, the average value of a further score dropped from 5.37 (like); 4.483 (between rather like - like) and to 3.95 (rather like). This may also be associated with a decrease in the average value of the taste test in line with the increasing of chips storage period.At different flour formulations ($R_{100}T_0$, $R_{80}T_{20}$ dan $R_{70}T_{30}$) panelists scoring average value respectively - helped 4.53 (rather like-like); 5.03 (like) and 4.217 (rather like). So in formulation $R_{80}T_{20}$, panelists give the highest score for the favorite. It is

also in accordance with the average value of flavor chips fillets of tilapia during storage were also the highest in the formulation $R_{80}T_{20}$.

3. CONCLUSION

Water content in all treatments still fulfill Indonesia National Standard or SNI (maximum 3%), except in $R_{100}T_0W_9$ slightly higher (3.1%) compared to the standard (max. 3%), protein content in all sample still fulfill Indonesia National Standard or SNI (minimum 5%), and TBA content show that there are two samples: $R_{80}T_{20}W_9$ and $R_{70}T_{30}W_9$ have levels above the threshold (max. 2 mg / kg), i.e 2.11 mg / kg and 2.279 mg / kg. Organoleptic analysis result: the diminishing amount of the rice flour in the dough, color of the chips will change from the brownish color to the yellowish; the highest average scores is in the sample $R_{80}T_{20}$ (3.8; savory, not rancid); the crispness doesn't show a big difference in all sample with the highest score of favor is in the sample $R_{80}T_{20}$ in a 3 weeks storage time (5.8; like very much). From this research, the conclusion is tilapia fish fillet chips have best chemical organoleptic characteristics is the chips with the formulation $R_{80}T_{20}W_3$, while the second best chips is with the formulation $R_{100}T_0W_3$. Nine weeks storage time is the starting point when chemical and organoleptic deterioration happens.

A big thank you goes to Susi Hartati (Sub offices Fisheries Klaten Regency), Mrs. Widatiningsih (village Daleman Tulung Klaten) and Dr. Iswan Riyadi (Head of the research center Universitas Widya Dharma Klaten) for their participation during the study.

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Degradation of Crude Oil-Contaminated Soil by Oxidoreductases

Muhamad Abdul Qorip[1], Nur Shabrina[1], Maharani Pertiwi Koentjoro[2], and Endry Nugroho Prasetyo[1]
[1]Biotechnology Laboratory, Department of Biology, Institut Teknologi Sepuluh Nopember, Gedung H 1st floor, Kampus ITS Keputih Sukolilo Surabaya, 60111 Indonesia
[2]Laboratory of Environmental Microbiology, Department of Biological and Environmental Science, Faculty of Agriculture - Shizuoka University & Structural Biology Research Center, Inter-University Research Institute Corporation - High Energy Accelerator Research Organization (KEK), Tsukuba-Ibaraki, Japan
*Corresponding author email address: maharanipertiwi@yahoo.com

Abstract

Crude oil is a mixture of hydrocarbons and other elements such as sulfur, nitrogen, oxygen, and metals. Crude oil is vastly used as gasoline, lubricating oils, solvents, and also applied in pharmaceutical industries. Although it is highly beneficial, it could also act as contaminants for the environment. Enzymes widely used as agents of biodegradation are oxidoreductases. This study combined oxidoreductases capability of laccase and cellobiose dehydrogenase (CDH) in the degradation crude oil-contaminated soil. Based on the assays of UV/Visible Absorption (UVVA) and Michaelis-Menten kinetics of enzymes, laccase-CDH-ABTS was the finest combination in degrading crude oil-contaminated soil with degradation percentage, $V_{max,}$ and K_m of 49.4%, 3.75 µg/s and $6{,}01 \times 10^6$ µg respectively.

Keywords: degradation, laccase, crude oil, oxidoreductases, cellobiose dehydrogenase (CDH)

1. INTRODUCTION

Crude oil is a complex mixture of hydrocarbons such as alkanes, aromatic hydrocarbons, resins, and asphaltenes with a few elements of sulfur, nitrogen, and oxygen (Speight, 2002). Environmental restoration of soil contaminated by crude oil may use physical, chemical, or biological approaches (Nugroho, 2006). Generally, biological treatment for restoring contaminated environment is by biodegradation (Cookson and John, 1995; Ilyina *et al*., 2003).

One group of enzymes which is widely used as agent of biodegradation is oxidoreductases (Baker and Herson, 1997). Some of the vastly applied examples of oxidoreductases for biodegradation are laccase and cellobiose dehydrogenase (CDH) (Nyanhongo *et al*., 2007; Nugroho Prasetyo, 2014). ABTS or 2,2-azinobis- (3-ethylbenzthiazoline-6sulfonic acid) is a standard laccase mediator that can be used to enhance the ability of laccase in oxidizing non-phenolic compounds such as alkyls by increasing the redox potential (Nyanhogo *et al*., 2006; Nugroho Prasetyo, 2014). Hence, this research aims to combine two enzymes of oxidoreductases reactions, laccase and cellobiose dehydrogenase (CDH), with the addition of ABTS in the degradation crude oil-contaminated soil.

2. METHODS

2.1 Enzymes preparation

Laccase produced by *Trametes hirsuta* was obtained from Prof. Georg M. Guebitz, Institute of Environmental Biotechnology, University of Natural Resources and Life Sciences (BOKU), Vienna, Austria. On the other hand, CDH was obtained from Dr. Eng. Desriani, M.Si., Laboratory of Molecular Health and Diagnostics, Biotechnology Research Center, Lembaga Ilmu Pengetahuan Indonesia (LIPI), Cibinong, Indonesia. The laccase acitivity was assayed following the method of Nugroho Prasetyo *et al.,* (2010). The reaction mixture was 0.8 ml succinate buffer pH 4.5 50 mM; 0.35 ml laccase; and 0.35 ml ABTS 10 mM at 37^{o}C. Change in absorbance of ABTS was observed at 436 nm for 5 min. Enzyme activity was calculated by the loss of molar coefficient (ε) (change of substrate mol^{-1} minute).

2.2 Crude oil-contaminated soil preparation

Soil sample was collected following the composite method described by Ramadhani and Oginawati (2006) during the visit to crude oil manufacture owned by local people in Hargomulyo Village, Kedewan, Bojonegoro, East Java, Indonesia on February 2015. The sample was obtained from the soil surface about 20 cm in depth using soil sampler. There were 8 plots of sampling locations in which each plot the soil weighed about 1 kg. The sample was homogenized and taken about one-fourth of it.

Crude oil extraction from the contaminated soil was conducted following the method as described by Li *et al.,* (2012). About 10 g of contaminated soil was added by 10 ml of chloroform and homogenized until the oil was dissolved. The mixture was filtered using Whatman® filter paper Grade 42. Chloroform was then slowly evaporated. The obtained oil was run in UV/VIS spectrophotometer to attain the maximum wavelength (λ).

2.3 Crude oil- contaminated soil degradation

The degradation of crude oil was conducted following the method of Nyanhongo *et al.,* (2002) and Latha and Kalaivani (2012). Varying enzymes and the combination with ABTS was done to degrade the crude oil-contaminated soil as follows: laccase (L), laccase-ABTS (LA), CDH (C), laccase-CDH (LC), laccase-CDH-ABTS (LCA). Degradation assay was conducted in room temperature with continued aeration for 48 h.

Degradation by L was conducted by mixturing 0.1 g of the contaminated soil, 2000 µl buffer sodium citrate (100 mM, pH 4.5), and 500 µl laccase. Degradation by LA was conducted by mixturing 0.1 g of the contaminated soil, 2000 µl buffer sodium sitrat (100 mM, pH 4.5), 500 µl laccase, and 250 µl ABTS 1mM. Degradation by C was conducted by mixturing 0.1 g of the contaminated soil, 500 µl CDH, and 2000 µl buffer sodium citrate (100 mM, pH 4.5). Degradation by LC was conducted by mixturing 0.1 g of the contaminated soil, 500 µl laccase, 500 µl CDH, and 2000 µl buffer sodium citrate (100 mM, pH 4.5). degradation by LCA was conducted by mixturing 0.1 g of the contaminated soil, 500 µl laccase, 500 µl CDH, 250 µl ABTS 1mM, dan 2000 µl buffer sodium citrate (100 mM, pH 4.5). The result of degradation was then dissolved in chloroform to further dissolve oil residues, followed by eliminating the buffer and enzymes. Degradation crude oil-contaminated soil was

determined using UV/Vis spectrophotometer by initially constructing the curve standard with maximum absorbance of the crude oil to obtain degradation percentage (%) (Evdokimov and Losev, 2007).

2.4 Enzyme kinetics

The enzyme kinetics was determined following the method of Kuchel and Gregory (2002) and Nyanhongo *et al.,* (2006). Of the five treatments, only three with high degradation percentage were chosen for the determination of the enzyme kinetics. The enzyme kinetics was constructed by varying the substrate concentrations on one type of enzyme concentration. Michaelis-Menten equation was constructed to form Lineweaver-Burk equation, thus resulting in linear line if one new variable was plotted against other variable to determine v_{max} and K_m (Kuchel and Gregory, 2002).

3. RESULTS AND DISCUSSION

3.1 Crude oil

Crude oil concentration in soil with chloroform as solvent resulted in 13.53 % of crude oil weight against soil weight. Based on the Decree of Environmental Life Minister No. 128 (2003), the maximum amount of crude oil allowed in soil for plantation is 1 %. The Decree also stated that oil content in soil below 15% should be treated biologically. Thus, enzymatic and eco-friendly treatment was conducted to address the oil contaminating the soil above the maximum amount of oil allowed in soil.

Result of crude oil extracted from contaminated soil was scanned using UV/Vis spectrophotometer to attain the maximum wavelength and presented in Fig. 1.

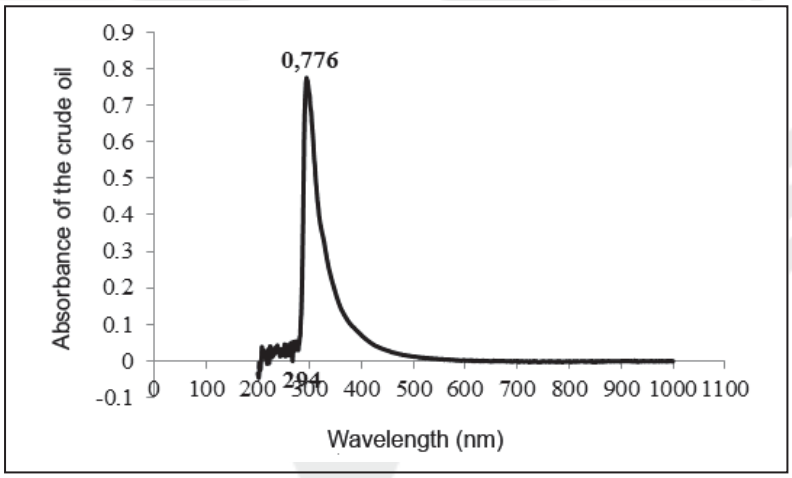

Figure 1. Chromatogram wavelength scan of crude oil from contaminated soil

Scan result showed that the maximum wavelength of the crude oil with chloroform as solvent in quartz cuvette was 294 nm. The wavelength value of the crude oil wasn't significantly different compared to the one reported by Akpoveta *et al.*, (2012) of 250 nm maximum wavelength. Speight (2002) stated that polynuclear aromatic hydrocarbon (PAH) in crude oil can be detected at the range of 200-350 nm, while total petroleum hydrocarbon (TPH) is in the range of 200-700 nm (Akpoveta *et al.*, 2012).

3.2 Oxidoreductases activity

Laccase activity against ABTS was 1200.061 $\frac{\text{Unit}}{\text{ml}}$ as presented in Table 1. That activity value was fairly good for oxidation of phenolic compounds (above 920.895 nkat/L (0.055 $\frac{\text{Unit}}{\text{ml}}$)) (Nyanhongo *et al.*, 2002; Couto and Sanroman, 2005).

Table 1. Oxidoreductases activity

Enzymes	ABTS (Unit/ml)	DCIP (Lactose) (Unit/ml)	Crude oil (µg/min/ml)
Laccase	1200.061	-	43.95
CDH	-	0.79	7.05

ABTS was a standard substrate for measuring laccase activity (Nyanhongo *et al.*, 2006). ABTS oxidation mechanism was initiated by attracting electron by laccase active site and followed by electron transfer to oxygen, leading to formation of water (H_2O) (Campos *et al.*, 2001). Hence, the reaction result was the amount of substrate oxidized per unit of time (Gomez *et al.*, 2005). Replacement of ABTS substrate with crude oil also showed laccase activity of 43.95 µg min^{-1} ml^{-1} (Table 1).

CDH activity of 0.79 $\frac{\text{Unit}}{\text{ml}}$ on Table 1 was obtained from lactose oxidation to lactonolactone with DCIP as electron acceptor. CDH activity result was in accordance with minimum activity for biobleaching of 0.05 $\frac{\text{Unit}}{\text{ml}}$ (Desriani *et al.*, 2012; Sygmund *et al.*, 2013). On the other hand, CDH activity against crude oil was 7.05 µg min^{-1} ml^{-1} (Table 1).

3.3 Strategy of enzymatic degradation of crude oil

Oxygen was fundamentally necessary for laccase and CDH. Laccase needs oxygen as electron acceptor to form H_2O, while CDH needs oxygen electron acceptor to form hydrogen peroxide (H_2O_2) (Nyanhongo *et al.*, 2002; Hou *et al.*, 2004; Thurston, 1994). Hence, to accelerate the reaction of crude oil degradation, oxygen supply is required. Fig. 2 shows the air supply provided by electrical aerator.

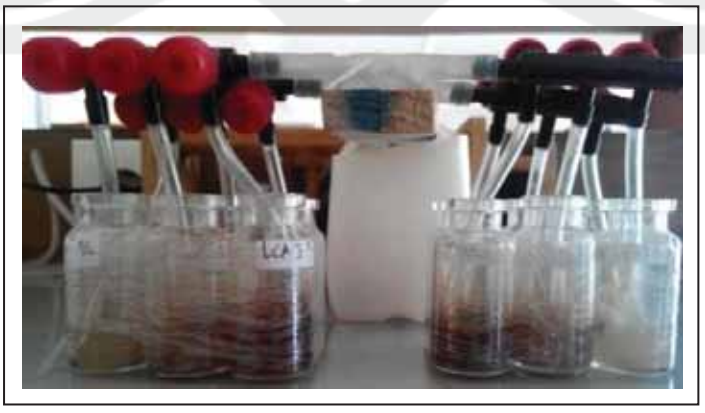

Figure 2. Air supply by electrical aerator in crude oil degradation

Crude oil degradation was conducted by laccase (L), laccase-ABTS (LA), cellobiose dehydrogenase (CDH), laccase-CDH (LC), and laccase-CDH-ABTS (LCA). Laccase and CDH are oxidoreductases vastly applied as degradation agents of phenolic compounds (Nyanhongo *et al.*, 2007). Fig. 3 shows the color change effect during degradation by oxidoreductases.

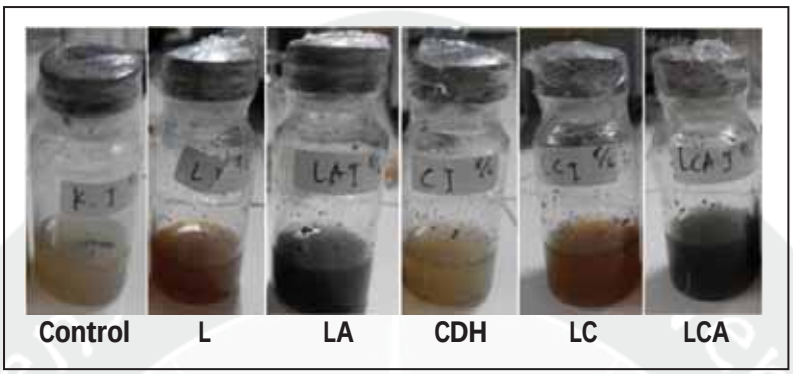

Figure 3. Color change as a result of enzymatic reaction

The color of the sample treated by LCA was slightly brighter than the color of sample treated by LA. This was because the oxidation of crude oil was more efficient with the formation of H_2O_2 as a result of CDH reaction with oxygen and further reaction forming peroxyl radical (Nugroho Prasetyo *et al.*, 2014; Thallinger *et al.*, 2013; Flitsch *et al.*, 2013; Nyanhongo *et al.*, 2013). H_2O_2 serves as biobleaching of aromatic compounds that gives color to the crude oil soil contaminant. Results of enzymatic degradation of crude oil soil contaminant are shown in Table 2.

Table 2. Degradation percentage of crude oil-contaminated soil

Enzymes combination	Degradation percentage ($\%\frac{gr}{gr}$)
Laccase	26.1
Laccase-ABTS	25.2
CDH	4.2
Laccase-CDH	22.2
Laccase-CDH-ABTS	49.4

Treatment by LCA resulted in highest degradation percentage of 49.4%, while treatment by L and LA was 26.1% and 25.2% respectively. Nugroho Prasetyo *et al.,* (2014) and Nyanhongo *et al.,* (2002) stated that the use of more than one enzymes with the addition of the mediator will provide a more effecient and effective degradation. The degradation efficiency was due to the use of ABTS mediator in laccase oxidation reaction (Thurston, 1994). The use of mediators facilitate laccase in oxidizing higher redox potential compounds and also oxidizing non-phenolic molecules such as alkyl chains in crude oil (Nugroho Prasetyo *et al.*, 2014; Nyanhongo *et al.*, 2002). Fig. 4 shows the mechanism of crude oil degradation by LCA in which ABTS is the key of efficiency during the reaction (serves as a donor and an electron acceptor).

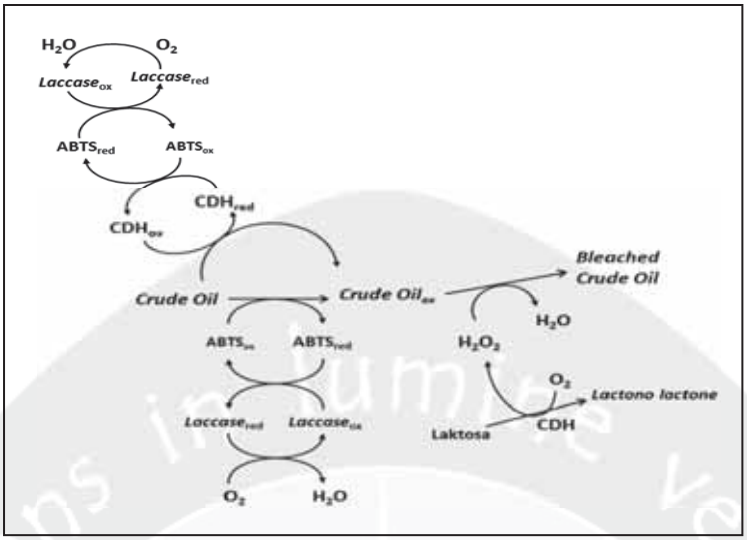

Figure 4. Mechanism of crude oil degradation treated by LCA (modified from Thurston (1994); Nyanhongo *et al.,* (2007); Nugroho Prasetyo *et al.,* (2014)

3.4 Enzyme Kinetics

Enzyme kinetics was determined for three different treatments (L, LA, and LCA) with the highest degradation percentage to obtain V_{max} (maximum velocity) and K_m (Michaelis coefficient). Table 3 shows the V_{max} and K_m of the three treatments.

Table 3. Enzyme kinetics of crude oil degradation

Enzymes	v_{max} (µg/s)	K_m (µg)
Laccase	3.472	6.84×10^6
Laccase-ABTS	2.139	4.84×10^6
Laccase-CDH-ABTS	3.750	6.01×10^6

Following the report by Nyanhongo *et al.,* (2006), the result of the Michaelis-Menten enzyme kinetics in the form of logarithmic graph was converted into Lineweaver-Burk graph to obtain K_m and V_{max}. K_m is an important indicator in enzyme kinetics showing whether the reaction rate (rate or v) is limited by excessive substrates ([S] $<K_m$) or excessive enzymes ([S]> K_m), while V_{max} is the maximum speed of an enzymatic reaction which indicates high substrate concentrations (Nyanhongo *et al.*, 2013).

The best treatment combination in degradation of crude oil is LCA with V_{max} and K_m of 3.750 $\frac{\mu g}{s}$ and 6.01×10^6 µg (6.01 g) respectively. Although LA has lower Km (4.84x106 g), the Vmax isn't high. Thus if Vmax and Km were to be inserted into Michaelis-Menten equation, it would have low v0 (initial velocity), indicating that low substrate concentration allows the reaction rate to reach maximum (Kuchel and Gregory, 2002).

4. CONCLUSIONS

Laccase-CDH-ABTS (LCA) was the best treatment combination with highest degradation percentage of 49.4%. LCA also has the best ratio combination of V_{max} and K_m of 3.750 $\frac{\mu g}{s}$ and 6.01x10^6 µg respectively. Further examination of detailed change of compound as a result of crude oil degradation by GC-MS (Gas Chromatography-Mass Spectrometry) or HPLC (High Performance Liquid Chromatography) is necessary to understand the formed or loss compound after degradation. In addition, the use of more enzymes combinations is also necessary to obtain optimum result.

5. ACKNOWLEDGEMENT

The authors are grateful to Prof. Georg M. Guebitz of Institute of Environmental Biotechnology, University of Natural Resources and Life Sciences (BOKU), Vienna, Austria and Dr. Eng. Desriani, M.Si., Laboratory of Molecular Health and Diagnostics, Biotechnology Research Center, Lembaga Ilmu Pengetahuan Indonesia (LIPI), Cibinong, Indonesia for the enzymes used in this study; Aunurohim, DEA and Nengah Dwianita Kuswytasari, S.Si., M.Si for advice and feedbacks during the study; Directorate of Higher Education, Ministry of Education and Culture of the Republic of Indonesia for funding through Bidik Misi Scholarship program (2011-2015); and Biomaterial and Enzyme Research Team (2014/2015) for financial support and assistance.

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Utilization of Wood and Bran Waste for Laccase Production by *Pleurotus ostreatus*

Febrian Mayang Arumingtyas[1], Maharani Pertiwi Koentjoro[2],
Endry Nugroho Prasetyo[1]
[1]Biotechnology laboratory Institut Teknologi Sepuluh Nopember, Gedung H Kampus ITS Keputih Sukolilo Surabaya 60111 Indonesia
[2]Laboratory of Environmental Microbiology, Department of Biological and Environmental Science, Faculty Agriculture-Shizuoka University.and Structural Biology Research Center, Inter-University Research Institute Corporation-High Energy Accelerator Research Organization (KEK), Tsukuba-Ibaraki, Japan.
*Corresponding author email address: febrianmayang@gmail.com

Abstract

Pleurotus ostreatus is a white rot fungi and is able to utilize phenol as carboun source. Phenolic compounds are toxic, carcinogenic or mutagenic potential. Phenols can be degraded enzymatically by fungi. Laccase produced by white rot fungi is an enzyme which is able to degrade phenol. Optimization of laccase production conducted in this study include varying temperatures and pH. *Wood and bran waste were used as carbon sources.* The enzyme was purified by ammonium sulfate precipitation and characterized by measuring the enzyme activity, protein content, isoelectric point, and molecular weight. Optimum enzyme activity was achieved through combination of temperature 25°C with pH 6 (278,45 U/ml) and protein content 0,102 mg/ml. Adding wood waste as carbon source to increase the enzyme activity to 534,21 U/ml. Laccase secreted by Pleurotus ostreatus has isoelectric point at pH 5 and molecular weight of 59 kDa and 43 kDa.

1. INTRODUCTION

Bacteria and fungi capable of utilizing phenol as carbon and energy sources in anaerobic or aerobic conditions (Nair *et al.,* 2000; Van *et al.,* 1998). Oxidoreductase are enzymes that can catalyze the transfer of electrons from one molecule to another, such as laccase (Tarr, 2003). Laccase produced by fungi has higher redox potential than bacteria and plants (Alcalde *et al.,* 2006). Laccase (benzenediol: oxygen oxydoreductase, EC 1. 10.3.2) is an extracellular enzyme containing copper and use oxygen as an electron acceptor (Thurston, 1994). Laccase has many benefits in industry including pulp delignification by separating lignin through oxidation reactions, as bleaching agent in the paper and textile industry, as well as bioremediation agents of industrial waste polyaromatic hydrocarbons (PAHs) (Madhavi & Lele, 2009; Kerovuo *et al.,* 2008; Han and Song, 2004). Therefore, large scale of efficient laccase production is necessary to meet industrial demand.

Laccase is commonly produced by fungi, mainly white rot fungi (Gianfreda *et al.*, 1999; Schmidt, 2006). White rot fungi are able to produce laccase which can oxidize the color of lignin (Pointing, 2001). Lignin is one of the natural wood biopolymer, consisting of phenol ether bond, alkyl ether, dialkyl ether, aryl ether glycerol-β-aryl and glycerol ethers guaiacyl-α-aryl (Chen, 2014). Laccase produced by white rot

fungi has high redox potential, especially those by *Trametes versicolor, Trametes villosa,* dan *Rigidoporus lignosus* (Kilaru, 2006). *Pleurotus ostreatus* is a white rot fungi highly potential of laccase production. However, laccase production by this species hasn't been produced large scale. The present study utilized sawdust and bran as carbon sources for laccase production by combining temperature and pH medium for optimization.

2. METHODS

2.1 Organism and cultivation condition

P. ostreatus was obtained from the collection of Laboratory of Micology of Institut Teknologi Sepuluh Nopember (ITS) (Surabaya, Indonesia). The fungi was grown on Potatao Dextrose Agar (PDA) at 25ºC for 24 hours and maintained at 4ºC until use.

2.2. Optimization and isolation of laccase

P. ostreatus was cultured in 250 ml Erlenmeyer flasks containing 100 ml of laccase production medium with the composition (g/100 ml): peptone 0.3 g, glucose 0.5 g, KH_2PO_4 0.06 g, $ZnSO_4$ 0.0001 g, K2HPO4 0.04 g, $FeSO_4$ 0.00005 g, $MnSO_4$ 0.005 g, MgSO4 0.005 g, $CuSO_4$ 0.0125 g as an inducer (Sivakami *et al.*, 2012; Chawachart *et al*., 2004), sawdust 1 g and bran 1 g. The fungi of 7 days old were transferred aseptically into 100 ml of laccase production medium with different pH (4, 4.5, 5, 5.5, and 6). The media were incubated at 25°C, 30°C, and 35°C for 7 days on rotary shaker 130 rpm. After 7 days, each culture was filtered by Whatman® filtration paper Grade 1 (Nyanhongo *et al.,* 2002) and centrifuged at 10.000 rpm for 15 min to collect the supernatant which was used as crude enzyme. The best combination of temperature and pH to produce high laccase activity will be used for producing enzymes in media with sawdust and bran waste as carbon sources.

2.3. Laccase Purification

Precipitation of crude enzyme was done by adding 0-30%, 30%-45%, 45%-60%, 60%-75%, and 75%-90% ammonium sulfate (Diah, 2007). The enzyme was subsequently centrifuged at 3000 rpm for 15 min.

2.4. Protein measurement (Bradford assay)

The protein concentration was determined following the Bradford (1976) method using bovine serum albumin (BSA) standard. Readings were carried out in a spectrophotometer at 595 nm.

2.5. Laccase assay

Laccase activity was measured using 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonate) (ABTS) as a substrate and standard. The reaction was initiated by the addition of 0.8 ml citrate buffer (pH 4.5), 0.35 ml ABTS (0.1 mM) and 0.35 ml laccase. The color change was read at 436 nm for 5 min (Irshad *et al.,* 2011).

2.6. Isoelectric point

Five test tubes were prepared and filled with 1 ml of laccase and 1 ml of acetate buffer pH 3, 4, 5, 6, and 7. Each tube was shaken and heated for 30 min. The isoelectric point was indicated by the most rapid or massive formation of sediment turbidity.

2.7. SDS-PAGE electrophoresis

SDS-PAGE was carried out following the method of Bollag and Edelstein (1991). The protein was stained by Coomassie Brilliant Blue.

3. RESULTS AND DISCUSSION

3.1. Optimization of Laccase production

Laccase activity was measured using azino-2,2'-bis (3-atilbenzotiazolin-6-sulphonic) (ABTS) as a substrate. Laccase is able to oxidize the ABTS radical cation ($ABTS^{+}$) and radical dication ($ABTS^{2+}$) and observed at 436 nm (Paul *et al.*, 2004; Mansur *et al.,* 2003). Optimum laccase activity was achieved through combination of temperature 25°C with pH 6 (278,45 U/ml). In nature, *P.Ostreatus* is able to grow at temperature range of 25-28°C (Chang and Miles, 2012). The pH medium 6 is the optimum pH condition that influenced the growth of *P. ostreatus* and also influenced the cells synthesize secondary metabolites (De Hui *et al.,* 2011). Table 1 presents laccase activity in varying temperatures and pH.

Table1. Laccase activity

	Activity (U/ml)		
pH	25°C	30°C	35°C
4	60,38	12,74	1,44
4,5	50,32	13,41	1,01
5	110,71	228,13	2,01
5,5	154,32	177,80	0,33
6	278,45	181,16	1,67

Table 1 showed that temperature above 30°C decreased laccase activity because fungi generally have optimum temperature range of 25-30°C (Minussi *et al.,* 2007). Fungi in nature generally live in the pH range of 4-6 (Arora & Gill, 2000) thus the highest laccase activity (130.84 U/ml) occurred at pH 6 (Table 1). These results correlated with other white rot fungi such as *Trametes pubescens* and *Trametes versicolor* which have pH optimum of 6 (McMillan *et al.,* 2008; Strong, 2008). Fungi are generally capable of producing laccase in lower range of pH than bacteria (Singh *et al.,* 2011).

Induction of laccase production can be increased by the addition of inducer. One of the inducer used is Cu^{2+} ions which is a constituent element of the laccase active site. The use of $CuSO_4$ in the laccase production medium can increase laccase activity up to 10 times (Bertrand *et al.,* 2013), hence this study used inducer of 0.1 mM $CuSO_4$ and had laccase activity average of 84.93 U/ml as a result.

3.2. Enzyme purification and characterization

Purification of laccase was done by ammonium sulfate purification (Diah, 2007). The result of laccase purification using varying salt concentrations is presented in Figure 2.

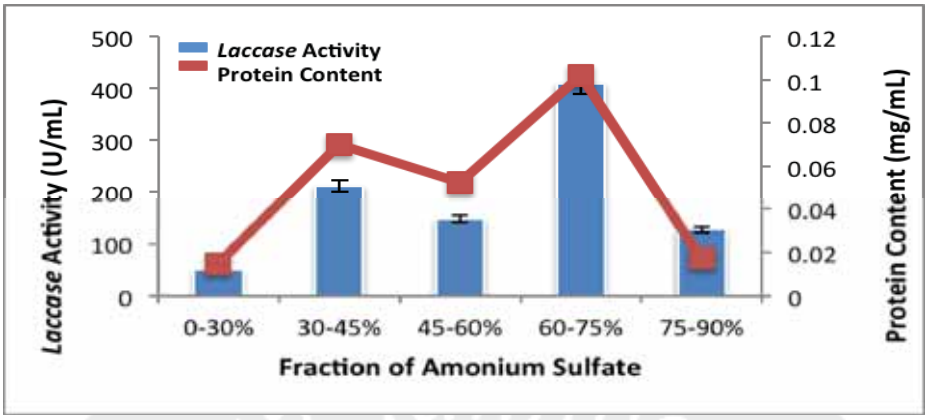

Figure 2. Protein content and laccase activity in each fraction of ammonium sulfate purification

Ammonium sulfate fraction of 60-75% showed the highest enzyme activity (409 U/ml) followed by fraction of 30-45% (212 U/ml). The two high activity values were due to the ability of *P.ostreatus* in secreting isozyme laccase. Isozyme is an enzyme which differs in amino acid sequence but catalyzes the same chemical reactison (Munoz *et al.,* 1997). The highest enzyme activity of 60-75% fraction showed high concentration of total protein (0,102 mg/ml), while the isoelectric point was reached at pH 5 (Fig. 3a). This result was in accordance with *Basidiomycetes* which have isoelectric point at pH 2,6-5,1 (Kilaru, 2006).

Result from SDS-PAGE electrophoresis showed that there are two bands of protein with molecular weight of 43 kDa and 59 kDa (Fig. 3b). Thus, the produced laccase was most likely an isozyme. The result from SDS-PAGE electrophoresis is consistent with the result of enzyme purification in which the highest laccase activity and protein content were presented in two fractions (30-45% and 60-75%). The molecular weight of laccase produced by the genera Pleurotus has molecular weight in the range of 55-59 kDa and is isozyme (Soden and Dobson, 2001).

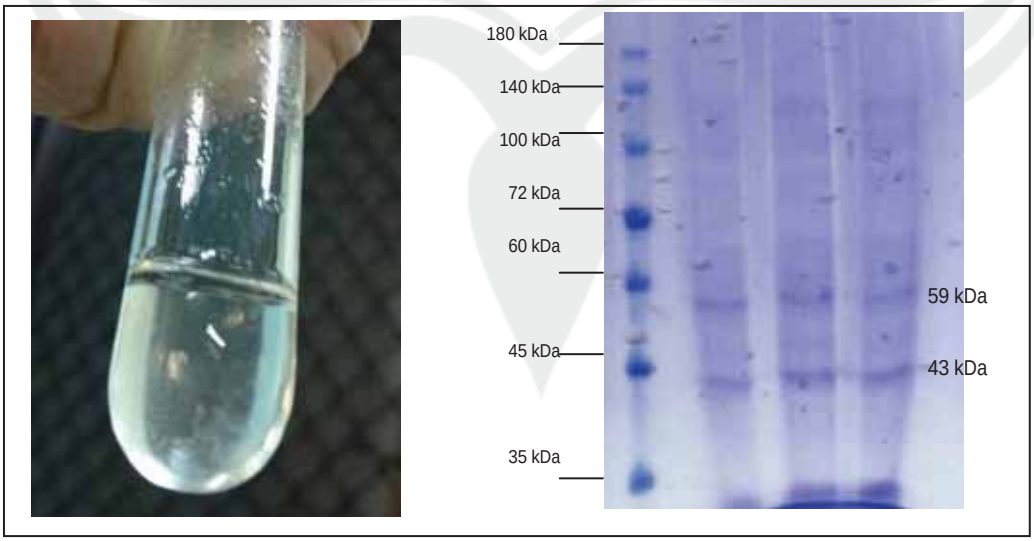

Figure 3. Characterizations of laccase. A) Isoelectric point of laccase indicated by coagulation; B) Protein bands visualized by Coomassie Brilliant Blue stain

3.3 Waste utilization

Results of the optimization by combining temperature and pH medium were used as formulations for producing laccase by utilizing sawdust and bran waste as carbon sources. Addition of 0.5 mM CuSO4 was also done to have more efficient production. Fig. 4 shows the laccase activity profile of sawdust and bran significant increase of laccase activity with sawdust as substrates occurred after 3 days of incubation. In contrast, laccase activity with bran as substrates decreased after 7 days.

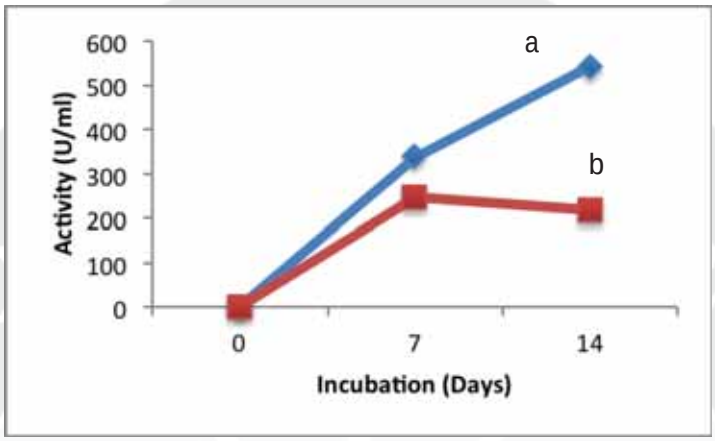

Fig.4. Laccase activity with different carbon source (a: sawdust, b: bran)

Laccase activity of both sawdust and bran as substrates after 14 days incubation was 543.21 U / ml and 246.91 U / ml respectively. This result was because lignin content in sawdust (25.7%) was higher than bran (22%) (Crini and Pierre, 2010; Mohd *et al.,* 2013).

4. CONCLUSIONS

Optimum condition for laccase production by *P. ostreatus* was at 25ºC and pH medium of 6 (278.45 U / ml). Higher activity of laccase production was generated by utilizing sawdust as substrate (543.21 U / ml). Ammonium sulfate purification can increase laccase activity by 47% in the 60-75% fraction with protein content of 0.102 mg / ml. Isoelectric point of laccase was reached at pH 5 with a molecular weight of 59 kDa and 43 kDa. The two molecular weights present a possibility that the laccase was isozyme.

5. ACKNOWLEDGEMENT

The authors are grateful for the academic grant provided by Biomaterial and Enzyme Technology Research Team (2014/2015) supervised by Dr. techn. Endry Nugroho Prasetyo, M.T. in which the study was conducted. Special thanks are also given to father Djoko Suprijono and mother Endah Poernawati for the motivation, support and prayers; to Nur Shabrina, S.Si. for proof-reading the manuscript; to family of *Scylla serrata* 2011 and Biomaterial and Enzyme Research Team (2014/2015) for the motivation, support and assistance during the study.

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Variation of Rice Husk, Corn Husk and Corn Hump Ratio as Alternative Growth Media for Pleurotus osteatus (*White Oyster Mushroom*)

Maria Goretti M. Purwanto*, Theresia D. Askitosari, Tjandra Pantjajani, Andreas L. Wijoyo
Faculty of Biotechnology, University of Surabaya
Raya Kalirungkut, Surabaya 60291 Indonesia
*Email: maria_gmp@staff.ubaya.ac.id

Abstract

Natural waste like rice husk, corn husk and corn hump are potential as alternatives substrates for Pleurotus ostreatus growth media because its nutrition. This research aimed to obtain the best variation of substrate composition for the growth of the mushroom. Parameters were wet weight of the fruiting bodies (yield), cap's diameter and fruiting bodies morphology. Based on the result, medium consist of 0.5 kg rice husk, 0.25 kg corn husk and 0.25 kg corn hump is the only composition gave the same yield as the control (100% sawdusk) did. There was also no significant difference on cap's diameter and morphology of the fruiting bodies.

Keywords: sawdust, rice husk, corn husk, corn hump, oyster mushroom

1. INTRODUCTION

Edible mushrooms are widely consumed in Indonesia where the climate is suitable for the natural growth as well as the cultivation of certain mushrooms. White oyster mushroom (*Pleurotus ostreatus*) is one of the most commonly cultivated edible mushrooms in Indonesia (Suriawiria, 2002). The cultivation process of white oyster mushroom usually makes use of a sawdust baglog as a substrate for the mushroom growth. Nowadays, the availability of sawdust tends to be limited, hampering the oyster mushroom cultivation in general. Therefore, search efforts for alternative substrates providing sufficient nutrition for the mushroom growth have gained a big interest.

According to Cahyana *et al*., (2006), the necessary nutrient composition for oyster mushroom growth more or less includes 27% lignin, 70% karbohidrat (selulosa dan glukosa), protein, nitrogen, fiber, and vitamins like tiamin at 100µg/L. All those nutrients are available in sawdust (Senyah, 1989). Rice husk, corn husk and corn hump are examples of agricultural solid waste obtained easily in huge amount in agricultural countries like Indonesia. They are used so far only as mix components for making fertilizer. The carbohydrate content in Rice husk, corn husk and corn hump has been reported to be high enough, making them potential as alternative growth substrates for mushroom growing on sawdust.

This work aimed to evaluate the capacity of those materials in substituting the sawdust portion in the common growth substrate. Their percentage in the total baglog composition was varied to see the influence on the fresh weight and morphology of the fresh mushroom yielded.

2. METHODS

2.1 Materials

The experiment was carried out at the Laboratory of Microbial Biotechnology, University of Surabaya, Indonesia, between September 2014 and February 2015. Sawdust was collected from the different sawmills in Mojokerto, East java. Dry rice husk, corn husk and corn hump used as substrate mixture were collected from Surabaya. Corn hump and corn husk were grinded before usage, while the rice husk was kept in the original shape and size. The experiment was laid out in a completely randomized design with 8 treatments and replicated 10 times.

2.2. Procedures

2.2.1Substrates preparation and spawn inoculation

Each sawdust (30% of volume) was supplemented with rice rice husk, corn husk and corn hump at certain ratio as written in Table 1, also mixed with calcium carbonate (0.02%) and water (50%) and thoroughly mixed. Polyprophylene bags (22.5 × 30 cm) were filled with 1 kg of each substrate, sterilized once at 121°C and 1 atm pressure for 3 h and for several hours allowed to cool down to the desired inoculation temperature in a dark room. Each baglog was stood up, the mouth was opened and the spawn (3% w/w) was placed into each bag at the approximate center using a sterilized metal implement. Bags were closed with paper.

2.2.2 Cultivation conditions

The bags were subsequently placed, longside down, into a spawn running room at 25 - 30°C in the dark and 65 - 70% relative humidity until completion of spawn running (approximately 30 days after spawn inoculation). After completion of spawn running the temperature and relative humidity was changed to 19 to 25°C and 80 - 90% RH, respectively. The baglogs were slit and the paper cover was removed. Water was sprayed for maintaining moisture up to the desired level in the form of fine mist from a nozzle.

2.2.2 Fructification and Harvest

During the fruiting process, temperatures of 19 to 25°C and 80-90% RH were maintained in almost total darkness. All mushroom fruit was harvested by pulling out the stalk and the yield was calculated based on the cumulative fresh weight of the mushroom fruits harvested from each baglog. In general, fresh weight, diameter of carps and the morphology of the mushroom fruits were observed. Production in each bag will gradually decrease until there is no more fructification because of degradation in the mycelium.

3. RESULTS AND DISCUSSION

3.1 Days to complete mycelium running

No remarkable differences were observed in mycelial running rate on spawn packets of the different substrates used, except for composition of rice husk:corn hump:corn husk (0.5kg:0.25kg:0.25kg). It ranged from 5-6 days, while others for 7 to 8 days. Variation in the time might be due to variations in log texture, where the rice husk

lowered the hardness and compactness of the logs. The result might be different if the rice husk would have been grinded before used as a substrate mix component.

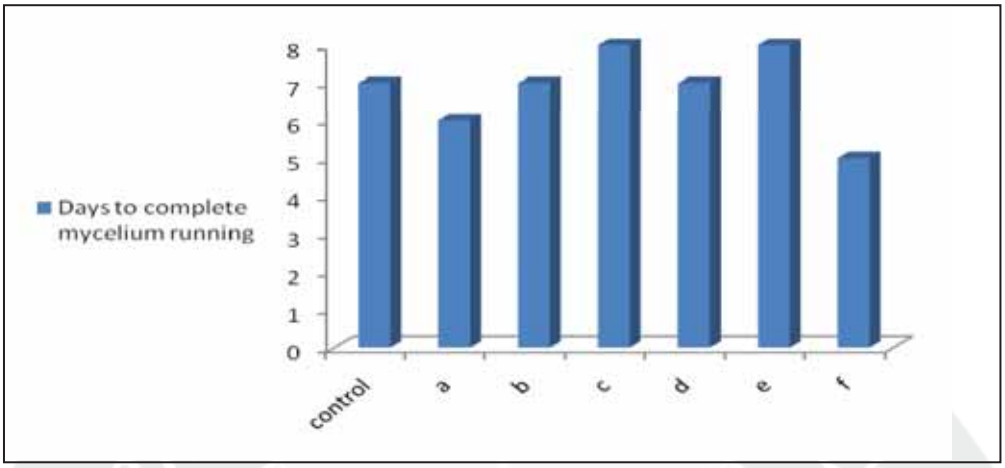

Figure 1. Time to complete the mycelium running affected by substrate composition
Control = 1kg sawdust, a = corn hump:corn husk (0.5kg:0.5kg), b = sawdust:rice husk (0.5kg:0.5kg), c = rice husk:corn hump (0.5kg:0.5kg),d = sawdust:corn hump:corn husk (0.5kg:0.25kg:0.25kg), e = sawdust:corn hump (0.5kg:0.5kg), f = rice husk:corn hump:corn husk (0.5kg:0.25kg:0.25kg).

3.2 Fresh weight of harvested mushroom fruits

Analysis of data reveals no significant effect of the substrate composition to the cap's diameter. Yet, in terms of fresh weight, it was clearly shown that the rice husk alone was not suitable for growing the mushroom, while the last composition (rice husk:corn hump:corn husk 0.5kg:0.25kg:0.25kg) performed as well as the control (sawdust) did.

Table 1. White oyster mushrooms growth on various media/substrate compositions

Substrate Composition				Average of mushroom fresh weight (g/baglog)	Average cap diameter (cm)	Color, size
Saw-dust	Rice Husk	Corn Hump	Corn Husk			
1 kg	-	-	-	235.70 ± 28.84 ^{a}	6.96 ± 0.94 ^{a}	white. normal size
-	-	0.5 kg	0.5 kg	123.30 ± 54.55 $^{c.d}$	6.46 ± 1.50 ^{a}	white. normal size
-	1 kg	-	-	-	-	-
0.5 kg	0.5 kg	-	-	159.70 ± 48.00 $^{b.c}$	7.06 ± 1.442 ^{a}	white. normal size
-	0.5 kg	0.5 kg	-	84.00 ± 4.24 $^{c.d}$	7.14 ± 0.27 ^{a}	white. normal size
0.5 kg	-	0.25 kg	0.25 kg	118.50 ± 27.64 $^{c.d}$	6.204 ± 0.953 ^{a}	white. normal size
0.5 kg	-	0.5 kg	-	92.63 ± 29.67 ^{d}	6.857 ± 1.584 ^{a}	white. normal size
-	0.5 kg	0.25 kg	0.25 kg	196.43 ± 24.78 $^{a.b}$	6.695 ± 1.695 ^{a}	white. normal size

3.3 Number and morphology of effective fruiting bodies

Number of well-developed fruiting body was recorded and presented in Table 1. Dry and pin headed fruiting body was discarded but twisted and tiny fruiting body was included during counting. The percentage of effective fruiting body did not vary significantly in different substrates. They were rather similar in numbers, size, color and also cap's diameter.

3.4 Yield per packet

Significant variation was found in yield of white oyster mushroom grown on different substrates used. The maximum biological yield was recorded with control (1kg sawdust) and also with the composition of rice husk:corn hump:corn husk (0.5kg:0.25kg:0.25kg). The lowest biological yield was observed in composition of rice husk:corn hump (0.5kg:0.5kg). Chaudhary *et al*., (1985) explained the process of break-down of lignin. There is an apparent correlation between the ability to degrade lignin and the production of phenolases, which oxidize phenolic compounds to simple aromatic compounds that can be absorbed by mushroom mycelium and is used for it growth. The product of cellulolytic action in simple and soluble carbohydrates and the end products being glucose was absorbed by the fungal mycelium for growth and energy. Therefore, cellulose rich organic substrates are good for the cultivation of mushroom (Gerrits and Muller, 1965; Quimio, 1987). High cellulose content in wood results in enhanced cellulose enzyme production and increased yield of mushroom (Ramasamy and Kandaswary, 1976). The substrates with high lignin and phenolic content should decrease the activity of the enzyme, hence slow growth and low yield.

4. CONCLUSIONS

Variation on substrate composition did not give different results on the morphology (color, size and the cap's diameter of white oyster mushroom, except the fact that the rice husk alone did not showed any growth of mycelia. Yet, in terms of fresh weight, it was clearly shown that the rice husk alone was not suitable for growing the mushroom, while the last composition (rice husk:corn hump:corn husk 0.5kg:0.25kg:0.25kg) performed as well as the control (sawdust) did. Thus, that composition can be considered as an alternative growth media for white oyster mushroom.

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Enhancing Production of Woody Edible Mushrooms by Modifying Nitrogen Source Components of The Medium

Aris Mumpuni and Purnomowati
Faculty of Biology Jenderal Soedirman University
arsmpn@yahoo.com; purnomowati53@yahoo.com

Abstract

Pleurotusostreatus (white oyster) and *Auriculariapolytricha* (black jew's ear) are the two common easily available edible mushrooms in the market. Their main substrate components are wood sawdust, and rice bran, both of them act mainly as the sources of carbon and nitrogen, respectively, and some other important component such as gypsum, limestone, phosphate fertilizer, and water. As the availability of rice bran highly depends on harvest season of rice, yet the price fluctuates, this can be problems to mushroom farmers. On the other hand, there are some other organic materials available in the surroundings, such as vermicompost which can be easily found and stored in a quite long period. Vermicompost can be used to substitute the role of rice bran as nitrogen provider in the medium. This research was done to find out the influence of combinations of rice bran and vermicompost (100%:0%; 75%:25%; 50%:50%; 25%:75%; and 0%:100% of the main formula for originally rice bran portion) toward the enhancement of the mushrooms production and find out the optimum combination. The result showed that the treatment did not influence fresh weight of the white oyster production, all treatment combinations delivered approximately equal yield. Nevertheless, it highly significant influenced the jew's ear mushroom production. Treatments of 75%:25%; 50%:50%; 25%:75% significantly different to the rest combination (100%:0%; and 0%:100%), and highly enhancing the yield of fresh weight production of the jew's ear mushroom.

Keywords: *Pleurotusostreatus; Auriculariapolytricha;* rice bran; vermicompost; mushroom production enhancement.

1. INTRODUCTION

White oyster and black jew's ear mushrooms are two of many edible mushrooms easily found in the market and are considerably sold as daily vegetables. Both mushrooms are nutricious.According to Miles & Chang (1989) the former contains protein (25-50%), fat (2-5%), sugar (17-47%), mycocellulose (7-38%), mineral (8-12%) and some vitaminssuch as niacin, riboflavin, vitamin D, C, B1, B5, dan B6; while FAO (1982) stated that the nutricious components of the later are 0.70% fat; 7.25% protein; 18.70% fiber; and 71.50% carbohydrate. Within 100 g black jew's ear mushroom also contains energy 321.50 calori; 332.60 mg calsium; 14.30 mg iron; 122.10 mg phosphorus; 0.008 mg vitamin B1; 1.17 mg vitamin B2, 0.38 mg vitamin C;and 0.43 mg niacin.

The two mushrooms grow naturally on lignocellulosic substrate, and in term of cultivation, the substrate are then enriched using either organic material such as rice

bran and inorganic ones such as gypsum, limestone, and phosphate fertilizer. Rice bran is important as its role mainly on providing nitrogen element. Nitrogenon the medium support the growth of mushroom mycelium. Increasing levels of nitrogen makes mycelium becomes thick and compact. The use of vermicompost combined with rice bran in specific compositionis expected toincrease the production ofthe mushroom.

High nitrogen concentrations can accelerate the growth ofmycelium. Thick and compact mycelia growth influences increasing mushroom production (Miles, 1993). Moore and Landecker(1996) adds that the appropriate nutrient contentand ratio of CandN accelerate the growth of mycelium.

Rice bran as the main ingredient of the medium is not always available in the market, this is caused bythe availability of abundant bran only during rice harvest season, besides the branis alsoutilized by peopleforother needs, such as animal feed, and it can not be stored.On the other hand, there are some other organic materials available in the surroundings, such as vermicompost which can be easily found and stored in a quite long period. Vermicompost can be used to substitute the role of rice bran as nitrogen provider in the medium.

According to Zahid (1994), vermicompost is an organic fertilizer made of degradation of organic materials by microorganism and earth worm. Susanna & Arisandi(2010) reported that vermicompost contains high Nitrogen compound; within 100 g it has 1.4% Nitrogen. It is expected that introduction of vermicompost in specific compositions with rice bran, together with other components, into the media will create adequate nutrient to enhance the mushrooms to grow optimally.

This applicative research was aimed to find out the effect of utilization of vermicompost and rice bran in specific composition of growth media to enhance production of white oyster and black jew's ear mushrooms; and to find out the most appropriate composition.

2. METHODS

White oyster and black jew's ear mushrooms were grown on media formulated by Susilawati and Raharjo (2010), and Marlina and Djarijah (2001) respectively, each with 20% rice bran content as the basic formula. The basic formula was then modified by introducing vermicompost combined with rice bran with composition as follows:

A1: Growth media with 100% rice bran and 0% vermicompostof the basic formula
A2 : Growth media with 75% rice bran and 25% vermicompostof the basic formula
A3: Growth media with 50% rice bran and 50% vermicompostof the basic formula
A4: Growth media with 25% rice bran and 75% vermicompostof the basic formula
A5 : Growth media with 0% rice bran and 100% vermicompostof the basic formula

Media composition was the dependent variable; the independent one was mushrooms production. The main parameter was mushroom fresh weight with supporting parameter were mycelial growth speed, Biological Efficiency Ratio (BER), and C/N ratio of the media.

3. RESULT AND DISCUSSION

Production of both white oyster mushroom and black jew's ear mushroom varied in each medium, stated by the fresh weight ranged between 158.7 to 215.22 g; and 72.7 to 109.3 g respectively.

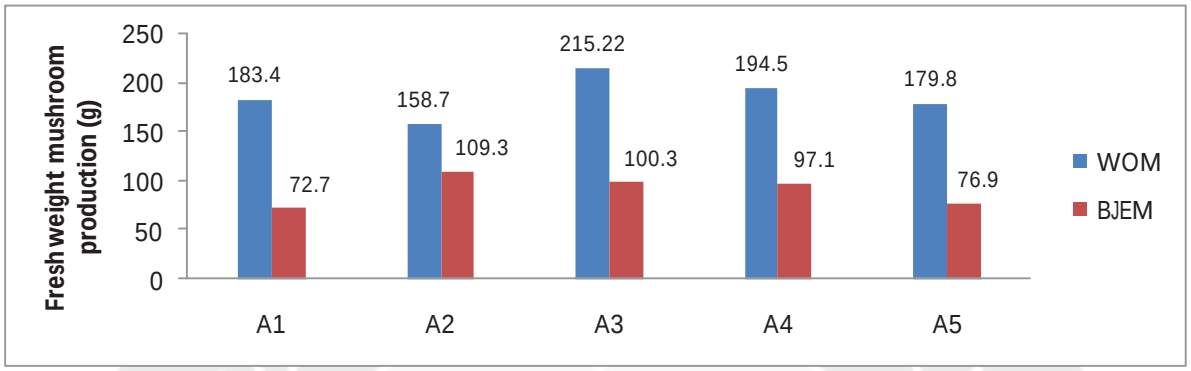

Figure 1. Growth of white oyster mushroom and black jew's ear mushroom in different media

Analysis of variance of white oyster mushroom production (Table 1) revealed that different combination of the media had no influence on production of the white oyster mushroom.

Table 1. Analysis of variance of white oyster mushroom production

Sources of Variance	DF	Sum of square	Mean square	F calc	F Tab	
					0,05	0,01
Treatment	4	8,580.12	2,145.029	1111.381^{ns}	2.87	4.43
Error	20	38,601.17	1,930.058			
Total	24	47,181.29				

ns = non-significant

Either in combination or stand alone, rice bran and vermicompost may act as nitrogen provider in the medium, however there was observed that the production of white oyster mushroom in media with higher nitrogen content (A5) was lower than that of the others, except A2 (Table 2.).The basic formula (A1) even gave higher yield than that of introduction 25% of rice bran to 75% vermicompostof the media (A2). Only that of 50% rice bran to 50% vermicompost (A3) gave the highest yield, yet statistically they showed no difference.

Table 2. C and N content and C/N ratio of the media

Treatments	White oyster mushroom media			Black jew's ear mushroom media		
	Organic C (%)	Total N (%)	C/N Ratio	Organic C (%)	Total N (%)	C/N Ratio
A1	44.24	0.43	102.42	26.0	0.32	81.0
A2	45.53	0.53	84.94	22.5	0.42	50.0
A3	40.67	0.55	73.28	16.5	0.61	27.0
A4	41.69	0.47	88.14	12.5	0.70	18.0
A5	40.85	0.67	60.79	9.5	0.82	11.5

The data showed that vermicompost can be used to replace or combined with rice bran as the component of media for white oyster mushroom with no difference in the yield. Vermicompost has a role as rice bran, as the source of nitrogen. This is waste of earthworm cultivation media. Zahid (1994) stated that vermicompost contains elements N, P, K, Mg, Ca, and Azotobactersp, an N non-simbiotic fixative bacteria.

Tjokrokusumo *et al*., (2004) stated that rice bran contains protein, carbohydrate, fiber, ash, and fat. Addition of rice bran is aimed to enhance nutrient content in the media, in order to support growth and development of mushroom fruit body. Protein, minerals, and vitamin B complex contents of rice bran could also stimulate mushroom to optimum growth.

Different composition of rice bran and vermicompost significantly influence production of black jew's ear mushroom. Anova of this treatment is as follow (Tabel 3.)

Tabel 3. Analysis of variance of black jew's ear mushroom production

Sources of Variance	DF	Sum of square	Mean square	F calc	F Tab	
					0,05	0,01
Treatment	9212.06	4	1239.94	5.48**	2.87	4.43
Error	4252.3	20	226.265			
Total		24				

** = highly significant

The analysis showed that difference in media delivered highly significant influence toward black jew's ear mushroom. On the other hand, the mushroom respond to the difference of carbon and nitrogen content differently compared to that of white oyster mushroom. The data showed that the production tend to adjust moderate nitrogen content of the media, in which optimum production occurred.

Table 4. Least Significant Difference (LSD) of black jew's ear mushroom production

Treatment	Mushroom production (g/*baglog*)
A1	72.7 a
A2	109.3 b
A3	100.3 b
A4	97.1 b
A5	76.9 a

The test revealed that average highest production was A2 (109.3 g/baglog), which was not different to that of A3 and A4; but significantly different to that of A5 and A1. It is assumed that the two of the later produced lower amount of black jew's ear mushroom due to their single source of nitrogen. A5 only have rice bran, meanwhile A1 only have vermicompost. This also revealed that combination of rice bran and vermicompost may support optimum growth of black jew's oyster mushroom.

Table 5. Comparison of some properties of white oyster and black jew's ear mushroom

Treatment	White oyster mushroom		Black jew's ear mushroom	
	Mycelial growth speed (cm/week)	BER (%)	Mycelial growth speed (cm/week)	BER (%)
A1	3.10	62.84	2.89	43.40
A2	2.38	64.77	2.88	64.87
A3	2.17	74.73	3.03	59.88
A4	2.04	68.45	2.28	57.97
A5	1.02	69.11	2.62	45.63

Table 5.revealed growth and yield properties of the mushrooms. Pattern of growth (mycelial growth speed) and yield (mushroom fresh weight and Biological Efficiency Ratio) of white oyster mushroom looks irregular compared to those of black jew's ear mushroom, with regular curve growth and yield pattern, explaining difference between the two mushroom in term of statistical analysis. The data also showed that black jew's ear mushroom tend to be more sensitive toward nutrient content in the media, especially carbon and nitrogen.

4. CONCLUSSION

As the treatments did not significantly influence of the treatments toward production of white oyster mushroom, it is assumed that vermicompost may substitute rice bran in any composition; meanwhile mixing rice bran with vermicompost with composition ranged between 75%:25% to 25%:75% of the main formula of cultivation media enhanced production of black jew's ear mushroom.

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Biochemical and Moleculer Characterization of Typical *Staphylococcus aureus* Isolates from Pasteurized Milk in Yogyakarta

Maria Irene Irwanto and Tri Yahya Budiarso
Faculty of Biotechnology, Duta Wacana Christian University Yogyakarta
Jl. Dr. Wahidin, S. No 5-25 Yogyakarta 55224 Telp: +62274563929, Fax: +62274513235
* yahya@staff.ukdw.ac.id

Abstract

Staphylococcus aureus is a known pathogen causing intoxication by producing enterotoxin in food. *Staphylococal enterotoxin* A in one of enterotoxins commonly implicataed in foodborne poisoning that is found in raw milk. During growth in milk, *S.aureus* strain are able to produce thermostable enterotoxins, that when ingested cause nauses, vomiting and diarrhea. The purposes of this reach is to characterization of biochemical and moleculer of *S.aureus*,isolates from pasteurized milk. A total of 25 samples collected randomly whichare sold by street vendors and café in Yogyakarta as most of the students consume it daily. Typical colonies *S.aureus*on*Baird Parker Agar* (BPA) were selected on*Manitol Salt Agar,* gram staining, Sugar test, *catalase* test and *coagulase* test. This result of the study only 79 colonies found typical *S.aureus*. Biochemical confirmation test used API-Staph obtained 4 isolates are identified as *S.aureus* and all isolates has staphylococal enterotoxin A based on moleculer test with PCR.

Keywords: *Pasteurized milk, staphylococcus aureus, enterotoxin A, PCR*

1. INTRODUCTION

Staphylococcus aureus is considered the most important of cause of diseases in the world among the according to the foodborne report. This bacteria is always implicated as a main agent of *foodborne illness* in the world especially in milkand dairy productsMilk is a very good subtrates for growing bacteria and also *S.aureus*, they have ability to grow in under a wide range of conditions and also produces the enterotoxin. (De Buyser *et al.,* 2001, Jorgensen *et al*., 2005). Contamination of *S. aureus* has occurred during milking process, transportation, equipment and environment. Milk contamination directly occur because of low system when producing the products such as poor hygiene during the process, the workers get sick because of influenza or etc. It also can adding the contamination, because workers handle the process directly. Retail and storage of products also affected the contamination. Pasteurization can kill the *S.aureus* cells, but the enterotoxins which is thermostable does still exist, inculuded of their biological activity (Morandi *et al.,* 2007).

Some isolates *S. aureus* can produce many variety of staphylococcal entertoxins (Ses, SEA to SEE, SEG to SEI, SER to SET). SEA is the most common cause of staphylococcal food poisoning in the worldwide (Argudin *et al.*, 2010). This

enterotoxin is very resisten to heat, it can't produces the toxins after 28 min at 121°C of heating.

Yogyakarta has so many kind product of foods especially *dairy products* such as milk. Milk is anutricious food containing animal proteins and very popular and became one of the main objective of a culinary tourism in Yogyakarta. A lot of people consumed it daily specially students in Yogyakarta.The objective of the present study was tocharacterizationof biochemical and moleculer of *S.aureus* isolated from pasteurizedmilk are sold by street vendors and café in Yogyakarta.

2. MATERIALS AND METHODS

2.1. Milk samples

A total 25 samples from street vendors and cafe were aseptically collectedon a random basis from different localities in Yogyakarta city. All the samples were placed in the sterile plastic bags and immediately in a container containing ice cubes to the laboratory of microbiology for bacteriological analysis.

2.2. Enumeration and Selection of *S.aureus*

Twenty-five ml of each samples was placed in to a sterile *Enrichment culture Brain heart Infusion* (BHI Broth). The medium was incubated at 37°C for 24 hours. The culture was inoculated onto*Baird Parker Agar* (BPA) plates with 5% eggyolk tellurite emulsion (Oxoid) and incubated on 37°C for 24-48 hours.Typical colonies of *S.aureus*were gray to black (potassium tellurite reaction) and were surrounded by clear zones (egg yolk reaction) were submitted for the following test: gram staining, *Manitol Salt Agar* (MSA), Maltose fermentation, catalase, and coagulase (Bennet and Lancette, 2001;Thaker *et al*., 2013).

2.3. Biochemical Identification of *S.aureus*

API-STAPH system used for biochemical test for *Staphylococcus, Micrococcus* and *Kocuria* which is consists of 20 mediums to test an homogenous bacterial suspension at 0,5 McFarland turbidity added. Incubated at 37°C for 18-24 hours, and metabolism produces colour changes or revealed by the addition of reagents (Langlois *et al*., 1983).

2.4. Detection of enterotoxins gen with PCR

For DNA isolation use commercial kit Genejet DNA kit. The primers used for the detection is SEA from Johnson et al. (1991) sea1 F 5'- ttggaaacggttaaaacgaa- 3' andSEA-2 R5'- gaaccttcccatcaaaaaca-. Each reaction contained of 5µl ddH_2O, 1 µl Primer F, 1 µl Primer R, 3 µl DNA. DNA was performed in 1,5 % gel agarose (Sigma-Aldrich) contained of ethidium bhromide dan buffer to elektroforesis for 1 hours at 90 V (Enduro Power Supply, EPD 300 V, Labnet International Inc. Amplication of DNA was performed by PEQ-Lab PCR with conditions : *pre-denaturasi* for 3 min at 95 °C*, denaturasi* for 2 min at 94 °C *,annealing* 30 cycles for 1 min 30 sec at 55 °C and *final extension* for 1 min at 72 °C (Rall *et al.,* 2008). Fragment of DNA will be visualized with (AlphaImager, Alpha Innotech Corporation), used Alpha Ease FC Software.

3. RESULTS AND DISCUSSION

Sample collected from pasteurized milk from different places which is distinguished related with conditions of sanitation and environment. Sample was innoculated in BHI broth as a enrichment culture consistedbrain infusion solids, beef heart infusion solids, proteose peptone and glucose. After 24 h incubation, sample was streak on BPA plate and incubate in at 37°C for 24-48 h. Baird Parker Agar is a selective medium for the isolation and presumptive identification of coagulase-positive staphylococci. This medium is used extensively for detecting Staphylococcus aureus in foods, dairy products, and other materials.Baird Parker Agar is a selective medium for the isolation and presumptive identification of coagulase-positive staphylococci. Lithium chloride and potassium tellurite act as selective agents. Egg yolk is the substrate to detect lecithinase production and lipase activity. Staphylococci produce dark gray to black colonies due to tellurite reduction; staphylococci that produce lecithinase break down the egg yolk and cause clear zones around respective colonies. An opaque zone of precipitation may form due to lipase activityof *S.aureus* (Bennet and Lancette, 2001; Thaker *et al*., 2013). Based on the typical colonies on BPA were found 79 of the total 2779 colonies. On every sample of milk by the streak which is dominated by the emergence of the colony BPA medium brown to black suspected the group of *Proteus*.The second is that it is to be the main target groups of *S. aureus*. The group is the latest colonies large brown to dark suspected *E. coli* (Parker, 1992).

Typical colonies then were selected on the MSA medium. The next stage after the purification skreening is the stage in biochemistry. Medium Mannitol Salt Agar consisting of 7.5% salt sodium chloride so that can be used as media selective according to the reference of Marshall, R. (1992). A number of studies rose by the culture of bacteria, most bacteria can 't grow in the concentration of salt on 7.5 % except group with staphylococcus bacteria.Other composition that supports is a substrate mannitol sugar in the form of indicators and also phenols red. Staphylococcus group especially staphylococcus aureus will be able to change the color of the medium red originally becomes yellow even around a colony also changed colors become yellow.

The stage of differentiation that can be done is with coloring *gram staining*. This test aims to distinguish a group of bacteria positive and negative. A group of *S. aureus* is the gram positive. Cells previously stained with crystal violet or gentian violet. This procedure produces "purplecolored iodine-dye complexes" in the cytoplasm of bacteria. The cells that are previously stained with crystal violetand iodine are next treated with a decolorizing agent such as 95% ethanol or a mixture of acetone and alcohol. The difference between Gram-positive and Gram-negative bacteria is in the permeability of the cell wall to these "purple colored iodine-dye complexes" when treated with the decolorizing solvent. While Gram-positive bacteria retain purpleiodine-dye complexes after the treatment with the decolorizing agent, Gram-negative bacteria do not retain complexeswhen decolorized.Gram-positive bacteria will retain a purple color because it can absorb crystal violet, so when observed to be purple (Elsa *et al.,* 2010). The test continued with the coagulase and catalase test. Be drawn second test was done very differentiates the *S.aureus* with clusters of other *Staphylococcus*. Aimed at ascertaining the coagulation activity are capable of performing the activity of a clumping of bacteria. Coagulation also associated with the

process protein in denaturing if does not will exactly gave bad impacts for humans. Of the nature of coagulan can also indicated that bacteria is able to react in concentration a salt is quite high (Elsa *et al.,* 2010). The final test for biochemical identification of candidates *S. aureus*used API Staps system (Fig.1). Isolates transferred under the kit, continued with the vortex untilthe turbidity equal to 0.5 mcfarland.The original color of medium is red, when isolates can do fermentation activity in carbohydrates medium such as fruktosa, manitol, or laktosa xylitol. It going to change the color of red to yellow. But there are three types of medium to certain regaen to know whether he is a positive or negative reaction. Medium of NIT, PAL and VP reagen to see the colours changing. As the results 3 isolates were identified as *S. aureus* (97,8%) and 1 isolates (86,7%). All isolates positip as *S.aureus* from *pasteurized milk* sampel of street vendors.

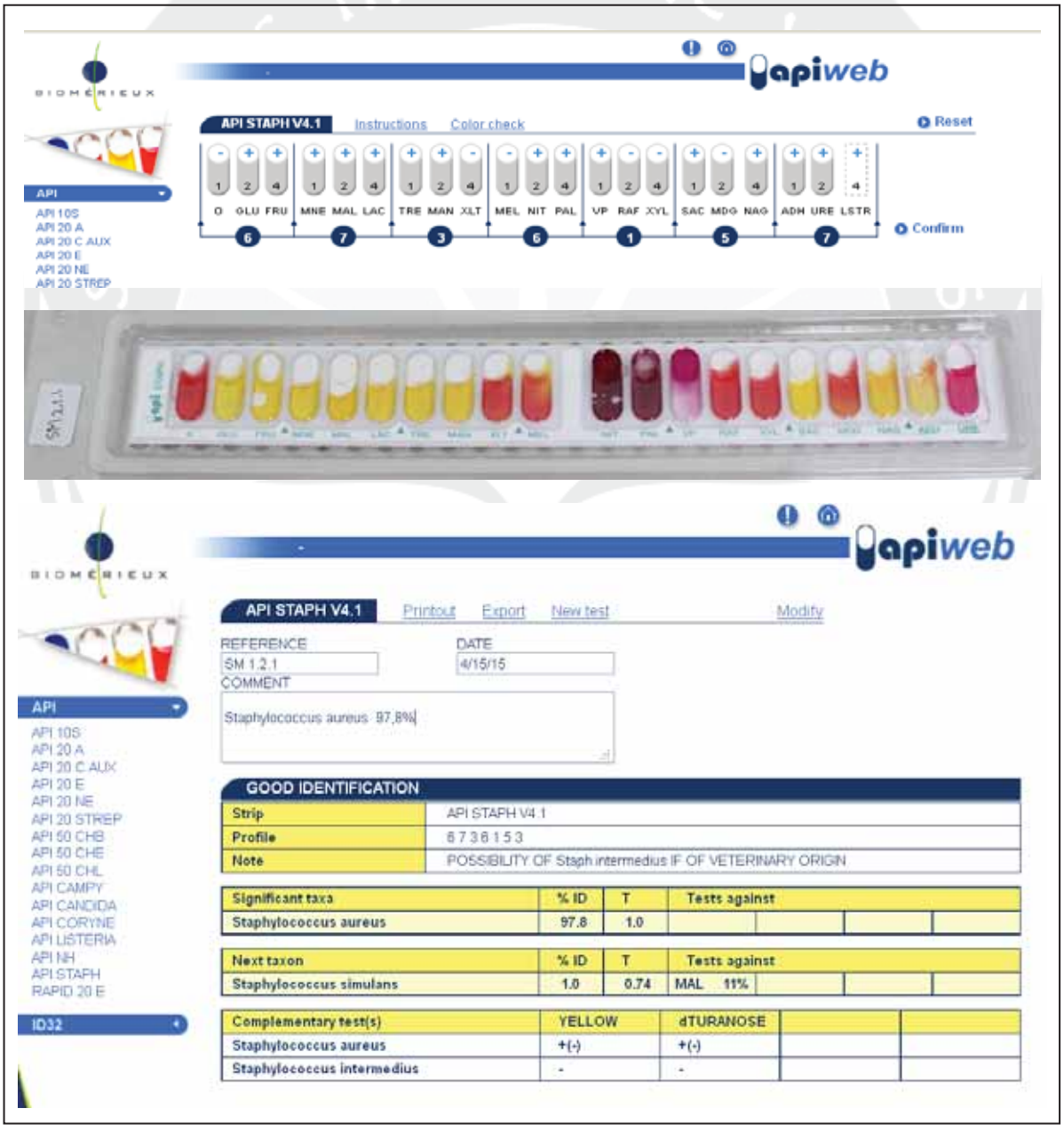

Figure 1. API-STAPH Results

Twenty five samples has been collected just only 3 sample milk street vendors there are bacteria have the *sea.*All detected at the location of the target of 120 bp with a primer sea1 F 5'- ttggaaacggttaaaacgaa- 3' dan sea-2 R5'- gaaccttcccatcaaaaaca--. Isolation of the stages performed a modification of DNA on how it works with the addition of two types of antibiotic catrimaxasol and lysostaphin. After that by using pcr , conducted the stage in *pre-denaturasi* for 3 min at 95 °C*, denaturasi* for 2 min at 94 °C ,*annealing* 30 cycles for 1 min 30 sec at 55 °C and *final extension* for 1 min at 72 °C (Rall *et al.,* 2008). Fragment of DNA will be visualized with (AlphaImager, Alpha Innotech Corporation), used Alpha Ease FC Software (Figure 2).

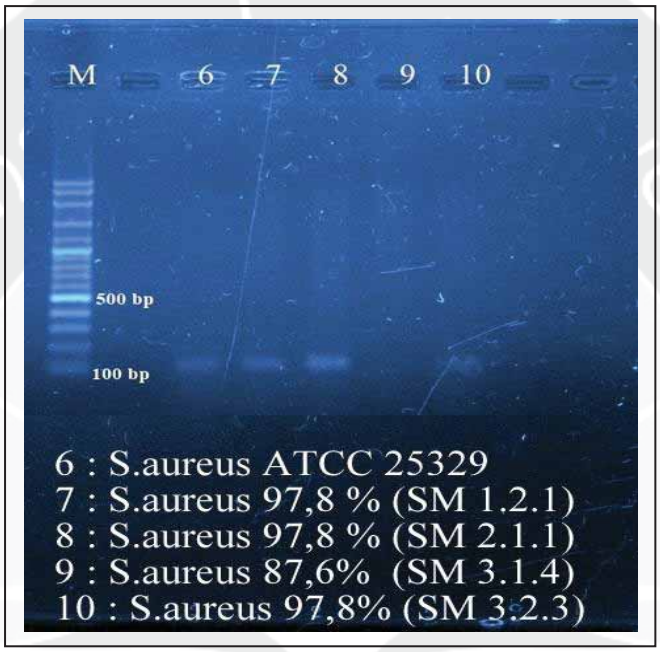

Figure 2. Amplification Result of PCR

4. CONCLUSSIONS

This result of the study 25 samples of *pasteurized* milk from street vendors and café in Yogyakarta, obtained 79 colonies typical as *S. aureus*. Based on biochemical confirmation test used API-Staph obtained 3isolates are identified as *S. aureus* (97,8 %) and 1 isolates (86,7 %). All isolates has staphylococal enterotoxin A based on moleculer test with PCR.

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Variability and Intraspecies Classification of Gembolo (*Dioscorea bulbifera* L.) in Yogyakarta and Surronding Areas Based on Morphological Characters

Purnomo
Laboratory of Plant Systematics, Faculty of Biology, Universitas Gadjah Mada.
Jl. Teknika Selatan, Sekip Utara Yogyakarta
Hand phone: 08156858078 and 087738340009
E-mail address: pakkencur@yahoo.com

Abstract

Research on genetic variability and intra-species classification of yam (*Dioscorea bulbifera* L.; Dioscoreaceae) based on morphological characters aimed to find genetic variability resulting the morphological variability, and classification into groups cultivars of *D. bulbifera* are reviewed based on morphological characters for the cultivation and conservation of plants. Searching the distribution and cultivation information of *D. bulbifera* in Yogyakarta and surrounding areas. Based on the information that's found, it were conducted the samples collection (accession) of cultivars and variants of *D. bulbifera*, with exploration survey methods. Samples were collected includes, tubers, roots, stems, leaves, flowers, fruits, and seeds. Morphological characterization performed to obtain a range of morphological variation of *D. bulbifera* population. Scoring data is done by IPGRI / IITA and CTCRI descriptors of *Dioscorea* crops, with binary and multistate techniques, and continued with the standardization of data into tables 0-1 (presence and absence). Clustering was done with UPGMA method or the average linked, and carried out with the help of soft ware NTSYS pc2.1, to form a dendrogram. Dendrogram illustrates intra-species classification, with cluster formation ad indicator, similarity coefficient values between 0.60 to 0.80 indicated that it's group under the species category. The results showed that based on morphological characters the population of *D. bulbifera* in Yogyakarta and surrounding areas had a lov morphological variability. This is shown also by the dendrogram with similarity index range between OTU's were 0.81, that's means having a low variability. *D. bulbifera* have anomositik stomata type, and amylum granules tuber starch with an oval form. Classification of intra-species of *D. bulbifera* cannot be done because it did not forming a specific cluster on the dendrogram.

Keywords: *Dioscore bulbifera*, genetic variability, morphological, intra-species classification

1. INTRODUCTION

Dioscorea bulbifera L. (Dioscoreaceae) is a plant species include to the section *Combilium* from the genus of *Dioscorea* that forms tubers (van Steenis, 1954), and local name in Java known as gembolo. Gembolo origin from geographic regions of Asia, and now split up in Africa through human cultivation (Burkill, 1935). Tubers used for food-based carbohydrates, starches derived from tubers processed into various types of food, and can be fermented into ethanol (Bimantoro, 1981; Wanasundera and Ravindran, 1994; Ariesta, 2004; Jayakodi *et al*., 2007;

Balakhrisant *et al*., 2007). Tuber of *D. bulbifera* is a food that is used by the local rural communities karsts ecosystems in the dry season (drought), for example in Gunung Kidul, Bantul and Kulon Progo (Purnomo, 2009). Morphological, physiological, and cytological species concept is drawn from population genetic morphologically uniform, have the same basic chromosome number, crosses individual fellow citizens are fertile species, and populations occupy a certain geographical area.

Adaptation of sub-populations of species population to ecosystems within the geographical area will form an ecological variant as ecotypes. Embodiment ecotypes in the study biosystematics can be as sub-species if related to geographic areas (*topodeme*), varieties when associated with a variety of habitats within the same climate (*ecodeme*), nor the forma, if variation morphology is plastic (plastodeme) (Stace, 1989; Singh , 1999). Likewise, Allopatric and sympatric speciation events may be the cause of new varieties within a species (Butlin *et al*., 2008). Crosses between individuals resident species can also bring a new variety (Hawkes, 1986, Harlan & de wett, 1986).

Varieties in the formal of a botanical classification known as sub-populations of species that are still diverse (Elkington, 1986), in contrast to cultivated varieties (cultivars) that have a high degree of similarity by cultivation. Classification of crops aimed to identify the direction of evolutionary variation, the main problem is not in line with the botanic classification, and the natural relationship between varieties and cultivars (Brandenburg, 1986; Parker, 1986; Lewis, 1986). Pure strains and clones are a technique to obtain a uniform population of cultivated plants with good results (Parker, 1986).

Cultivation requires genetic variability data of a species, intra-species classification (sub-species, varieties, forms, cultivars) as holding a cross and natural heritage, as well as classification crop on cultivars groups (Hasan *et al*., 2008) including crop of water yam (*Dioscorea alata* L.). The formation of cultivars groups are useful to predict the shape and the benefits of new cultivars that will be created at the will of the people.

Population of *D. bulbifera* in Yogyakarta and surrounding areas, showed morphological variation, especially in the form of tubers, tubers color, and leaves (Purnomo, 2009). On this species, found in karsts Society which often indicate the presence of morphological forms of cultivars. Thus, research on genetic variability and classification intra-species yam of *D. bulbifera* based on morphological characters important to be done to get cultivars and cultivar groups for cultivation, which also are important for conservation. This study has a novelty in terms of characterization and classification, to identify the genetic diversity D. bulbifera based on morphological characters in Yogyakarta and surrounding areas.

The study of genetic variability and intra-species classification of *D. bulbifera* was carried out with two objetives were to Identified the magnitude of genetic variability based on morphological characters, and to create intra-species classification of *D. bulbifera* also based on morphological characters, as a first step of cultivation in Yogyakarta and surrounding areas.

2. METHODS

Samples were collected from the region of Yogyakarta and surrounding areas include tubers, stems, leaves, and flowers. Tubers planted as a living collection at the experiment station are 13 accessions. Morphological characterization and scoring of each accession by following the instructions of the Center of Tuber Crop Research Institute (Anonymous, 1991) and International Plant Genetic Resources Institute (Anonymous, 1997). Data of morphological description of each accession of *D. bulbifera* were analyzed descriptively to obtain a range of genetic variability based on morphological characters and made a key to the identification of a group of *germplasms*.

Based on the data calculated morphological similarity scores between accessions (Otus) using Jaccard formula (Sokal and Sneath, 1963), to obtain a matrix of similarity between OTU's in the form of an accession number. Based on the similarity matrix between OTU's performed a cluster analysis using UPGMA method (Unweighted Pair-Group Method using arithmetic Average) to create a dendrogram with the help of soft ware Numerical Taxonomy and Multivariate Analysis System (NTSYSpc2.1) (Rohlf, 1993). Dendrogram will describe the cluster group germplasm, and rasionalisai formation of clusters based on similarity values, the expected grouping at a value below the rate of 0.75 (Sokal and Sneath, 1963; Davis and Heywood, 1972; Singh, 1999).

3. RESULT AND DISCUSSION

3.1. Morphological Variability of *D. bulbifera*

The identification was based on the 13 accession; 5 accessions from Gunung Kidul, 4 accessions from Bantul, 1 accession from Sleman, 2 accessions of Kulon Progo, and 1 accession of Purworejo all accessions is included in *Dioscorea bulbifera* L. species with local names gembolo or jebubuk. Morphological characters of D. esculenta from Yogyakarta and souronding areas were: Habit; herb or as geophyte plants, the vegetative period September to March, generative period in February-March, a period of tubers dormancy in the ground from April to August. Ground tubers in every single stem, round-shaped elongated with ends have 2-3 branches (lobes), the surface of the tuber roots rare to densely, light brown tuber skin to brown color, white to yellowish flesh tubers, Tubers slightly rough texture of the meat, 10-20 cm long, 10-15 cm wide, with a weight of 3-5 kg (natural conditions not cultivation). Growth directions of stems twining clockwise (Figure 1.a.), stem length of 5-8 meters, from the nodes 8-10 of stems forming aerial tubers (bulbils; aerial tubers) or jebubuk, spherical with diameter of 3-6 cm, dark brown tuber skin was equipped with light brown buds (Figure 1.b.). *D. bulbifera* have a single leaf, the leaf spread position, long blade and petiole nearly equal, cylindrical petiole, thicker in base, 9-12 cm long, smooth surface, green or reddish green color. Leaf blade heart shaped (cordate), 8-12 cm long, 7-11 cm wide, cordate base, entire margin, short tapered ends, leaf tape with hydathodes structure, leaf nervation curved structure, secondary venation is pinnate, veins (venation) is also in the arrangement of pinnate, light green leaf color. The male flowers are arranged in a compound spikes 15-20 cm long, each spike supports 3-10 flowers,3 sepals, 3 petals, stamens 6 (3 + 3). Female flowers on the observation period was not obtained. The specific morphological characters (traits) of *D. bulbifera* in the field is the formation of aerial tubers ball like shaped with

irregular buds, leaf shape cordate, and cylindrical stem with soft spines at the base of the leaf petiole.

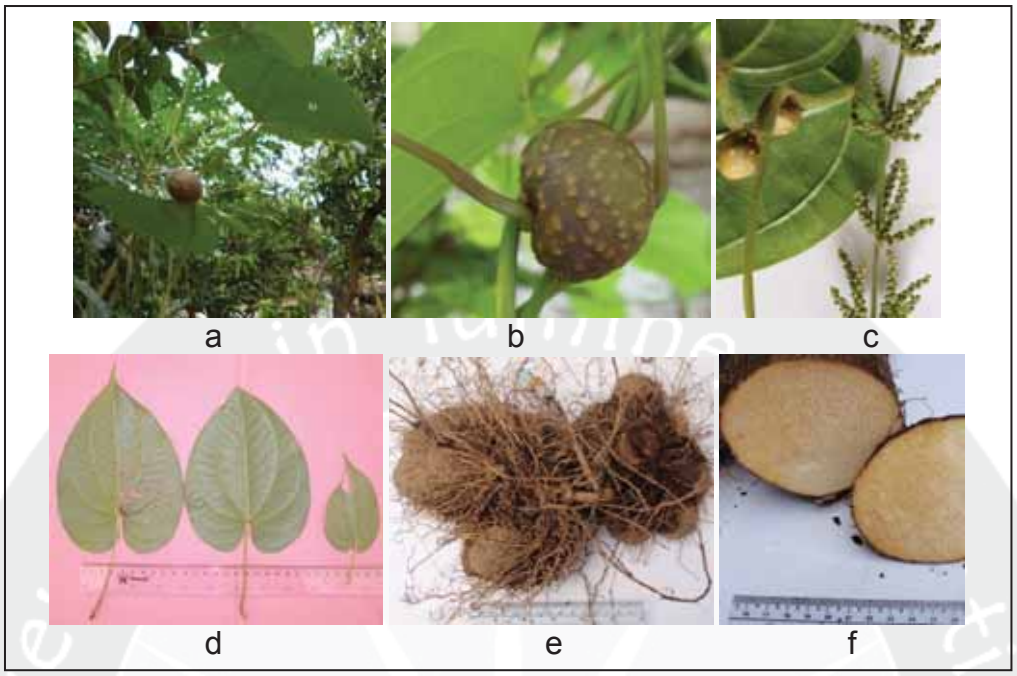

Figure 1. Morphological characters of *D. bulbifera* collected from Yogyakarta and souronding areas

Notes: a. Habit and *bulbil* (aerial tuber), b. *bulbil* position in stem node or axill of leaf, c. *Bulbil* and staminate flower, d. leaf shaves and their size variation, e. ground tuber, f. yellowish white tuber flesh color.

Based on observations of leaves micro-morphology *D. bulbifera* have epidermal derivate as trachoma with umbrella-shaped form, with single-celled feet, and a crown with 5-8 cells. anomositic stomata type with a 3- 4 cells (Figure 2.b.). The starch of the tuber (ground tubers) oval with a size of 0.5-1 .µm (Figure 2.c.).

The Leaves of *D. bulbifera* surface was smooth, almost equal to the leaf surface Uwi (*Dioscorea alata* L.). This is because the trichomes of both the Dioscorea species have the same structure, but the shape of starch of D. bulbifera and D. alata differ in size, resulting in relationships analysis based on morphological characters of both species are closely related, it is in line with the results of the study Purnomo (2012).

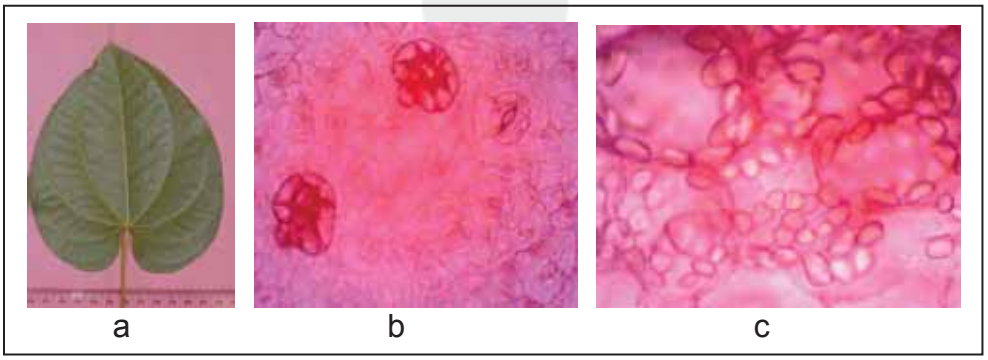

Figure 2. Micro-morphological characters of *D. Bulbifera*

Notes: a. leaf shape and size , b. tricomes and stomata, c. amilum in tuber fleh

D. bulbifera population in the region D.I. Yogyakarta and the surrounding area has rarely, it is because rural communities very few bulbs utilize land and air bulbs (bulbils) for foodstuffs, medicines, and others (Purnomo, 2009). To foodstuffs constrained premises bitter taste of boiled tubers, bulbs especially air. In the era of 1960 to 1970 are still prevalent tubers *D. bulbifera* used by rural people as food additives. Conditions of the population and usability are not developed in the community may be the cause of the erosion of population *D. bulbifera* in nature, leading to extinction. Thus, the utilization of carbohydrate-based innovation bulbs and secondary metabolites contained in *D. bulbifera* need to be assessed for the welfare and biological conservation.

In countries other than Indonesia utilization of the tuber *Dioscorea* spp. has grown rapidly. Nigeria (Africa) note that *D. bulbifera* utilized for carbohydrate-based food ingredients tubers (Sartie and Robert, 2011). Bulbs *Dioscorea* spp. containing glucose, maltose, and sorbitol (Balakhrisan *et al*., 2007). Starchy tuber *Dioscorea* spp. Maker is a basic ingredient of food, the nutritional value is similar to sweet potato or sweet potato (*Ipomoea batatas* L.) (Bressan *et al*., 2007). The molecular structure, composition and physico-chemical flour of *Dioscorea* spp. Sri Lanka is known is similar to sweet potato flour (Jayakodi *et al*., 2006). And informed by Gao *et al*., (2007) that *D. bulbifera* contains an active part in the anti-tumor constituents JB6 epidermal cells of mice.

Knowledge mentioned above illustrate that the real root gembolo material can be developed to be some products that are beneficial to human welfare. Based on this, the data necessary genetic variability and classification based on morphological characters intra-species if it is still possible to detect.

3.2. Genetic Variability and Intraspecies Classification of *D. bulbifera*

Samples were obtained in Yogyakarta Special Region and surrounding areas are 13 specimens, automatically this accession as an OTU's were compared in this study, considering the small frequency *D. bulbifera* that can be found in the research area. The OTU's *D. bulbifera* the data presented in Table 1 below.

Table 1. Accession number, origin accession, habitat description, and population of *D. bulbifera*

Accession number	Accessions origin	Local name in Yogyakarta and sourronding areas	Habitat and population
01/BIO/DB	Wonosadi, Gunungkidul	Jebubuk	Wonosadi forest,rare, in the shade of a tree and shrubs..
02/BIO/DB	Patuk, Gunungkidul	Jebubuk	On the cliff settlements, rarely, under an acacia tree.
03/BIO/DB	Karang Mojo, Gunungkidul	Jebubuk	On the cliff settlements, rarely, in the shade of bamboo.
04/BIO/DB	Tepus, Gunungkidul	Jebubuk/gembolo	On the cliff settlements, rarely, in the mahogany tree trunk.
05/BIO/DB	Panggang, Gunungkidul	Jebubuk/gembolo	On the cliff settlements, rarely, in the mahogany tree trunk.

Accession number	Accessions origin	Local name in Yogyakarta and sourronding areas	Habitat and population
06/BIO/DB	Sendang Sari, Bantul	Jebubuk/gembolo	On the cliff settlements, rarely, under an acacia tree.
07/BIO/DB	Sedayu, Bantul	Jebubuk/gembolo	On the cliff settlement, the banks of the River Progo, rarely, in the shrubs.
08/BIO/DB	Pundong, Bantul	Jebubuk/gembolo	On the cliff settlements, rarely, in the acacia tree trunk.
09/BIO/DB	Imogiri, Bantul	Jebubuk/gembolo	On the cliff settlements, rarely, in the acacia tree trunk.
10/BIO/DB	Kali Bawang, Kulon Progo	Jebubuk/gembolo	on a cliff settlements, rarely, on the banks of the river in shrubs.
11/BIO/DB	Kokap, Kulon Progo	Jebubuk/gembolo	On the cliff settlements, river cliff, rarely, in the acacia tree trunk.
12/BIO/DB	Kepuh Harjo, Sleman	Jebubuk/gembolo	On the cliff settlements, tebung river, rarely, in the acacia tree trunk.
13/BIO/DB	Purworejo	Jebubuk/gembolo	On the cliff settlements, rarely, in the acacia tree trunk.

The local name of *D. bulbifera* in Yogyakarta and surrounding areas were not varied, namely gembolo or jebubuk. The crop condition is as a wild type population of the remnants of the past cultivation, now occupies around settlements with sparse populations, and if converted at the rate of scarcity eroded condition (vulnerable).

Scoring was done on the morphological characters tubers, stems, and leaves. Flowers organ cannot be found on any accession continuous, so it cannot be compared, and will be presented as a description. Scoring is based on the instructions of the CTCRI (Anonymous, 1991) and IPGRI / ITTA (Anonymous, 1997). as *Dioscorea* spp. descriptors, because descriptors of *D. bulbifera* specifically yet. Scoring morphological character data was performed using binary and multistate. Scoring results after the standardization of data obtained the present and absent characters on 0-1 tables.

Based on the present and absent table calculated similarity index between OTU's use of the formula Jaccard similarity coefficient (Sokal and Sneath, 1963), to obtain morphological similarity coefficient, to obtain a matrix table of similarity between OTU's.

Based on the similarity matrix between OTU's, performed a cluster analysis with UPGMA clustering method (Unweighted Pair-Group Method using arithmetic Average) or Average Linkage with the help of software Numerical Taxonomy and Multivariate Analysis System (NTSYSpc2.1) (Rohlf, 1993), obtained dendrogram presented in Figure 3 below.

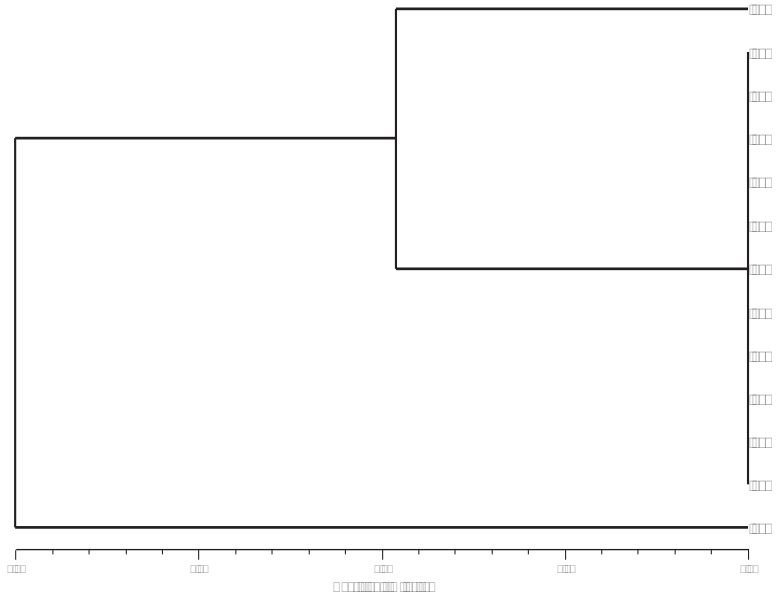

Figure 3. Dendrogram indicate the phonetic relationship between OTU's based on morphological similarity of *D. bulbifera* in Yogyakarta and surrounding areas

Accession of Purworejo (13) separates the general population on the similarity coefficient of 0.81, so it has desimilaritas 0.19. This is likely due to differences in the substrate. In accordance with the opinion of Green & Charity (2009) which states that different substrates can affect the character size bulbs. In the dendrogram also seen that the accession of Wonosadi Gunung (01) split with other populations on the similarity coefficient of 0.93 or 0.07 desimilaritas. Similarly to this accession likely because the substrate is clay. This is in accordance with the opinion of Jayakody *et al*., (2007) that the starch content of tubers affected by the condition of the substrate.

Separation of clusters or variation within the species, the similarity coefficient value above 0.80 is a phenomenon of low diversity. According to Singh (1999) and Stace (1986) stated that the variation above 0.80 the species had low diversity. Thus, the classification intraspesies D. bulbifera can not be done because of the similarity between accessions are very high, so it seemed obvious morphology (the difference is vague).

Accession No. 02, 03, 04, 05, 06, 07, 08, 09, 10, 11, 12 clustered on the similarity of 100%, this happens because the accession number is a clone. The origins of the accession of the region D.I. Yogyakarta, whereas plants dispersal were more dominant through tubers, thereby offspring phenotype nearly equal. In scoring the morphological characters of plants produce 100% similarity (similarity coefficient). Based on this it does not show clustering on dendrogram meaningful, except by the quantitative characters (size).

4. CONCLUSIONS

Vegetative morphological variation in *Dioscorea bulbifera* L. was identified include to the low category of Genetic variability. The population of *D. bulbifera* in Yogyakarta

and surrounding areas cannot be clustered in smaller groups by vegetative morphological characters.

5. ACKNOWLEDGEMENT

Thank you for Faculty of Biology Research Management which has provided funding of research through a research contract number UGM/BI/1885/M/05/01.

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Potentiality of Ketapang (*Terminalia catappa* L.) Leaf Extract as Antimicrobial Agent against Ice-ce Disease

Siti Luthfiyah[1], Isdiantoni[2], Maharani Pertiwi Koentjoro[3*], and Endry Nugroho Prasetyo[1]
[1]Biotechnology Laboratory, Department of Biology, Institut Teknologi Sepuluh Nopember, Gedung H 1st floor, Kampus ITS Keputih Sukolilo Surabaya, 60111 Indonesia
[2]Agrobusiness Department, Faculty of Agriculture, Universitas Wiraraja Jalan Raya Sumenep-Pamekasan Km. 5 Patean, Sumenep, 69451, Indonesia
[3]Laboratory of Environmental Microbiology, Department of Biological and Environmental Science, Faculty of Agriculture - Shizuoka University & Structural Biology Research Center, Inter-University Research Institute Corporation - High Energy Accelerator Research Organization (KEK), Tsukuba-Ibaraki, Japan
*Corresponding author email address: maharanipertiwi@yahoo.com

Abstract

Antimicrobial test of fallen leaf and young leaf of ketapang (*Terminalia catappa* L.) conducted on ice ice microbes on *Eucheuma cottonii* using disc diffusion method and dilution method(MIC). Extraction of fallen leaf and young leaf using maceration method with ethanol 96%. Antimicrobial test for ice-ice microbes was ed by the disc diffusion method using 100% concentration. Dilution method using MIC value from extract's concentrations of 0%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100%. The highest antimicrobial activity continued using clear zone method with carrageenan medium.The result of the study showed antibacterial activity against *V. alginolyticus* from fallen leaf extract that formed inhibiton zone of 11.65 mm as a largest diameter and classified as weak inhibitory response. Young leaf extract formed smaller inihibtion zone of 6.77 mm and classified has not inhibitory response. MIC values for fallen leaf extract is 60%. Confirmation test showed that the fallen leaf extract is able to inhibit ice-ice microbes though the extract was not strong enough toward ice-ice microbes on *E. cottonii* thalli.

Keywords: antimicrobial, *Eucheuma cottonii*, ice-ice, *Terminalia catappa* L.

1. INTRODUCTION

Indonesia is one of country which produce *Eucheuma cottonii* and ranked second in the world after Philippines (Sievanen *et al*., 2005). *E. cottonii* is one type of red seaweed which produced many of carrageenan (Soenardjo, 2011). Carrageenan is a group of gelling and thickening polysaccharide obtained from the extraction of certain species of red algae that is commonly used as a compactor, thickening and stabilizing agent, especially in food production (Necas and Bartosikova, 2013). However cultivation of *E. cottonii* currently experiencing a decrease in the quality and quantity because of the factors which inhibiting the growth of seaweed such as stress, then followed by the disease, especially ice-ice (Hurtado *et al*., 2006). Ice-ice disease is triggered by unfavorable ecological conditions in cultivation area such as changes in salinity, water temperature and light intensity, resulting stress. Stress makes *E. cottonii* easily infected with ice ice microbes such as *Vibrio alginolyticus* bacteria (Aris, 2011). Ice-ice microbes will depolymerized the carrageenan and

reduce seaweed's carragenan (Mendoza *et al*., 2009). Widiastuti (2009) states that ice-ice disease decrease the production of seaweed as big as 60% to 70%, even in areas of chronic till crop failure. The use of natural materials as antimicrobial agents become an alternative solution to handling ice-ice disease such as Ketapang (*Terminalia catappa* L.) (Hardhiko *et al* ., 2004). This study aims to utilize and the ketapang leaf extract (*T. catappa* L.) as an antimicrobial agent that is expected to obtain the proper formulation and practical for farmers to prevent and resist this endemic diseases.

2. METHODS

2.1. Sampling of *E. cottonii* and Environmental Measurement

Infected *E. cottoni* taken from the beach of Palasa village, Poteran Island, Sumenep district. Young *E. cottoni* taken as the sample (25 days after planting) and placed in plastic containing sea water. Then the samples were brought to the laboratory for ing. *Environmental parameter measured* in cultivation area such as pH, salinity, and currents.

2.2. Extraction of ketapang (*T. catappa* L.) leaf

This research used two types of ketapang leaf i.e. leaf and fallen leaf. Ketapang leaf washed and dried at room temperature. The dried leaf are cut into pieces and mashed into simplisia. Simplisia were stored in closed containers and not exposed to light. The simplisia soaked by ethanol for 3 days at room temperature for 72 hours then filtered. The filtrate was separated and leaf is soaked back into the new solvent, then maceration repeated as much as ± 2 times. The filtrate which obtained was concentrated in a rotary evaporator (40 ° C) (Ginting, 2008). Extracts made into 11 different concentrations (0%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100 %) with a sterile distilled water as solvent.

2.3. Antimicrobial Activity of Ketapang (*T. catappa* L.) Leaf Extract against Ice-ice Microbes

2.3.1. Disk Diffusion

V. alginolyticus bacteria obtained from The Development Center of Brackish Aquaculture (BBPBAP) Jepara. 0,1 ml bacterial suspension was inoculated on MH (Mueller Hinton) medium. Paper discs with a diameter of 10 mm was soaked in the extract concentration of 100% for 15 minutes, while using distilled water for negative control and chloramphenicol as positive control. Afterwards, paper disc placed on the surface of the MH medium and incubated at room temperature for 24 hours. The diameter of inhibition zone formed was measured with a ruler and classified as inhibitory response (Greenwood, 1995).

2.3.2 MIC (Microbial Inhibitory Concentration)

The type of extract with the largest inhibitory diameter in disk diffusion furthermore ed by MIC (dilution). 4.5 ml of medium TSB 2% poured into sterile tube. Then 0,5 ml fallen leaf extracts with varying concentrations added into each tube. 0.25 ml bacterial suspension was added (10^4) then mixed with vortex until homogeneous. Then incubated at room temperature for 24 hours (Boyd, 1995).

2.4 Confirmation test of anti ice-ice disease

The effectiveness of fallen leaf extracts using carrageenan medium consisted of 15 g of carrageenan in 1000 ml of sea water. *V. alginolyticus* suspension were inoculated in medium's wells (Hu *et al*., 2008). Furthermore, infected *E. cottonii* were soaked by fallen leaf extract and laid into medium's wells. Then the plates incubated at 30 ° C for 24 hours and rinsed with Lugol's iodine for 3-5 minutes. Effectiveness of extract proved with a small size of clear zone on the medium.

3. RESULTS AND DISCUSSION

3.1. Selection of infected *E. cottonii* by ice-ice disease

Infected *E. cottoni* taken from the beach of Palasa village, Poteran Island, Sumenep district on the coordinate point S 07°04'08.3" dan E 114°01'49.7". Figure 1 presents the infected *E. cottonii* characterized by **whitening** and softening of the **thalli.**

Figure 1. Infected *E. cottonii* thalli

E. cottoni which taken as the sample were young thalli aged 25 days after planted. Young thalli were more susceptible to ice-ice disease than older thalli, since younger talus has a thin layer of the epidermis and sensitive to environmental changes (Arisandi, 2011). The environmental condition data obtained in planting area when *E. cottonii* sampling shown in Table 1.

Table 1. *Environmental parameter of* Palasa Beach- Poteran Island

Parameter	Value	Optimum value
Tempature	28-30°C	27°C-30°C (Setiyanto, 2008)
pH	8,5 – 9	8-8.9 (Aslan ,1998)
Salinity	30-31‰	33-35‰ (Luxton, 1999)
Currents	0,04 m/s	0,2-0,4m/s (Indriani, 1991)

Based on data in Table 1 conditions of temperature, pH, salinity, and current is quite good and optimum for seaweed growth (Setiyanto, 2008; Aslan, 1998). However, salinity and currents classified as not optimum for seaweed (Luxton, 1999; Indriani, 1991). Strong water currents is able to preventing the pathogen invades the surface of seaweed (Largo *et al*., 1997).

According to correspondence in the Palasa village-Poteran Island, ice-ice disease usually occurs at the beginning of the rainy season, which is about December. Declining salinity conditions during the rainy season will increase the ice-ice disease

infection. Environmental stress will reduce the durability of seaweed and easily penetrated by ice-ice microbes (Fresco, 2004). At first the bacteria stick to the seaweed then begin its lytic activity and able to digest the cells of the epidermis, destroying the plastids, hold the pigment, so the thalus were bleached (Largo, 2002).

4.2 Antimicrobial activity test

Disc diffusion of ketapang leaf extract toward *V. alginolyticus* growth shown in Figure 2.

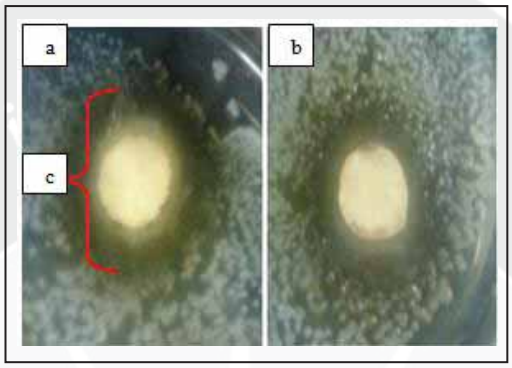

Figure 2. Disc diffusion of ketapang leaf extract toward V*. alginolyticus* growth (a. fallen leaf; b. young leaf; c. inhibition zone)

Inhibition zone of ketapang leaf extract against *V. alginolyticus* is shown in Table 2.

Table 2. Diameter of inhibition zone of ketapang leaf extract against *V. alginolyticus*

Type of extract	The diameter of inhibitory zone on a 24-hour incubation (in mm)				Inhibitory response
	1	2	3	Average	
Fallen leaf extract	13.67	11	10.3	11.6	Weak
Young leaf extract	6.66	7.33	6.33	6.77	None

In Table 2 the diameter of the largest inhibition zone produced by the fallen leaf extract with a diameter of 11.65 mm and classified as weak response. Young leaf extract with a diameter of 6.77 mm and did not have inhibitory response (Greenwood, 1995). The size of the inhibition zone in accordance with the effectiveness of antimicrobial agents (Okeke, 2011). The differences of inhibitory response between fallen leaf and young leaf cause by the differences in the compound of each leaf type. Based on research (Riasari *et al*., 2014) fallen leaf has the highest flavonoid content than the other leaf types in breadfruit (*Artocarpus altilis*). Flavonoid able to inhibit the function of the cytoplasmic membrane of bacteria by reducing the fluidity of cell membranes and damage the hydrogen bonds in nucleic acids thus inhibiting the synthesis of DNA and RNA (Cushnie and Lamb, 2005). The young leaf extract produce smaller inhibition zone than fallen leaf may be cause by the content of flavonoid. Ketapang young leaf which extracted with ethanol produce small inhibition zone against *V. alginolyticus* when compared with old leaf in the disc diffusion and MIC (Nadirah *et al*., 2013).

Antimicrobial test using the disc diffusion method showed that the type of extract that has the grea antimicrobial response was fallen leaf extract. Furthermore, fallen leaf extract was ed by dilution method using MIC (Minimum Inhibitory Concentration). MIC values will show the lowest concentration of an antibiotic or an agent for inhibiting the growth of microbes (Madigan *et al*., 2012). MIC values for each concentration shown in Table 3.

Table 3. MIC values of fallen leaf extract of ketapang (*T. catappa* L.) against *V. alginolyticus*

Extract concentration	∑ Bacterial colonies			Average
	1	2	3	
0%	>300	>300	>300	>300
10%	>300	>300	>300	>300
20%	>300	>300	>300	>300
30%	>300	>300	>300	>300
40%	>300	>300	>300	>300
50%	>300	>300	>300	>300
60%	3	9	9	7
70%	0	6	4	3.34
80%	0	1	2	3
90%	0	0	1	1
100%	0	0	1	1
kloramfenikol	1	4	0	1.67

: MIC (Minimum Inhibitory Concentration) value

Data in Table 3 shows huge number of *V. alginolyticus* in concentrations of 0%-50%. MIC values of fallen leaf extract against *V. alginolyticus* was 60% and continued to decline until the highest concentration. The higher concentration of extract resulted high antimicrobial substances in the solution, thus increase antimicrobial effectiveness (Vilas, 2011). Chloramphenicol used as positive control is bacteriostatic and high sensitivity to *V. alginolyticus* (Costinar *et al*., 2010).

4.1 Confirmation test of anti ice-ice disease

The result of antimicrobial activity test continued with confirmation test of anti ice-ice disease. Fallen leaf extract (60%) used in this test with carrageenan medium**.** Clear zone around culture indicate the production of certain enzymes (Devasia and Usha, 2012). According to Sie *et al.,* (2009) clear zone may be visible in 16-18 hours on *Alcaligenes* bacteria to degrade agar. The observation of clear zone is shown in Figure 3.

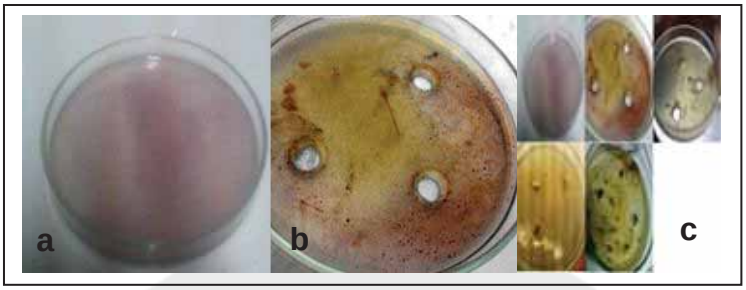

Figure 3. Clear zone by fallen leaf extract against ice-ice microbes on carrageenan medium. (a) carrageenan medium without microbes (control), (b) Fallen leaf extract (60%) against *V. alginolyticus*, (c) Infected *E. cottoni* which soaked by fallen leaf extract (60%)

Based on Figure 3. carrageenan medium without microbes (positive control) was dark purple after coloured by Lugol's iodine. The purple colour signifies that no occurrence of hydrolysis of starch (negative) so that there is no clear zone (Meurant, 1987). In Figure 3(b) indicated that fallen leaf extract inhibited the growth of *V. alginolyticus* evidenced by a dark color (purple) around the wells though the clear zone formed too in Figure 3(b). Araki (1999) states that *Vibrio* sp. that isolated from the surface of marine algae is able to producing *k*-carrageenase. Carrageenase is an enzyme that cuts internal bond (1-4) of carrageenan, forming a chain of neocarrabiose or neagaraobiose oligogalactan and belong to a group of 16 glycoside hydrolase (Liu and Chi, 2011). In Figure 3(c) *E. cottoni* which soaked with fallen leaf extracts formed clear zone, indicated that the extract was not strong enough toward ice-ice microbes on *E. cottonii* thalli. It was likely because of weak antimicrobial activity in MIC test.

4. CONCLUSIONS

The result of the study showed antibacterial activity against *V. alginolyticus* from fallen leaf extract that formed inhibiton zone of 11.65 mm as the largest diameter and classified as weak inhibitory response and 60% as MIC values. Young leaves extract of ketapang (*T. catappa* L.) does not have inhibitory response. Afterward, confirmation test showed that the fallen leaf extract is able to inhibit ice-ice microbes though the extract was not strong enough toward ice-ice microbes on *E. cottonii* thalli.

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Effect of Drying Methods on Hygiene Level and Seaweed (*Eucheuma cottonii*) Quality

Risanda Martalina Astuti[1], Maharani Pertiwi Koentjoro[2*], and Endry Nugroho Prasetyo[1]
[1]Biotechnology Laboratory, Department of Biology, Institut Teknologi Sepuluh Nopember, Gedung H 1st floor, Kampus ITS Keputih Sukolilo Surabaya, 60111 Indonesia
[2]Laboratory of Environmental Microbiology, Department of Biological and Environmental Science, Faculty of Agriculture - Shizuoka University & Structural Biology Research Center, Inter-University Research Institute Corporation - High Energy Accelerator Research Organization (KEK), Tsukuba-Ibaraki, Japan
*Corresponding author email address: maharanipertiwi@yahoo.com

Abstract

The effect of drying methods on hygiene level and seaweed, *Eucheuma cottonii*, quality has been studied. *E. cottonii* was dried using sunlight, solar heat, ultraviolet (UV), and oven. Hygiene level was analyzed by Total Plate Count and Most Probable Number. Moisture content measured based on seaweed biomass. Chlorophyll, total protein, vitamin C, vitamin A, and tannin content were quantified using UV-Vis Spectrophotometer. Whilst content of flavonoid, terpenoides, saponin, and carrageenan measured by percentage of residual weight to sample. UV dried resulted highest hygiene level (18,250 cfu/g and negative coliform). Phytochemical analysis also revealed that extract obtained from UV dried contain higher flavonoid (0.18 mg/g), saponin (0.52 mg/g), and terpenoides (0.05 mg/g), except in tannin where oven dried biomass is the highest (1.032 AU ±0.08). Oven dried showed greater chlorophyll, total protein (12.28 mg/ml ± 2.25), and vitamin A (1.848 AU ± 0.02) content, respectively, compared to others. Seaweed extracted from solar dried yield the biggest carrageenan (45.2%). Drying methods had no significant effect on vitamin C content. This result is expected can become reference as future plan for seaweed drying process.

Keywords: drying methods; Eucheuma cottonii; hygiene; phytochemical; quality

1. INTRODUCTION

Eucheuma cottonii is one of the largest species of seaweeds cultivated in Indonesia (Sedayu and Basmal, 2011). According to data from the Food and Agriculture Organization (FAO) in 2010, Indonesia is the largest exporter of *E. cottonii* (Cai, Hishamunda and Ridler, 2013). Dried seaweed dominates the export market as widely used as industrial raw materials (Zatnika and Istini, 1985).

Drying of certain crops is a very ancient and relatively easy technique for food preservation (Mathlouthi, 2003). Preservation of foods by drying is based on the concept of lowering the availability of water for the activity of microorganisms in food (Prabhakar and Mallika, 2014). Microbial contaminant levels that exceed the upper limit value is one of strong factor for the industry to refuse raw materials offered by farmers (Hernani, 2009). Thus drying can helps to conserve the desirable qualities by decreasing total microbial contaminant (Gupta *et al*., 2011).

Drying methods have different effects on microstructure and quality of dehydrated products (Gutierrez *et al*., 2008). Processing conditions, like high temperature, have great effect on retention of biological activities of biochemicals. It can cause partial or total damage of bioactive compounds due to breaking of vital bonds or initiation of side reactions (Hammed *et al*., 2013). Chan *et al*., (1997) reported that different drying methods have been found to affect the nutritional composition of the brown seaweed.

In Indonesia almost all seaweed dried under the sun (Neish, 2013). This type of drying is simple and cheap but generates poor quality of dried product (Mathlouthi, 2003). The risk of contamination during open drying also increases with the opportunity to grow in poorly designed driers as well as with the concentration of the microorganisms in the environment (Ramesh and Chakkaravarthi, 2013). Chan *et al*., (1997) showed that sun dried products have lower levels of ash, mineral, and vitamin C content compared to oven and freeze drying suggested that micronutrients are very sensitive to condition during process.

Consumption of food product containing pathogens such as bacteria and parasites or the food contaminated by bio-toxins can cause foodborne illnesses (Jahan, 2012). Besides, Harsojo *et al*., (2000) said that contamination of coliform bacteria can cause food poisoning to consumers. Studies are less available in literature which study of drying effect on the hygiene level and quality of seaweed including nutritional properties and phytochemical content constituents. This research conducted to analyze hygiene level and nutritional also phytochemical contents of *E, cottonii* in variety drying methods (sun drying, solar drying, uv-light radiation, and oven).

2. METHODS

2.1. Samples Preparation

Samples of *E. cottonii* were collected from Palasa Village, Poteran island, Madura, Indonesia (S: 07°04'08.3" dan E: 114°01'49.7") in January 2015. Fresh materials of *E. cottonii* were thoroughly washed with seawater ant their epiphytes removed. The cleaned samples were divided into four groups. One group was dried under direct sunlight, the second group was dried using solar dryer, the third group radiated using UV-light, and the last group was dried in a 60°C oven. All samples were dried until the moisture content reaches about 30%. The dried samples were ground using food processor to get seaweed powder and then stored in air-tight plastic bags at room temperature for further analysis.

2.2. Hygiene analysis

Hygiene level of the samples was analyzed using Most Probable Number and Total Plate Count Method (Harley and Prescott, 2002).

2.3. Quality determination

2.3.1. Moisture determination

Moisture content was analyzed by weighed the biomass of *E. cottonii* used analytical balance. Wet biomass determined as W_0 and dried biomass was determined as W_1. Then the percentage of moisture content in seaweed calculated with following formula.

$$Moisture(\%) = \frac{W_0 - W_1}{W_1} x\ 100$$

2.3.2. Determination of protein

2 g of *E. cottonii* powder were grinded in mortar with 5 mL of phosphate buffer (pH 7). The extract was transferred into centrifuge tube then phosphate buffer (pH 7) was added to 10 mL. The homogenate was centrifuged at 8000 rpm for 20 minutes. After extraction, 0.1 mL of different samples were taken out in separate test tubes. 5 mL of protein reagent (Coomasie Brilliant Blue G-250) were added to the test tube and the contents mixed by vortexing. The absorbance at 595 nm was measured after 2 min and before 1 h in cuvettes against a reagent blank. A standard curve was made using Bovine Serum Albumin in varies concentrations. Protein content in the extracted samples was determined from the standard curve and the amount of protein in mg/ml was calculated (Bradford, 1976; Pandey and Budhathoki, 2007).

2.3.3. Determination of vitamin C

2.5 g of *E. cottonii* coarse powder and 2 mL glacial acetic acid was mixed. The mixture was stirred about 20 min and rapidly filtrate. The volume of sample is made up to 10 ml with distilled water. Then 50 µL of 0.4 $mmoll^{-1}$ Methylene Blue solution and diluted up to 10 mL with distilled water. Decrease of absorption was measured at λ_{max} = 665 nm. Results are expressed in mg of ascorbic acid per 100 g of dry sample (Tahirovic *et al*., 2012).

2.3.4. Determination of β-carotene

A representative portion of *E. cottonii* powder sample (1 g) was weighed in a test tube. Then 5 mL of chilled acetone was added and the tube was held for 15 min with occasional shaking at 4 ± 1°C, vortexed at high speed for 10 min, and centrifuged at 1370 × g for 10 min. Supernatant was collected into a separate test tube and the compound was re-extracted with 5 mL of an acetone followed by centrifugation once again as above. Both of supernatant were pooled together and then passed through the Whatman filter paper No. 42. The absorbance of the extract was determined at 49 nm wavelength in UV-Vis 10 Genesys spectrophotometer (Biswas *et al*., 2011).

2.3.5. Determination of carrageenan

10 g of dried material was washed with tap water to remove sand and salt and then incubated in 6% KOH solution in an 80°C water bath for 3 h. Ratio between samples and solution is 1:3 (g/mL). The samples were washed overnight in slowly tap water. The samples were then stirred tightly in 1 L of distilled water and boiled for at least 1 h. Carrageenan was precipitated in ethanol with the ratio between filtrate and solution is 1:3. Precipitates carrageenan was then soaked in chloroform and dried at room temperature for 2 days.

2.3.6. Phytochemical determinations

2.3.6.1. Determination of flavonoids

5 g of *E. cottonii* powder was extracted repeatedly with 50 mL of 80% aqueous methanol at room temperature. The whole solution was filtered through Whatman filter paper No. 42 (125 mm). The filtrate then evaporated into dryness over a water bath and weighed to a constant weight (Biradar and Rachetti, 2013).

2.3.6.2. Determination of saponins

5 g of samples powder was put into conical flask and 25 mL of 20% aqueous ethanol were added. The samples were heated over a hot water bath for 3 h with continuous stirring at about 55°C. The mixture was filtered and the residue re-extracted with another 50 mL 20% ethanol. The combined extracts were reduced to 20 mL over water bath at about 90°C. The concentrate was transferred into a 250 mL separating funnel and 5 mL of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. Purification process was repeated. 30 mL of n-butanol was added. The combined n-butanol extracts were washed twice with 5 mL of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation, the sample were dried in the oven to a constant weight (Biradar and Rachetti, 2013).

2.3.6.3. Determination of terpenoids

5 g of seaweed powder were taken separately and macerated in alcohol for 24 h then filtered. The filtrate was extracted with petroleum ether. Then the ether extract was treated as total terpenoids (Biradar and Rachetti, 2013).

2.3.6.4. Determination of tannins

500 mg of the sample was weighed into 50 mL plastic bottle then distilled water was added and shaken for 1 h in a mechanical shaker. This was filtered into volumetric flask then 5 ml of the filtrate was pipette out into a test tube and then mixed with 2 mL of 0.1 M $FeCl_3$ in 01 N HCL and 0.008 M potassium ferricyanide. The absorbance was measured at 700 nm within 10 minutes using UV-Vis 10 Genesys spectrophotometer.

3. RESULTS AND DISCUSSION

3.1. Hygiene Level of Dried *E. cottonii*

Food products are the primarily vehicle responsible for the transmission of microbial disease of the gastrointestinal system (Harley and Prescott, 2002). For this reason, it needs to be examined by standard microbiology test including most probable number and total plate count. The microbial counts of dried *E. cottonii* from each method stored at ambient temperature are presented in Table 1.

Table 1. Total enumeration of bacteria from MPN and TPC test in each dried E. cottonii samples

Drying method	TPC (cfu[a]/g)	MPN index/100 ml	Confirmed test[b]
Sun drying	137,750	350	+
Solar drying	56,500	17	+
UV radiation	29,000	7	-
Oven drying	105,500	180	+

[a] colony forming unit
[b] (+) = positive coliform
(–) = negative coliform

Data from Table 1 shows that drying exerted UV radiation has the lowest microbial contaminated *E. cottonii* compared to others (29,000 cfu/g). Despite the highest microbial contaminated *E. cottonii* showed by sun drying method which reach 137,750 cfu/g. Furthermore, the result showed that UV radiation method contains no coliform after confirmed test done, based on MPN index for five tubes series. Contrary, another three drying methods indicated coliform containing in samples. This observation agrees with the study of Wakjira (2010), which mentioned that the product from sun drying is often unhygienic as a result of microorganisms and insect such as flies. UV Radiation is divided into three segments: UV-A, UV-B, and UV-C. The UV-C (λ = 200 – 280 nm) has lethal germicidal effect and has long been used as an effective method to disinfect water and to decontaminate surfaces and packaging in the food industry (Turtoi, 2013). It has been reported that UV-C damages microbial DNA. UV-C radiation from 05 – 20 kJ/m^2 inhibited microbial growth by inducing the formation of pyrimidine dimers which alter the DNA helix and block microbial cell replication (Lado and Yousef, 2002; Ribeiro *et al*., 2012).

3.2. Quality of Dried *E. cottonii*

3.2.1. Chlorophyll

Chlorophyll level in dried *E. cottonii* from various dehydrated method are summarized in Figure 1.

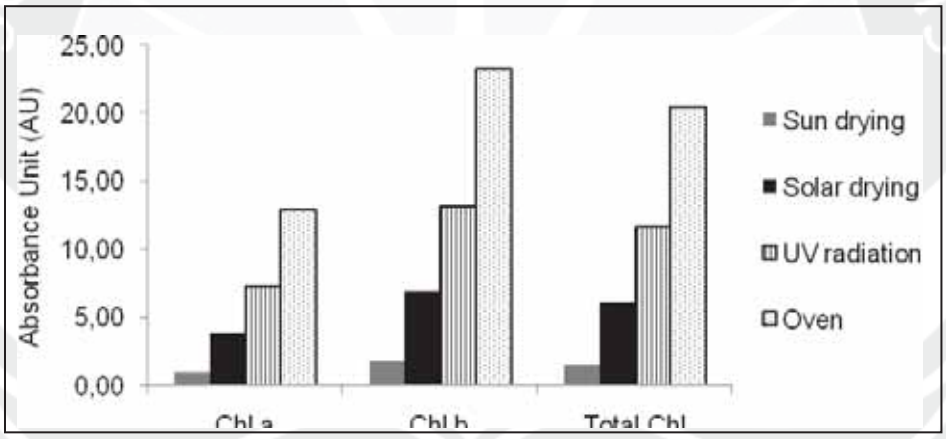

Figure 1. Chlorophyll content in E.cottonii from varied drying method

Processing of any kind will affect chlorophyll content in plant. The level of chlorophyll *a* (12.9 AU ± 082), chlorophyll *b* (23.27 AU ± 1.47), and total chlorophyll (20.52 AU ± 1.30) was found to be higher in oven dried *E. cottonii*. Meanwhile, the lower level of chlorophyll content indicated by sun drying method with amount of chlorophyll *a* is 0.98 AU ± 1.3, chlorophyll *b* 1.77 AU ± 0.35 and total chlorophyll 1.56 AU ± 0.31. Chlorophylls are known to be easily degraded by conditions such as diluted acids, heat, light, oxygen (Tonucci and Von Elbe, 1992). Thermal processing induces structural and chemical variations to the tissue of vegetables that often result in color changes (). The reason for the green color is the formation of pheophytins during heating that cause the conversion of chlorophylls to pheophytins (Erge *et al*., 2008).

3.2.2. Protein

The influence of drying methods on protein content presented in Figure 2. Oven dried *E. cottonii* preserved the most protein compared to the other drying methods. An amount 12.28 mg/ml ±1.908of protein was found in oven dried seaweed powder. Whilst *E. cottonii* in solar drying had the smallest amount of protein (4.27 mg/ml ±0.696). The heating cause denaturation of protein cells leading to loss of protein due to weakening of three-dimensional conformation of the protein cells (Boateng, 2013).

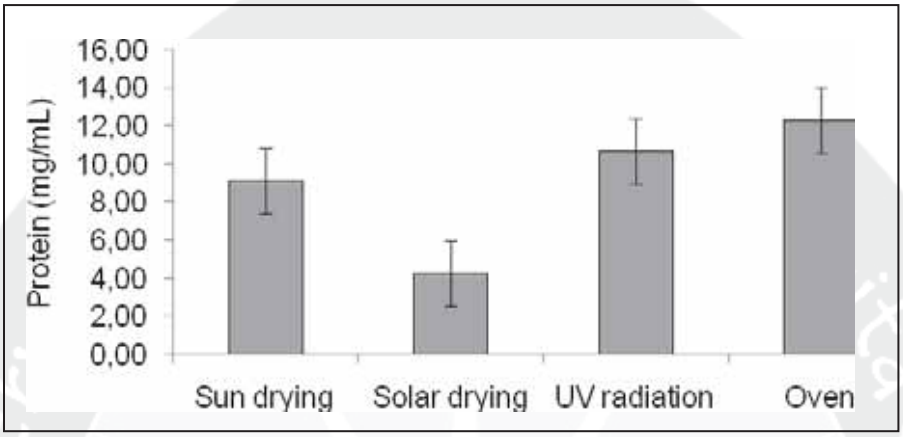

Figure 2 Total protein content in dried E. cottonii from each methol

3.2.3. Vitamin C (Ascorbic Acid)

Spectrophotometric methods use in this study involves reduction of colored methylene blue (MB^+) dye by ascorbic acid where as a result of the reaction colorless leucomethylene blue (CMB^+) product is formed (Mowry and Orgen, 1999).The reaction system is a basis for indirect spectrophotometric determination of AA when added in increasing amount, consume MB^+ and decreasing the concentration of MB^+ (Tahirovic *et al*., 2012). Figure 3 shows the proximate ascorbic acid in *E. cottonii* from various drying method.

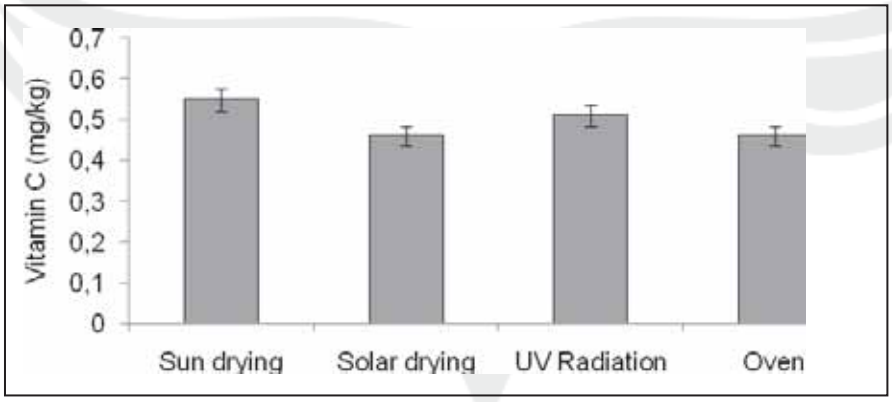

Figure 3. Ascorbic acid content in each E. cottonii from varied drying method

The result obtained from sun dried *E cottoniii* shows the highest ascorbic acid content with an amount 0.55 mg/kg ±0.011 mg/kg followed by UV radiated *E. cottonii* which is 0.51 mg/kg ±0.022, while the smallest ascorbic acid content found in solar dried *E. cottonii* as much as in oven dried *E. cottonii* that is 0.46 mg/kg ±0,004.

However, various drying method do not indicate significant effect on ascorbic acid content in *E. cottonii*. These biological material are simply dehydrated in forced air dryers heated by solar energy (Santos and Silva, 2008).

Vitamin C is also known as ascorbic acid is a heat-sensitive bioactive compound in the presence of oxygen and gets degraded by oxidative processes, which are stimulated in the presence of light, oxygen, and enzymes like ascorbate oxidase and peroxidase (Alothman *et al*., 2009). Moreover, during the drying process, ascorbic acid degradation was found to be moisture and temperature dependent (Santos and Silva, 2008).

3.2.4. β-carotene

Vegetables were analyzed for their beta carotene content because it was the precursor of vitamin A (Benedich & Higdon, 2004), including seaweed. Absorbance at λ = 449 nm is a representation of total β-carotene content in dried *E. cottonii* measured using UV-Vis 10 Genesys spectrophotometer. The influence of various drying method shown in figure 4.

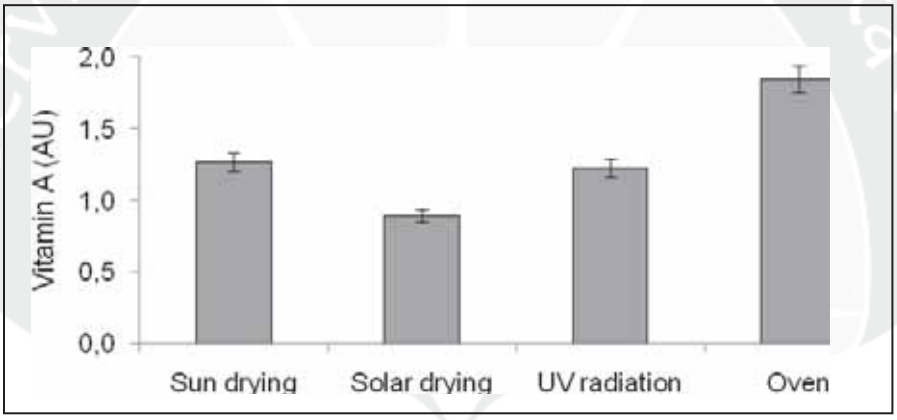

Figure 4 Total β-carotene content in various drying method in AU[1]

The β-carotene content varied from trace amount in sun dried, solar dried, UV radiated, and oven dried *E. cottonii*. From the data it was evident that oven dried *E. cottonii* contains the highest amount of β-carotene (1.848 AU ±0.02) compared to others method. *E. cottonii* with significant level of β-carotene after oven dried is sun dried (1.3 AU ± 0.25). Solar dried *E. cottonii* has the lowest mean amount of β-carotene (0.894 AU ± 0.09). However, solar drying caused the most loss indicating a probable greater heat exposure of the drying seaweed under this method. According to Penicaud *et al*., (2011), β-carotene is a very reactive compound due to its highly unsaturated structure, especially at high temperature, and oxidation, due to the occurrence of oxygen in food.

3.2.5. Carrageenan

The result of carrageenan yielded from *E. cottonii* under the four drying methods are summarized in Figure 5.

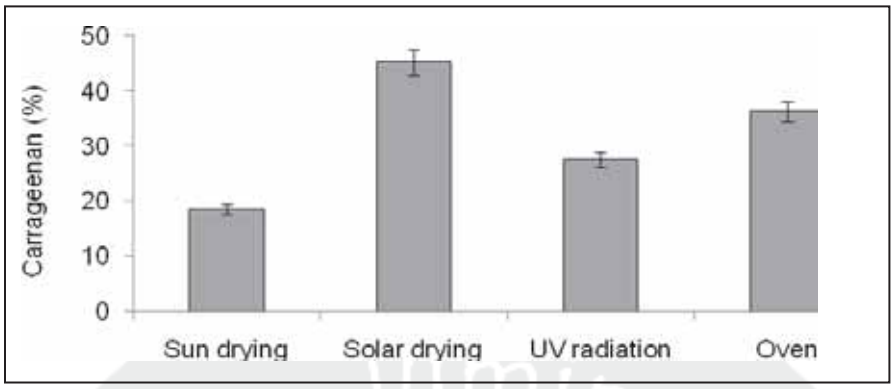

Figure 5 Total carrageenan yielded from varied drying method

Samples obtained from solar dryer has the highest amount of carrageenan yield which is 45.2% followed by oven dried *E. cottonii* (36.31%). Meanwhile, samples from sun dryer has the lowest carrageenan yield with total amount only 18.45%. Similar observation have been reported earlier by Ekman and Peterson (1990) that the photon irradiance and day length seriously alter the carbohydrate yield and gel strength in *Gracilaria sordida* and *Gracilaria verrucosa* (Eswaran and Rao, 2001). *E. cottonii* is usually dried under open air over 5 days; however, this process is totally dependent on weather conditions such as temperature, relative humidity, and the velocity of the air. The seaweed can be damaged compromising its phycocolloid quality because those factors can't be controlled in open sun drying (de Faria *et al*., 2014).

3.2.6. Phytochemical content

The phytochemical constituent extracted from *E. cottonii* samples are summarized in Figure 6 and 7.

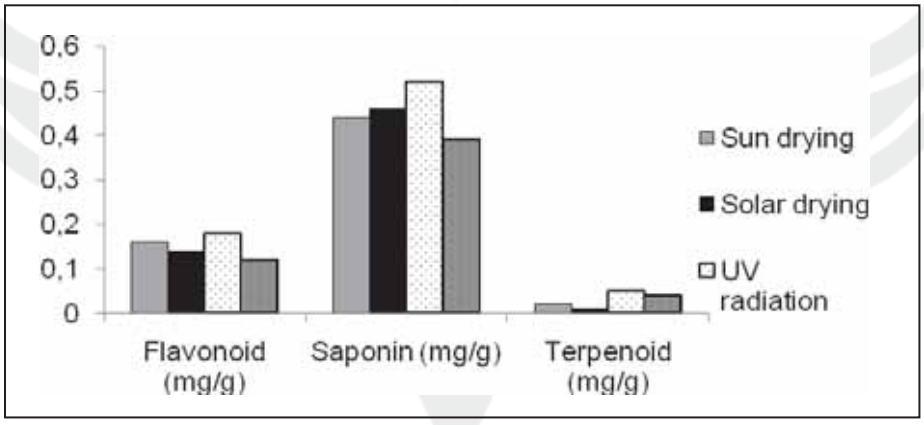

Figure 6. Total flavonoid, saponin, and terpenoid content from varied drying method

It was observed that *E. cottonii* powder gained from UV radiation drying process retain highest amount of flavonoid (0.18 mg/g), saponin (0.52 mg/g), and terpenoid (0.05 mg/g). This finding agreed with previous finding on extracts of flavonoid content from fresh-cut tropical fruits. It has been reported that the longer UV treatment time, greater was the increase in the overall flavonoid content (Alothman *et al*., 2009). UV

light induces both biological stress and defense mechanisms of plant tissues. These inducible effects include the accumulation of antimicrobial compounds, cell wall modification, and an increase in the activity of defense enzymes, also increased antioxidant activity (Mercier, 1997).

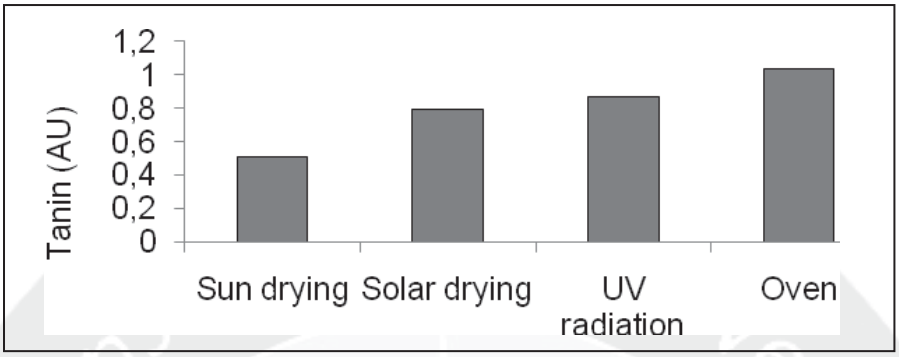

Figure 7. Total tannins content from each varied drying methods

Contrarily, the levels of tannins were higher in the oven dried *E. cottonii* powder than those of solar, sun, and UV dried powder. Study done by Rwubatse *et al*., (2014) reported that oven dried orange peel flour has higher tannin content than solar and sun dried. This may cause by the absence of sunlight and oxygen in the oven which lead to preserve the formation of tannin. According to Schieber *et al*., (2001), the thermal processing can affect the phytochemicals by thermal breakdown that affect the integrity of the cell structure which then results in the migration of components, leading to losses by leakage or breakdown by various chemical reactions involving enzymes, light, and oxygen.

4. CONCLUSIONS

The result obtained in this study showed that, drying had effect on the hygiene level and quality of *E. cottonii*. UV radiation was observed to generate relatively better products in term of hygiene level compared to other drying methods. Moreover, UV radiation also showed the highest level of phytochemical content except for tannin. This because UV light is inducible for antimicrobial activity. Besides, chlorophyll, protein, and β-carotene level was found to be the greatest amount in oven dried *E. cottonii*. In contrary, there is no significant effect in vitamin C level from varied drying methods.

5. ACKNOWLEDGEMENT

The authors are thankful for the academic grant provided by Biomaterial and Enzyme Technology Research Group (2014/2015) supervised by Dr. techn. Endry Nugroho Prasetyo, M.T. in which this study was conducted. Author would also like to thanks to Wirdhatul Muslihatin, S.Si and Nur Hidayatul Alami, S.Si, for comments and constructive suggestion provided for improving the manuscript; Isdiantoni, S. P., M. P. for assistance and technical support in Palasa village, Poteran island, Madura, Indonesia. Special thanks are also given to member of Biomaterial and Enzyme Technology Research Team (2014/2015) for assistance and support which can't mentioned one by one here.

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Acute Lung Toxicity of Juice and Soup of Katuk (*Sauropus Androgynus*) Leaves as Breastmilkbooster Related to Bronchiolitis Obliterans

Amelia Lorensia[1], Oeke Yunita[2], Andreas Kharismawan[3], Cindy Edelweis[3]
[1]Departement of Clinical Pharmacy-Community, Pharmacy Faculty, Surabaya of University, Surabaya, Indonesia
[2]Departement of Pharmaceutical Botany, Pharmacy Faculty, Surabaya of University, Surabaya, Indonesia
[3] Student of Pharmacy Faculty, Surabaya of University, Surabaya, Indonesia
amelia.lorensia@gmail.com

Abstract

Sauropus androgynus (SA) (katuk) as a traditional medicine to supplement breast milk production, has been widely used by the people of Indonesia to improve and accelerate the production of breast milk (air susu ibu/ASI). However based on some research said katuk suspected to cause constrictive bronchiolitis obliterans because of toxin exposure. Some adverse events katuk leaves in some countries could reduce the sale value of katuk, either as food or food supplements in Indonesia, if not further investigated. Indonesian society can consume leaves katuk direct way in the form of salad or soup can be cooked in advance. Therefore in this study will be conducted in vivo toxicity tests on the leaves katuk originating from East Java, Indonesia, is given in the form of vegetable juice and stew katuk orally, in Wistar females.SA extract was separated into eight parts, namely 500, 1000, 3000, and 5000 mg/kg for jus and soup groups. After 14 days, each rat was observes in macroscopic and microscopic of the lung. Then, It analyzed with ANOVA. The administration of juice and soup of leaves SA don't cause changes in the physical condition of the rats. And katuk (Sauropus androgynus) leaf juice cause significant differences in the results of macroscopic observation that lung volume as well as the results of microscopic observation that the bronchial lumen ratio between treatment groups katuk leaf juice 5000 mg / kg and negative controls. Katuk leaf soup (Sauropus androgynus) causes significant variations in the macroscopic conditions (lung volume) and microscopic (the size of the bronchial lumen) in female Wistar rats. Our results indicate that the toxic necrosis of SA is dose-independent. More evidence is needed to clarify the incidence of necrosis in chronic used.

Keywords: Acute toxicity, *Sauropus androgenus*, lung histopathology

1. INTRODUCTION

Sauropus androgynus (SA) (katuk) has been widely used by the people of Indonesia to improve and accelerate the production of breast milk (air susu ibu/ ASI). Where ASI is useful for the growth and development of the baby, because it contains many growth factors and can boost the immune system because it contains many antibodies (Hapsari, 2000; Playford *et al.*, 2000). Katuk leaf infusion can increase milk production in mice, but the extract ether and ether-petroleum fractions did not

show an increase in milk production significantly. Supplement products containing katuk leaves have many in the market, which is intended to increase milk production (Sa'roni *et al.*, 2004; Azis and Muktiningsih, 2006).

But since 1994 has been raised as a result of side effects on the respiratory katuk consumption in Taiwan for a long time and without the rules of use, for the purpose of body slimming. The whole treatment kontroversional done, either steroids or bronchodilators does not give effective results to overcome these side effects. Even some of the patients died and the patients were still alive, there has been a chronic respiratory failure That is irreversible and require a transplant to overcome these side effects (Lin *et al.*, 1996; Chang *et al.*, 1998). A similar incident also raised in Japan, which was reported byOonakahara *et al.,* (2005).Katuk can cause constrictive bronchiolitis obliterans (CBO), the syndrome is fatal respiratory failure due to exposure to toxins. Lung biopsy of some patients who died showed that indicate obstruction of the bronchioles bronchiolitis obliterans (Lai *et al.*, 1997; Chang *et al.*, 1998). Bronchiolitis obliterans is a pulmonary obstructive disease caused by persistent inflammation in the bronchioles which lead to the proliferation of fibroblasts, resulting in obstruction of the lumen of bronchioles and can be demonstrated by several kinds of tests such as pulmonary function test (PFT) and high-resolution computed tomography (HRCT). Bronchiolitis obliterans is characterized by obstruction of the bronchioles, bronchiectasis, water-trapping and decrease in diffusion capacity(Laohaburanakit *et al.*, 2003). Histopathological changes in patients with bronchiolitis obliterans is the presence of inflammation, necrosis and fibrosis of the bronchioles (Wang *et al.*, 2000). Necrosis is cell death due to extreme stimuli from the environment that is characterized by swelling of the cytoplasm, decreased cell integrity and cell lysis. In the necrotic area are fibrosis and shrinkage of cartilage, fibroblasts and smooth muscle cells. Cell death can also be caused by programmed cell death (apoptosis) due to morphological and biochemical changes. In pathological conditions, apoptosis and necrosis often occur together (Yu *et al.*, 2007).

The severity of bronchiolitis obliterans due to consumption katuk leaves is influenced by several factors such as the amount, duration and manner of consumption leaves katuk (Oonakahara *et al.*, 2005; Yu *et al.*, 2007). On average, patients consumed more than 150 g leaf fresh katuk every day and breathing problems after 4-12 months (Oonakahara *et al.*, 2005; Yu *et al.*, 2007).Most patients taking katuk leaves in the form of juice. Only a small proportion of patients who consume the leaves by boiling prior katuk (Hsiue *et al.*, 1998; Yu *et al.*, 2007). Leaf consumption katuk way affect the prevalence of onset of bronchiolitis obliterans. Consumption katuk leaves with cooked first known to decrease the prevalence of bronchiolitis obliterans (Ger *et al.*, 1997). Katuk leaf juice consumption due to the long-term, at least 300 patients in Taiwan and eight patients in Japan experienced bronchiolitis obliterans (Sawahata *et al.*, 2010).

Some adverse events katuk leaves in some countries could reduce the sale value of katuk, either as food or food supplements in Indonesia, if not further investigated. Indonesian society can consume leaves katuk direct way in the form of salad or soup can be cooked in advance. In addition to salad and soups made, leaves katuk also been consumed in the form of dosage extract(Sa'roni *et al.*, 2004).Therefore in this study will be conducted in vivo toxicity tests on the leaves katuk originating from East

Java, Indonesia, is given in the form of vegetable juice and stew katuk orally, in Wistar females.

2. METHOD

This research is an experimental laboratory that tested the acute toxicity of juice and soup of the katuk leaves in female rats wistar strain. Observable toxic effects of changes in the physical condition of rats as well as changes in the macroscopic and microscopic lung conditions. Animals used in this study were female, because most consumers who consume katuk leaves is women, who are often used to facilitate breastfeeding. Wistar female rats were healthy and sexually mature weighing 150-200 g, aged 6-8 weeks, were obtained from Pusvetma (Pusat Veterinaria Farma, Surabaya).

2.2 Procedure

2.2.1 Preparation of Katuk Extract

Katuk leaves obtained from the TOGA garden, Faculty of Pharmacy, University of Surabaya, Surabaya, East Java, Indonesia. Katuk leaves used are katuk leaves dark green stalk and all the leaves (petiolus). Samples leaves cleared of impurity particles with water, dried at room temperature without heating so as not to damage the metabolites in the leaves.Then leaves katuk water content removed by freeze dry method. Samples katuk leaves dark green, further prepared into vegetable juice and vegetable stew (food processing).

2.2.2 Preparation of Animal experiments

Female rats chosen by reason of leaf consumption katuk leaves been done by women that is as facilitating breastfeeding. Also note that female rats are more sensitive to toxicity compared to male rats. Animal ages between 8-12 weeks with a weight variation of no more than 20% (OECD, 2001).Experimental animals are conditioned for at least seven days prior to treatment (DG POM, 1991). The whole mice were fed pelleted starter and drink ad libitum during the adaptation period. Maintenance space has an optimal temperature 22 ± 3□C and relative humidity 30-70% with 12-hour lighting cycle of light and 12 hours dark. The physical condition of the experimental animals include covering body weight, presence / absence of hair loss, eye clarity, presence / absence of mucus in the nose, presence / absence of diarrhea and motor activity was observed. After normal ascertained, rats were fasted overnight (18 hours) before administration of treatment (OECD, 2001).

2.2.3 Preparation of Experimental Animals for Katuk Leaves Juice dan Soup

Experimental animals were randomly divided into ten groups:

Negative control : negative control group
J_{500} : katuk leaves juice 500 mg/kg group
J_{1000} : katuk leaves juice 1000 mg/kg group
J_{3000} : katuk leaves juice 3000 mg/kg group
J_{5000} : katuk leaves juice 5000 mg/kg group
S_{500} : katuk leaves soup 500 mg/kg group
S_{1000} : katuk leaves soup 1000 mg/kg group
S_{3000} : katuk leaves soup 3000 mg/kg group
S_{5000} : katuk leaves soup 5000 mg/kg group

with each group consisting of three rats. Each animal in each group was given a dosage katuk leaf juice orally one time and then observed for 14 days (OECD, 2001). Administration of the test substance should correspond to the expected route of administration to humans. Test substances that can not be given once and must be divided into several times of administration, time of administration should not be more than 24 hours. Giving done orally using oral sonde. Test animals were fasted from food overnight (18 hours) before administration of the test material. Experimental animals were fed again after 3-4 hours of administration of katuk extract juice/soup (DirJen POM, 1991; OECD, 2001).

2.2.4 Physical observation

After 30 minutes of administration of the test preparation, test animals were observed carefully for the presence of toxic symptoms and death. Special attention is given during the first four hours after administration and periodically observed every hour on the first day. Furthermore, observations were made every day until day 14 to determine the beginning, intensity and duration of symptoms occurring toxic (DirJen POM, 1991; OECD 2001).

2.2.5 Macroscopic Observation

Macroscopic observation conducted to observe the presence/ absence of morphological changes in the organs. Pulmonary morphological observations include the presence/ absence of discoloration and lesions on the surface of the lung. Weighing lung organ is done in an indirect way by using the measuring cup, by weighing each organ in NaCl 0.9% solution. Immediately after macroscopic observation, the lungs were fixed in 10% buffered formalin. Furthermore, the procedure of making preparations and tissue staining using *Hematoksilin-Eosin*.

2.2.6 Microscopic Observation

Coloring process and making preparations for the lung tissue performed at the Faculty of Science and Technology University of Airlangga, Surabaya, East Java. Microscopic observation was done by measuring the ratio of the diameter of the bronchial lumen using a light microscope at 100x magnification and ocular micrometer. Diameter is the average diameter of the vertical and horizontal measurements. Observations were made on the field of view for each of the three preparations were then averaged. Bronchial lumen ratio is obtained by comparing the bronchial lumen diameter with diameter bronchioles and set in terms of percent (%).

2.2.7 Data Analysis

Analysis data used in this study is the method of one-way ANOVA was used for normally distributed data analysis and homogeneous while the data were not normally distributed and normally distributed data but not homogeneous analyzed by Kruskal-Wallis test. $p < 0.05$ indicates significant differences.

3. RESULT AND DISCUSSION

3.1. Observation results of Lung Macroscopic in Katuk Extrak Juice Intervention

Morphological observation of lung, lung weight, and lung volume throughout the experimental animals were observed. Did not reveal any morphological changes in

the lung. By Kruskal-Wallis test, known weights lung test group did not experience a significant difference (p (0.274)> 0.05) as compared to the negative control group. Lung volumes while the test group was significantly different (p (0.025) <0.05) with the negative control group. Results of lung volume measurements showed a downward trend with increasing dose proportional katuk leaf powder.

In this study found no lesions on the surface of the lung. Damage, discoloration or pleural surface morphological changes can indicate the presence of structural damage(Greaves, 2012). Determination of the presence / absence of lesions in the lungs can cause bias in the interpretation of research results as the observation presence/ absence of lesions is very subjective. Moreover, determination of lesion-related information must also be supported by the diagnosis, distribution, and severity of the pathological condition should therefore be made to ensure the histological observation of pathological changes that occur (Dua dan Jackson, 1988).

Lung weighing results showed no significant difference between the negative control group to the treatment group. The big difference in organ weights may be indicative of toxic effects on the organs involved in this case lung organ (Greaves, 2012). Generally, the proportion of lung weight is directly proportional to the size and weight of experimental animals (Suckow *et al.*, 2006; Greaves, 2012). Although it does not show significant differences, it can not be concluded that there are no toxic effects arising from treatment. Past studies have concluded that the weighing of organs is not recommended to be taken into consideration in the acute toxicity test because the weight of the new organ will be affected if it continues to be exposed to toxic substances for at least seven days (Sellers *et al.*, 2007).Based on the measurement results, there is any significant difference in lung volume between the animal test group and negative control group. This may be caused by trapping water in the lower respiratory tract so that outside air can not get into the lungs (Greaves, 2012).

3.2 Observation results of Lung Macroscopic in Katuk Extrak Soup Intervention

Normality test is done using the Shapiro-Wilk test to determine normality pulmonary organ weights. Normality test results that the data are normally distributed weight p> 0.05 (p = 0.095). Homogentitas test using Levene Test, obtained p> 0.05 (p = 0.135). Statistical parametric analysis was then performed using one-way ANOVA, showed no significant difference in lung organ weights, the value of p> 0.05 (p = 0.154). Test for normality using the Shapiro-Wilk test to determine the normality of the data volume of lung organ. Volume of data normality test results were not normally distributed p value <0.05 (p = 0.024). Non-parametric analysis performed using Kruskal-Wallis, obtained significant difference in lung volume, p <0.05 (p = 0.038).

In this study found no changes in lung morphology. Lung surface looks smooth, shiny and smooth. Changes in lung morphology showed toxic effects. Macroscopic observation was also made by weighing the lungs and lung pengkuruan organ volume, if there is a change may exhibit toxic effects which will affect respiration (Sellers *et al*., 2007). There is no significant difference between lung weights between negative control group with the test group, whereas lung volumes are significant changes between the negative control group to test group.

3.3. Observation results of Lung Microscopic in Katuk Extrak Juice Intervention

Histological observation of lung tissue was observed using a light microscope with a magnification of 400x. A layer of fibroblasts in the group of leaf juice katuk 5000 mg/kg thicker than the layer of fibroblasts in the other group.

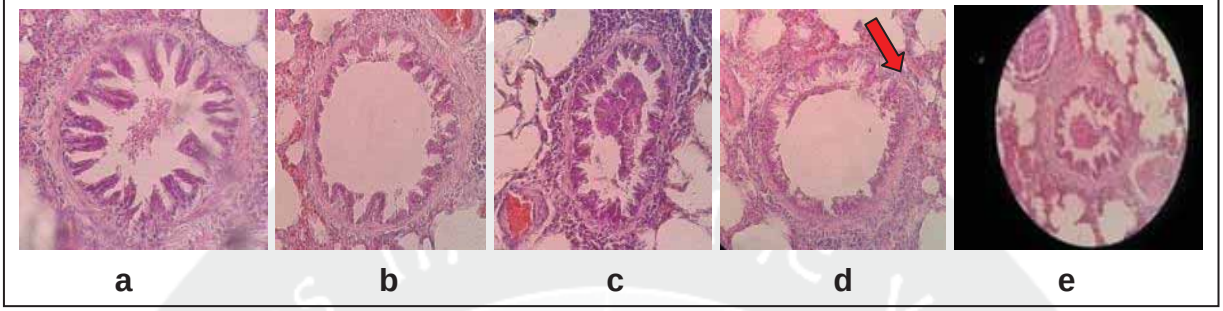


Figure 1. Lung Tissue Preparation: (a) Negative control group; (b) J_{500}; (c) J_{1000}; (d) J_{3000}; (e) J_{5000}

Description: ➡ :Fibrosis in the bronchial lumen

Diameter measurement is done using ocular micrometer on a microscope with a magnification of 10x power magnification 10x objective lens. Bronchial lumen ratio in the test group were significantly different (p (0.006) <0.05) compared to the negative control group. To determine the groups that provide meaningful difference Post Hoc analysis. From the analysis of Post Hoc test obtained the bronchial lumen ratio difference is significant between the negative control group with group leaf juice katuk 5000 mg / kg (p (0.022) <0.05) and the treatment group leaf juice katuk 1000 mg / kg with group leaf juice katuk dose of 5000 mg / kg (p (0.018) <0.05).

On microscopic observation found a decrease in bronchial lumen ratio were significant in animal groups that received leaf juice preparations katuk 5000 mg / kg. The results are consistent with bronchiolitis obiterans characteristic is the existence of fibrosis in the bronchial lumen which causes a narrowing of the bronchial lumen (Laohaburanakit *et al.*, 2003). Thickening layer of fibroblasts is due to the increase in cytokines including interleukin (IL-1, -2, -4, -6, -8, -10, -12, -13), tumor necrosis factor, fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), insulin growth factor (IGF), transforming growth factor-β (TGF-β), endothelin-1 (ET-1) produced by leukocytes and cells that undergo necrosis (Cui *et al.*, 2003; Laohaburanakit *et al.*, 2003; Yu *et al.*, 2007; Pappas *et al.*, 2010). Increasing the number of cytokine that induces fibrogenesis process that led to the formation of excess collagen matrix that will narrow the lumen of bronchioles and thicken the layer of fibroblasts (Wang *et al.*, 2000; Greaves, 2012).

Compounds in the leaves that cause toxic effects katuk still unknown. Research conducted byYunita (2011) suspect phytol is a compound that has the potential to cause toxic effects. Phytol classified terpenoid compounds which are metabolized to a reactive epoxide group (Klaassen, 2008). The existence of these reactive compounds can cause cell damage resulting in necrosis of the cells that trigger an inflammatory reaction (Kumar and Robbins, 2007).

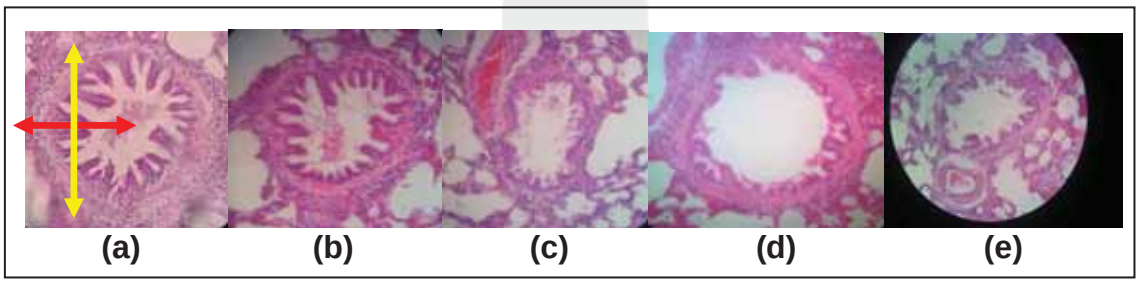
Not found any cell necrosis and leukocyte infiltration around the bronchial lumen. Cells undergo necrosis will difagosit by polymorphonuclear cells (PMN) such as

neutrophils, eosinophils, and basophils. Infiltration of PMN cells induced by cytokine and chemokine produced by cells of necrotic (Parslow *et al.*, 2001). Leukocytes in the necrotic area has undergone lysis or absorbed back into the lymph and so can not be observed during the end phase. Leukocyte infiltration can be found at the beginning of acute inflammation and the chronic inflammation which there are many chemokine produced by necrotic cell to attract leukocytes to areas of inflammation. In this study, the dosage is only given once and observations were made 14 days after the treatment so that the infiltration of leukocytes can not be observed at the end phase (Wang *et al.*, 2000).

Microscopic changes only occurred in experimental animals in the group receiving a dose of leaf juice katuk 5000 mg / kg, marked by a decrease in bronchial lumen ratio. This may be due to the lower dose of 5000 mg / kg is not enough to induce cell damage. Total exposure to toxic substances affect the presence / absence of damage to the cells because the cells have the capacity to adapt to exposure to toxic substances(Kumar dan Robbins, 2007).Bronchial lumen ratio dose group 1000 mg / kg dose group was significantly different to 5000 mg / kg body weight while those with larger doses of 3000 mg / kg body weight did not differ significantly with dose group of 5000 mg / kg. One of the factors that influence the genetic factors of each animal that can affect the immune system so the body's defense reactions expressing different. All the variables that can affect the results of the research have been equated and controlled. Genetic factors are one of the variables that can not be controlled so that anomalies in the results is most likely due to genetic factors of each animal.

3.4. Observation results of Lung Microscopic in Katuk Extrak Soup Intervention

Results of microscopic observation by measuring the diameter of the lumen of bronchioles. Microscopic observations were analyzed statistically using one-way ANOVA (p = 0.05) to detect significant differences bronchial lumen ratio between the control group and the test group. One-way ANOVA statistical results obtained significance value of p <0.05 (0.001) which means that there are significant differences between the groups. To find out which groups are having a significant difference, followed by statistical analysis using the Post Hoc Test. Post Hoc Test results obtained statistically significant difference between the control group and the dose of 500, 1000, 5000 mg/kg, whereas a dose of 3000 mg/kg found no significant difference against the control group.



Gambar 10. Lung Tissue Preparation: (a) Negative control group; (b) S_{500}; (c) S_{1000}; (d) S_{3000}; (e) S_{5000}

diameter of the lumen of bronchioles
diameter of bronchioles

Microscopic observations done by measuring the ratio of the bronchial lumen diamaeter on 3 field of view. Results of the bronchial lumen ratio for each animal the negative control group at 89.96. Lumen ratio for the group at a dose of 500 mg/kg (79,80); a dose of 1000 mg/kg (80,77); a dose of 3000 mg / kg is 84.94 and dose 5000 mg / kg is 82.14 µm. Bronchial lumen ratio were analyzed statistically using one-way ANOVA ($p < 0.05$) note that the significance value of $p < 0.05$ ($P = 0.006$), the results of statistical analysis showed that there were significant differences between negative control group and 4 test groups. Then proceed statistical analysis of data obtained Post Hoc test that between the negative control group at a dose of 500, 1000, and 5000 mg/kg had significant differences, whereas the dose of 3000 mg / kg there was no significant difference.

Observations lung tissue using light microscopy 400x magnification, the observation has not been the infiltration of leukocytes. Leukocyte infiltration will occur when there is inflammation. In this study does not happen dikarena leukocyte infiltration of leukocytes can undergo apoptosis (Parslow e*t al*., 2001).

On the network also has not been found necrosis, this may be caused by granting katuk leaves as much as one that has not been exposed to many toxic compounds. Increasing number of leaves katuk consume, the more doses of toxic compounds that are exposed. The toxic compound can lead to tissue damage that will trigger the inflammatory mediators such as cytokines and chemokines (Parslow *et a*l., 2001). Cytokines and chemokines will attract leukocyte cells such as T-lymphocyte cells and neutrofril who was instrumental in bronchiolitis, mediators also trigger inflammation that will cause the proliferation of fibroblasts (Laohaburanakit *et al*., 2003). Fibroblast proliferation is one of the causes of fibrosis that occurs in the bronchial lumen. Increasing number fibrosis, the fibroblasts will increasingly thicken. Thickening of fibroblasts causes a narrowing of the bronchial lumen and cause airway obstruction (Myong *et al*., 2000). In the lung tissue were observed preparations have not yet experienced the occurrence of fibrosis or narrowing of the lumen, but has been unable to demonstrate the bronchial lumen size changes. This could be due to the influence of the length of time consuming katuk (Ngatidjan, 2006).

4. CONCLUSION

Based on the research that has been done, it can be concluded: Katuk leaf juice (*Sauropus androgynus*) does not cause a change in the physical condition of female Wistar rats. But it cause significant differences are observed macroscopic lung volume as well as the results of microscopic observation that the bronchial lumen ratio between treatment groups katuk leaf juice 5000 mg/kg and negative controls. There isn't changes in the physical condition of female Wistar rats after administration katuk soup. However it can causes significant variations in the macroscopic conditions (lung volume) and microscopic (the size of the bronchial lumen) in female Wistar rats.

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Mouse Sperm Agglutination with Total Protein Extracted from Endosperm Seeds of *Terminalia catappa*

Hery Haryanto[1] Aceng Ruyani[2], Selfianti[3]
[1]Biology Dept, FMIPA Universitas Bengkulu Indonesia
[2]Biology Education Dept, FKIP Universitas Bengkulu Indonesia
[3]Medical and Health, FKIK Universitas Bengkulu Indonesia
heryharyanto@unib.ac.id, ruyani@lycos.com , selfianti@gmail.com

Abstract

The aim of research was to agglutinate sperm of mice with total protein extracted from endosperm seed of *Terminalia catappa*. The total proteins contained a protein behaving like lectins. Lectins are glycoproteins that can bind to specific carbohydrate residues. Endosperm of *T. cattapa* seed was homogenazed with extracting buffer. The homogenate was stirred overnight with a stirring bar at 4°C, centrifuged at 2500 rpm at 4°C for 15 minutes. The supernatant was added with ammonium sulfate to a concentration of 50% (w/v), stirred with a stirring bar for 3 hours, then centrifuged 10,000 rpm at 4°C for 15 minutes. Precipitated protein was purified with 12,000 MWCO dialysis bag. The protein precipitate was dissolved in buffer and the concentation was quantified by Biuret method. Mice were killed by cervical dislocation, dissected to take out its epididymal duct. Sperm suspension was made by soaking the epididymal duct in 0.9% (w/v) NaCl, then the proximal part of epididymis was cut then pressed gently to expel sperm from of epididymal duct. Agglutination test was performed by mixing 5 μl sperm suspension with 5 μl total protein extract of dialysis fraction at concentration 4.23, 8.97, 13.2 , and 17.4 mg/dl, or fraction of ammonium sulfate, or crude extract fraction. The fastest agglutination was showed by dialysis fraction containing 17.4 g/dl protein. Endosperm of *T. Cattapa* seed contained protein-like lectin that could agglutinate mouse sperm.

Keywords: lectin, total protein, endosperm, Terminalia catappa, agglutination, sperm, mouse.

1. INTRODUCTION

"Ketapang" tree (*Terminalia catappa*) is one of plants used reforestation program along the coastal areas. The plant is highly tolerant to salinity, having aesthetic canopy with horizontal branches as shading cover, resistant to wind, preventing abrasion. Moreover, the seeds are edibel known as "tropical almond" (Mile, 2007).

Lectins are glycoprotein molecules that are found in plant parts (especially the seed endosperm), invertebrates, fish (Kumar *et al*., 2012; Sharon, 2007). Lectins are glycoproteins from plants and animals that can bind specifically with glicoconjugate residue on the surface of cells (Sharon, 2007). Lectins show variety of biological functions, such as : grouping blood group (phytoagglutinin) (Sharon, 2007), indicating of tumor cells (Muchtadi, 1989), showing spermicidal activity (Anuja *et al*., 2011; Nicolson, 1977). Lectins binds to the specific glycoconjugates on cell

membrane surface through non-covalent bonding. This bond is relatively weak, however if more than one bond are formed between specific glicoconjugates and lectins , they strong enough to agglutinate cells (Alroy *et al*., 1988).

The objective this research is to agglutinate sperms of mice (*Mus musculus*) with total protein extracted from seed endosperm of *T. catappa*. Previous research showed total protein of crude extracts agglutinated all blood types of human erythrocytes (Ratnasari, 2014). This implied that *T. cattapa* seed contained a protein behaving like lectins.

2. METHODS

2.1 Chemicals

Homogenizing buffer was made of 50 mM Tris-Hydroxymethil-Amino Methane, 50 mM $NaHCO_3$, 50 mM $MgCl_2$, 5 mM $Na_2S_2O_3$ at pH 7.4. Dialysis bag size was for 12.000 MWCO (molecular weight cut off). Sperm suspension liquid contained NaCl 0,9% (w/v). Bovine Serum Albumine as protein standard for quantitive determination of total protein with Biuret Method.

2.2. Procedures

Marure fruits of *T. catappa* fruit were peeled, and 50 grams of endosperm parts were homogenized in 150 ml cold buffer containing 50 mM Tris-Hydroxymethil-Amino Methane, 50 mM $NaHCO_3$, 50 mM $MgCl_2$, 5 mM $Na_2S_2O_3$ at pH 7.4. Firstly, the homogenation was stirred overnight at 4° C, secondly, homogenate was filtered using a cheese cloth, thirdly filtered liquid was centrifuged at 2,500 rpm for 15 min at 4° C. Finally, supernatant was added with ammonium sulfate 50% (w/v) (Singh *et al*., 2009) and centrifuged at 10,000 rpm for 30 min at 4° C to separate the precipitated total protein. Precipitated protein was dissolved in 2 mL buffer, then put in 12.000 MWCO dialysis bag, and equilibrated in buffer for 12 hours at room temperature with 2 changes of buffer. The protein quantitation was determined by Biuret method with Bovine Serum Albumin (BSA) as a protein standard by using a spectrophotometer at a wavelength of 540 nm. The protein quantitation was measured on crude extract, ammonium sulphate, and dialysis fractions.

Preparation of sperm suspension. Mice were 8-12 weeks old and with weight between 25-35 grams were killed by cervical dislocation, dissected, and cut epidydimal ducts , placed on a petri dish containing 1 mL 0.9 % (w/v) NaCl. Epidydimal proximal portion was cut and pressed gently until the liquid from epidydimal duct out and suspended in physiological saline (Suparni, 2009).

Sperm agglutination test procedure was as follows : 5 □ L of sperm suspension was dropped on a glass object was mixed to 5 □ L of protein extract, stirred with a toothpick 3 rounds, and timer was turned on, and then observed under a microscope for 5 minutes (300 seconds) and documented. When the sperm agglutination test more than 5 minutes no clumping was denoted as 300 seconds. Spermatozoa agglutination test was conducted on crude extract, and ammonium sulfate, and dialysis fractions. Data sperm agglutination test rate were analyzed with ANOVA. If there were significant differences and continued with Duncan test (DMRT).

3. RESULTS AND DISCUSSION

Protein quantitation of crude extract , ammonium sulfate, and dialysis fractions can be seen in Table 1. The fraction of ammonium sulfate showed a higher protein content than that of the fraction of dialysis. It gave a hint that the dialysis process excluded protein molecular weight of less than 12,000 kD dialysis out of the bag.

Table 1. Quantitation of total protein in endosperm seed of *T. catappa*

No.	Fraction	Protein Concentration (µg/dl)
1	Crude extract fraction	17.4
2	Ammonium sulfate fraction	26.4
3	Dialysis fraction	17.47

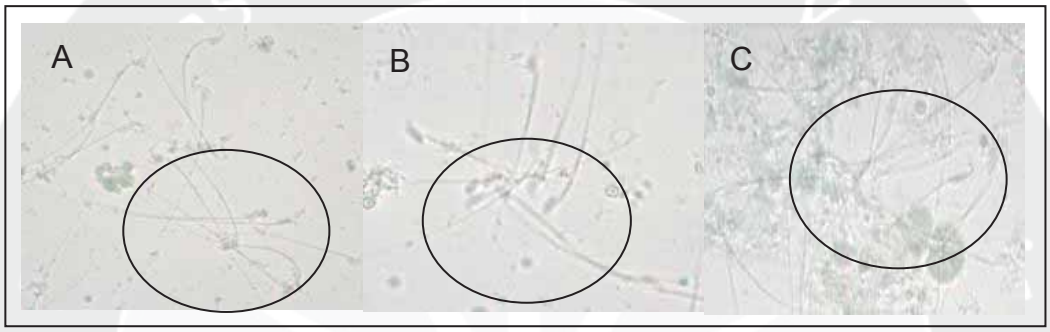

Figure 1. There were three type sperm agglutination with total protein extract of endospem seed of *T. catappa*. Head-head sperm aglutination (A), head-tail sperm agglutination (B), and tail-tail sperm agglutination (C)

The observation of sperm agglutination with a microscope showed three different types of binding between spermatozoa that were bridged by lectin proteins. There were three types agglutination, i.e : binding between the head-head, head-tail, tail-tail of spermatozoa (see Figure 1). The most type was head-head sperm aglutination. Because the head of spermatozoa, or acrosome part have more glycoconjugates than that of the tail. It showed that both the head and tail of mice sperms contained glycoconjugates that could bind to proteins extracted from endosperm of *T. Catappa*. This indicated that the protein behaving like lectins.

Sperm acrosome membrane contains □(1-4) galactocyl transferase which is the principal component of the acrosome and serves to initiate the bond between the head of the sperm with the egg zona pellucida particularly in the zona pellucida 3 (ZP3) (Youkim *et al*., 1994). Lectin protein from the endosperm of the *T. cattapa* seeds may have the same properties as ZP3, because both can bind to specific carbohydrate found in the cell membranes of sperm, especially on the head with the help of the bond of glycoproteins galaktosil transferase.

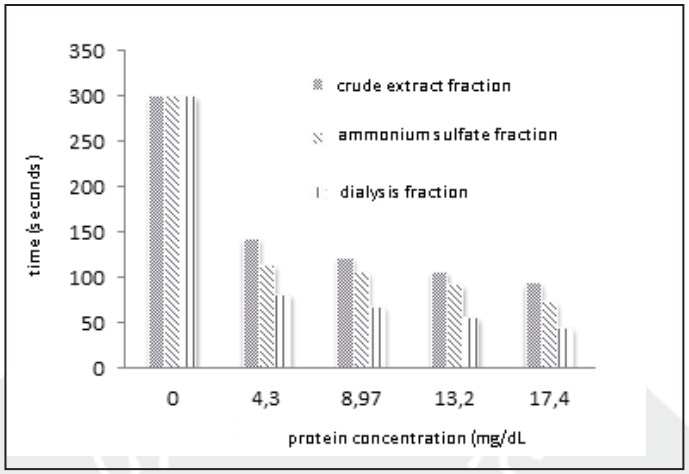

Figure 2. the rate of mouse sperm agglutination, the more concentrated total protein extracts show more faster in agglutination rate

3.1. The rate of sperm agglutination

Sperm agglutination rate of crude extract, ammonium sulphate, and dialysis fractions with 5 variations of protein concentrations (0, 4.23, 8.97, 13.2, and 17.4 mg/dl) can be seen on Figure 2. All control up to 300 seconds (5 minutes) mice sperm suspension showed no sperm agglutination. All concentrations of all fractions showed different agglutination rates. The higher the concentration of total protein in all fraction gave faster rates. Protein of dialysis fraction showed the fastest rate.

Analysis of variance of sperm agglutination of dialysis fraction showed significant difference, therefore it was continued with Duncan analysis to determine the least significant difference from the rate of sperm agglutination each treatment concentration (Table 2). This test showed a concentration of 17.4 mg/dL of dialysis fraction was significantly different from other concentration. While the concentrations of 13.2, 8.97, and 4.23 mg/dL were no real difference between these concentrations.

Table 2. Duncan least range test of sperm agglutiantion of dialysis fraction of protein extraxcted from endosperm *T. catappa*

No	Concentration mg/dl	The rate of agglutination (detik)	remarks
1	17,4	44.8	a
2	13,2	56.12	b
3	8,97	68.33	b
4	4,23	82.22	b

Endosperm of *T. catappa* seed contains protein that behaves like lectins. Therefore the protein can agglutinate mice sperms due to compatibility with glycoconjugates on the surface of sperm membrane.

4. CONCLUSIONS

The total protein extracted from *T. catappa* endosperm in crude extract, ammonium sulfate, and dialysis fractions can agglutinate sperm of mice. The fastest rate of mice

sperm agglutination is dialysis fraction ccontaining protein 17.4 g/dL, and showed significantly different from other concentrations.

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The Plankton Characteristic of Winong Lake in Gunung Kidul

*Agus Suyanto[1]
[1]Department of Environmental Engineering, Faculty of Environmental Engineering
Institute of Technology Yogyakarta-Indonesia
Email : sttlylh@gmail.com

Abstract

The biology issue has received much attention not only locally, nationally and regionally, but also globally with the declaration of the Rio De Janeiro Declaration 1992 about Sustainable Development. At local scale, Gunungkidul located in the southern part of Yogyakarta Special Province, Indonesia has one of the most unusual tropical karst areas in the world with very high concentration of limestone outcrops including the potential of lake biodiversity. The specified area of Winong lake is investigated the Biology potential of Winong Lake in GunungKidul. The objective of the research is to investigate the biology characteristic lake in term of plankton. The field survey and laboratory method is conducted to meet the objective.Theplankton parametersare both phytoplankton and zooplankton.The result shows that the number of plankton species found from lake water is as many as 73 species and dominated by *SphaeropilaAnnulina* and *Diplosigopsissideratheca.* The density of plankton in the lake water is as much as 1,275 x 10^4 individual / mL

Keywords: biology, flora, fauna, lake, winong

1. INTRODUCTION

Gunungkidul is located in the southern part of Yogyakarta Special Province, Indonesia which has an area of 1,486.84 Km^2 and the number of population is 677,998 people in year 2011. It also famous for its karst geology and it has very high concentrations of limestone outcrops.Water resource is a pivotal issue for the Gunungkidul karst. During the dry season, this area suffers water shortages for at least four (4) months. Annually it is reported that more than 41 per cent of the population of Gunungkidul have no access to safe drinking water (Kapedal 2006). In some localities, the population is forced to carry water from far distant springs or lakes (lake). Water resurgences from these springs emerge in the southern coastline which is far from settlements. Underground river course, on the other hand, are found at great depths (> 100 m) and not readily assessed by the local people. High investment and operational cost make underground water tapping infrastructure not affordable to local governments and private sectors.

Table 1. Lake Distribution condition showing dry and sedimentation conditions in Gunungkidul

Sub-district	Number of lake	Dry	Sedimentation
Purwosari	31	19	17
Panggang	22	12	11
Tepus	32	29	32
Tanjungsari	27	26	27
Semanu	42	37	41
Ponjong	21	14	4
Rongkop	49	36	31
Girisubo	27	22	26
Saptosari	21	15	12
Paliyan	10	7	4
Total	282	217	205

Source : Kapedal, 2006

The Gunung Sewu Karst Region is characterised by poverty and a lack of water. A long time ago, the village-based farming system has undergone little change. The population remains dependent on rain-fed farming. Since employment in the area is basically agricultural- based, most of the population's daily needs (dietary and income) are derived from farm produce. Although infrastructural developments have occurred, the two primary resources for farmers' survival, soil and water, have been only minimally developed. With biophysical limitations, i.e. no water for irrigation and a thin productive soil, land productivity in the area is low and levels of economic welfare are low. Low levels of social welfare are highlighted by the continued dependence of part of the population on cassava, a crop associated with the diet of the poor. Rice is viewed as a superior food in GunungSewu because it can be harvested only once a year, during the rainy season.

The biology issuesare not yet much attention from many stakeholders. The problem of lake are not only physical problem but also biology problem. The problem of dry, sedimentation, shallowing will impact to biology problem such as biodiversity problem. The objective of the research is to investigate the biology characteristic lake in term of plankton at Winong Lake in GunungKidul. It is important to provide a description of the biology resource of karstic lake ecosystem.

2. METHODS

The research is conducted at Winong Lake, Kepekviilage, Saptosari Sub-District, Gunung Kidul District. The sampling was conducted during rain season (November 2014). The biology parameters are total coliform, plankton and flora fauna. The unit analysis area of the research is below;

Figure 1. Sampling Location for total coliform and plankton at Winong Lake (S1-S10). Sn = Sampling location

Laboratory analysis was conducted in order to identify species and to calculate the density using haemacytometer.
Diversity index was formulated using Shannon dan Weaver formula (1949) in Ludwigdan Reynold (1988) as following formula ;

$$H' = -\sum_{i=1}^{S}\left[\left(\frac{n_i}{n}\right) ln\left(\frac{n_i}{n}\right)\right]$$

Information;
H' = Diversity Index Shannon-Winner
n = Total number of species
ni = Total number certain of species-i
ln = natural logaritma

Necton density was calculated using below formula ;

$$K = \frac{n_i}{A}$$

Information;
K= Necton density
ni = Total number certain of species-i
A= Net area

3. RESULTS AND DISCUSSION

Plankton are microscopic organisms (5-15µm) located on the surface of the water that their movement is influenced by water flow. Plankton is divided into two major groups, namely:

- Phytoplankton, the plankton that is like plants, can carry out photosynthesis and serve as a primary waters producer.
- Zooplankton, the plankton that is like animals, moves and serves as a secondary producer.
- Plankton observation taken from water sample was conducted by using *haemacytometer*. Based on the observation, some plankton species were found, namely;

Table 1. Species and Number of Plankton Phytoplankton

No.	Species	Numberof Fitoplankton		
		ATS	AT	ATH
Chlorophyta				
1	*Ankistrodesmusfalcatus*		1	
2	*Closteriopsislongissima*		4	
3	*Crucigeniaalternans*		85	
4	*Cylindrocapsageminella*		14	
5	*Desmatractumbipyramidatum*		1	
6	*Dictyosphaeriumpulchellum*		9	
7	*Disporacrucigenioides*		17	
8	*Elakatothrixviridis*		13	
9	*Euastropsisrichteri*		32	
10	*Gloeocystisgigas*		159	
11	*Kentrosphaerabristolae*	5	18	
12	*Kirchneriella obese*	1	6	1
13	*Lobocystisdichotoma*		51	
14	*Lobomonaspentagona*		7	
15	*Lobomonasrostrata*		5	3
16	*Mesotaeniumaplanosporum*		6	1
17	*Microthamnionstrictissimum*	1		
18	*Myrmecia aquatic*	1	5	
19	*Ophiocytiumcapitalum*		2	
20	*Ourococcusbicaudatus*			2
21	*Pleurogasterlunaris*	3		
22	*Prasiola Mexicana*		1	
23	*Pteromonas aculeate*		1	
24	*Pyramidomonastetrarhynchus*		1	
25	*Siderocelisornatus*		65	
26	*Sorastrumspinulosum*		1	
27	*Spermatozoopisexultans*	1		
28	*Sphaerocystisschroeleri*	6	7	
29	*Sphaeropilaannulina*		211	1
30	*Spinoclosteriumcuruatum*		1	
31	*Tetrallantoslagerheimii*		1	
32	*Tetraspora cylindrical*	2		
Chrysophyta				
1	*Bitrichiaphaseolus*		1	
2	*Bumillariaexilis*		1	
3	*Bumilleriopsisbreve*		4	1
4	*Chaetoceroselmorei*		1	
5	*Characiopsislongipes*		1	
6	*Chlorobotrysregularus*			2
7	*Chlorotheciumplorottae*		27	
8	*Pleurogasterlunaris*		1	
9	*Syncryptaadamsii*		2	
Cyanophyta				
1	*Spirulina major*	87		
2	*Merismopedia*sp.		24	
Euglenophyta				
1	*Ascoglenavaginicola*	4		
2	*Euglena deses*	1		
3	*Euglenamorphahegneri*	1		
4	*Menoidiumfalcatum*		1	
5	*Petalomonasmediocanellata*		2	2
6	*Trachelomonasvolvocina*	1		

7	*Urceoluscyclostomus*		2
Ochrophyta			
1	*Nitzschiacapitalia*		1
Phaeophyta			
1	*Heribaudiellafluviatilis*		11
Pyrrophyta			
1	*Audouinellaviolacea*		1
2	*Bangiafuscopurpurea*		73
3	*Glenodiniumkulczynskii*		1
4	*Gymnodiniumaeruginosum*	3	
5	*Hemidiniumnasutum*	1	

Table 2. Species and Number of Plankton Zooplankton

No.	Species	Numberof Zoooplankton		
		ATS	AT	ATH
Ciliophora				
1	*Blepharismalateritum*	2		
2	*Bryophryabavariensis*		1	
3	*Calyptotrichapleuronemoides*		1	
4	*Drepanomonas revolute*		2	
5	*Dysterianaviculla*		4	
6	*Leucophryspatula*		1	1
7	*Paravorticellacrassicaulis*		1	
8	*Prorodonteres*		1	
9	*Pyxidiumconthurnioides*		1	
10	*Strobilidiumgyrans*	1		
11	*Trimyemacompressum*		1	
12	*Vorticella campanula*		1	
Mastighopora				
1	*Cercobodoagilis*		1	
2	*Cladomonasfruticulosa*		1	
3	*Diplosigopsissideratheca*	23	183	108
4	*Multicilialacustris*		1	
5	*Parabodonitrophilus*	2		
6	*Sterromonasformicina*		2	
7	*Trigonomonascompressa*		4	
Rhizopoda				
1	*Bullinulariaindica*	9	1	
2	*Centropyxisaerophila*	3	12	2
3	*Centropyxishemisphaerica*		1	
4	*Centropyxisstellata*		12	
5	*Cyphoderiatrochus*		5	
6	*Diaphoropodon mobile*	1		
7	*Difflugia acuminate*	1	47	6
8	*Ditremaflavum*	1		
9	*Lesquereusiamodesta*		3	
10	*Thecamoebaverrucosa*	8		
11	*Trigonopyxisarcula*	1		
Turbellaria				
1	*Gyratrix hermaphrodites*		3	
2	*Mesostomacurvipenis*		1	
3	*Planariasimplicissima*		2	
4	*Prorhynchusstagnalis*		9	1

Note :ATS: existing treated lake water; AT: lake water; ATH: rain-fed water/household storage

Based on above table, plankton species found in the water sample tested are as many as 57 species of phytoplankton and 34species zooplankton. The number of plankton species found from treated lake water is as many as 24 species and dominated by the *Spirulina major* and *Diplosigopsissideratheca*. On the lake water is found as many as 73 species and dominated by *SphaeropilaAnnulina* and *Diplosigopsissideratheca*. In rain-fed water is found as many as 13 species dominated by*Diplosigopsissideratheca*. The number of plankton species is mostly found in the lake water. By referring to the species that dominate, there are similarities among the three water sources, namely the existence of types of zooplankton *Diplosigopsissideratheca*. Itshows that these species can adapt to existed environmental conditions. The plankton density of the three water sources is presented in Figure 2.

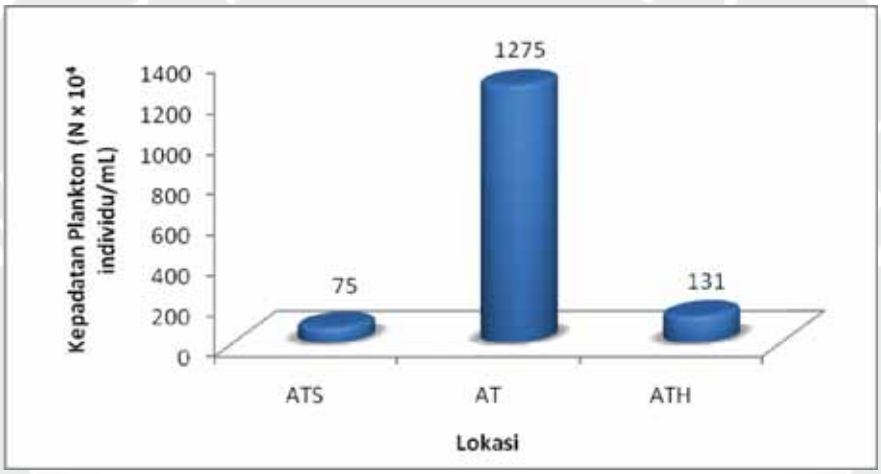

Figure 2. Plankton Density in Each of the Three Water Sources

Based on above figure, it shows that the highest density of plankton in the lake water is as much as 1,275 x 10^4 individual / mL while the lowest is the treated lake water as much as 75 x 10^4 individual / mL. The density of plankton in the lake water is much higher than other water sources due to physical and chemical quality of the water that supports the growth of plankton. The existence of plankton in wateris much supported by the availability of nutrients and optimum water condition. Sastrawidjaya (2009) stated that if the density is high, the algae are also harmful to the environment because after their lush growth, then the rest of the rotting algae will spend the oxygen in the water, so the water will be in a bad smell, no taste, and polluted. It is seen on the physical condition of Winong lake water;it is in a foul smell, greenish.There is a lot of garbage on the edge of the lake water area. The garbagae contained in the lake is inorganic waste.

In fact, the number of species and plankton density is higher than the lake water. This is due to lack of sunlight that enters the treated lake water and rain-fed lake water. In rain-fed water, the containeris made of a large reservoir which is only open slightly at the top so it only allows a little sunlight to enter to it. As we know that the main factor for the growth of phytoplankton is sunlight to support the plankton photosynthesis process. The treated lake water containeris made ofopen permanent reservoir which allows sunlight to get in the tank, but then the water is processed using certain techniques and flowed through the water faucet. This water treatment

technique can eliminate the plankton that the amount of plankton is found less.Based on the laboratory result, It is found that phytoplankton species is more commonly found. It is grouped into the division of *Chlorophyta* or green algae. Green algae that live in fresh water are characteristically cosmopolitan, especially the ones living in a place with enough light such as lakes, lakes, puddles of rain, the water flows (river or ditch). Types of plankton in the form of phytoplankton are far more commonly found in the three sources of water compared to zooplankton. It can be seen in Figure below

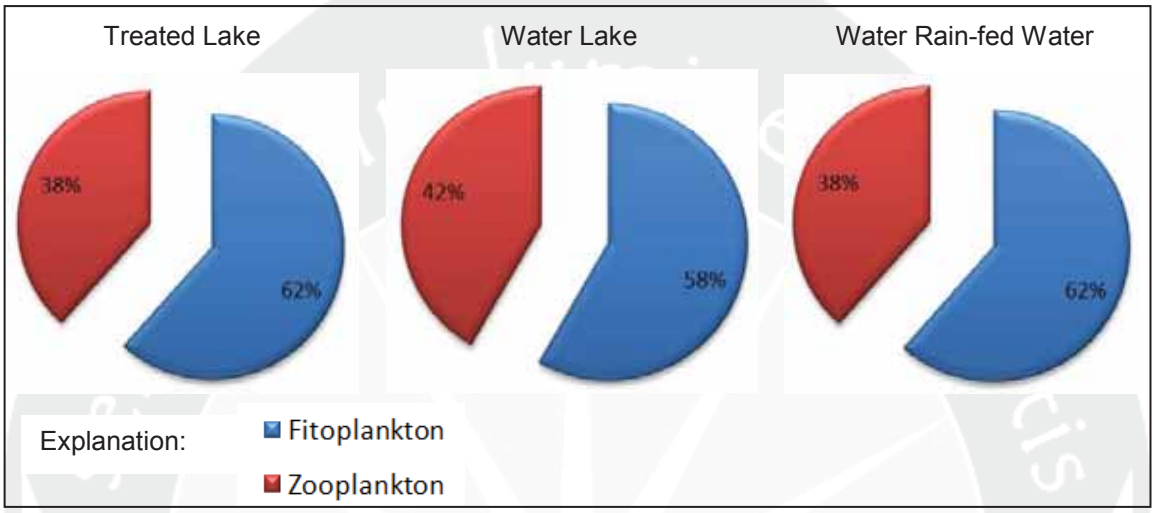

Figure 3. Comparison of phytoplankton and zooplankton species

From the calculation of the index of plankton species diversity using the Shannon index, the highest diversity value is the lake water of 2.98. Diversity index value (ID) of Shannon plankton are presented in Figure below;

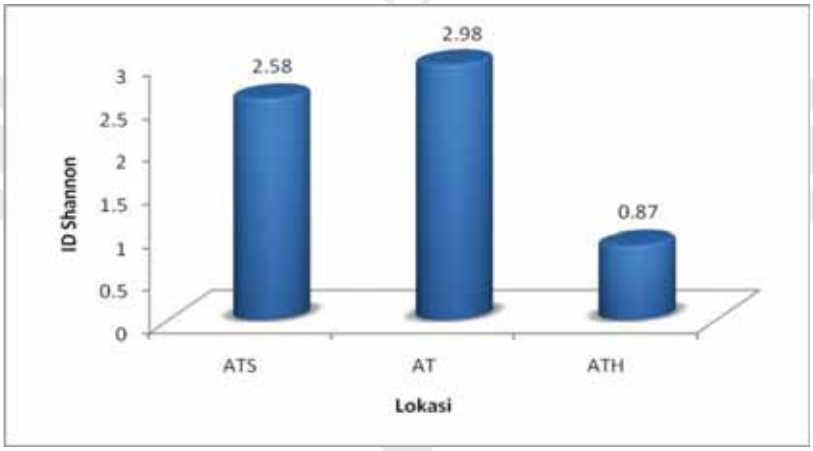

Figure 4. Shannon Plankton Diversity Index inEach Water Source

The diversity value of plankton in the treated lake water and lake water is includedinto moderate criteria (score range of 1-3) while the diversity index value on rain-fed water criteria is low (<1). The diversity value of the rain-fed water is much smaller than the treated lake water. This is due to the amount of plankton species that are found in rain-fed water less and there is one species with the number of

individuals who are very much the type of *Diplosigopsissideratheca*. According toIndriyanto (2006), a community is said to have low species diversity if the community is arranged by fewer species and only dominated by a few species.

High diversity index in a location indicates that the site is suitable for the growth of plankton. If the low diversity index showninthe location is low, it will be less suitable for the growth of plankton (Odum, 1994). Referring to the index species diversity and the abundance of plankton, the lake water is quite suitable for the growth of plankton. The plankton abundance can be used as an indicator of waterfertility (Mardani *et al*., 2013). The higher abundance of plankton, the more fertile the water is.

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Cloning of cDNA Encoding Membrane Protein of Tachyzoite of *Toxoplasma gondii* In pUC19

*Mufasirin[1] , Wayan T. Artama[2] , Sumartono[3], Sri Hartati[4]
[1]Department of Veteriner Parasitology, Faculty of Veterinary Medicine, University of Airlangga , Surabaya, Indonesia
[2]Department of Biochemistry, Faculty of Veterinary Medicine, University of Gadjah Mada, Yogyakarta, Indonesia
[3]Department of Parasitology, Faculty of Veterinary Medicine, University of Gadjah Mada, Yogyakarta, Indonesia
[4]Department of Internal Medicine, Faculty of Veterinary Medicine, University of Gadjah Mada, Yogyakarta, Indonesia
mufasirinfkhunair@gmail.com

Abstract

The aim of the research is to clone cDNA encoding membrane protein of Toxoplasma gondii using pUC19 for developing molecular diagnosis and vaccines. Total RNA and messenger RNA were isolated from tachyzoites of Toxoplasma gondii that growing up in Swiss strain mice using PolyAtract®mRNA Isolation Systems (Promega) and synthesis of cDNA using Riboclone®cDNA Synthesis Systems (Promega). Complementary DNA was ligated with *Eco*RI adaptor using Riboclone®*Eco*RIAdaptor Systems (Promega). preparation pUC19 vector, pUC19 ligated with cDNA was transformed in *E. Coli* DH5α. Plasmid recombinant was analyzed using endonuclease restriction enzyme (*Eco*RI and *Hind*III), total protein isolation and immunoblotting. Results of transformation using the pUC19 vector obtained two recombinant clones carrying genes encoding membrane proteins.

1. INTRODUCTION

Toxoplasmosis is a parasitic disease caused by *Toxoplasma gondii*. The disease is spread all over the world, and is more common in cold climate regions[1]. In acute infections, most are asymptomatic, and that really shows just a slight pain symptoms and is usually not diagnosed[2]. Infection in adults showed no clinical symptoms but sometimes showed lymphadenitis in cervical region and other regions, accompanied by a mild fever[3]. In pregnant women and animals, severe infections can lead to miscarriage or congenital defects in children born[1].Toxoplasmosis can be transmitted from animals to humans, so it requires a good disease control strategies. Among other disease control strategies include knowledge of toxoplasmosis, treatment and vaccination.

The use of the vaccine in endemic areas is one appropriate control measures. Toxoplasmosis can be done to control through vaccination of pets, especially cats as the definitive host, *Toxoplasma*-free cattle feeding and limiting contact between humans and cats. *Toxoplasma gondii* vaccines that have been used in the form of a live vaccine (tachyzoite), the surface protein[4], and nucleic acids[5]. Constraints development of live vaccines is the development of the culture and handling of

tachyzoite relatively insecure and expensive, while the surface protein is use vaccine as an antigen supply is not easy. The use of inactivated vaccine and sub unit vaccine of *Toxoplasma* are one alternative to vaccination, but the obstacles faced are contaminants, cost prohibitive in the provision, as well as toxic effects when administered in large quantities[6].

Diagnosis of toxoplasmosis is often based on the presence of antibodies against *Toxoplasma*. Humans and animals showed seropositive for *Toxoplasma* is not necessarily suffering from toxoplasmosis, although once infected with *Toxoplasma*. Diagnosis is confirmed by finding cysts on the brain or tissue and the presence of *Toxoplasma* circulating in the form of bradyzoite or tachyzoite[7]. Presence of antigens in the body can be detected by Enzyme Linked Immunoassay (ELISA) technique[7], Polymerase Chain Reaction (PCR)[8,9,10], Polymerase Chain Reaction - DNA Enzyme Immunoassay (PCR-DEIA)[11]. PCR diagnostics constraint-DEIA or hybridization techniques tracker is available *Toxoplasma* protein for the manufacture of antibodies and DNA is used as a tracer.

Toxoplasma gondii is an obligate intracellular protozoan that infects several types of nucleated cells. Contacts among the receptors on host parasite is an initial entry of the parasite into the host[13]. P30 is a major membrane protein is a essential ligand in *Toxoplasma*[12]. P30 is located on the membrane surface and rhoptry of tachyzoite[14]. The development of recombinant DNA technology allows solving the problem of providing a surface protein that is immunogenic in a large amount, so that the manufacture of the vaccine may be cheaper. Recombinant DNA technology can also provide a solution providing both nucleic acid used as a vaccine or material for hybridization tracers in the diagnosis of disease. pUC19 vector is a plasmid often used in engineering is a *Escherichia coli*. The aim of the research is to clone cDNA encoding membrane protein of *Toxoplasma gondii* using pUC19 for developing molecular diagnosis and vaccines.

2. METHODS

2.1 Total RNA Isolation

Some 10^{10} tachyzoites coupled with 1.5 ml of guanidine thiocyanate and mixed by means of resuspended, then transferred into a sterile homogenizer RNase-free tubes. Homogenization is done with the homogenizer 6 times, each with a speed of 1200 rpm for 30 seconds. Homogenization is done in a cold state in order to avoid damage to RNA. Results of homogenization transferred into RNase-free sterile tube, then added with Na-acetate-tenth of the volume and mixed until blended. The solution was put in ice for 5 minutes and added with phenol chloroform as much. Mixing is done by flipping and turning the tube and incubated on ice for 15 minutes. Aquaeus phase obtained by centrifugation with 4000 rpm on 4^{o}C for 25 minutes. Aquaeus phase was transferred into a new sterile RNase-free tubes and added isopropanol (1: 1) and mixed with way of flipping through the tube. The solution was incubated at -20^{o}C overnight. The solution was centrifuged at 4000 rpm at 4^{o}C temperature for 25 minutes. Obtained pellet was washed with cold 75% ethanol and centrifuged at 4000 rpm at 4^{o}C for 25 minutes. The supernatant was discarded and the pellets was dried until completely dry at room temperature. The next pellet resuspended with 250 µl RNase-free water.

2.2. mRNA Isolation

1) Annealing probe. Messenger RNA was isolated using PolyAtract®mRNA Isolation System. Two hundred and fifty microliters of total RNA coupled with RNase-free water until the volume becomes 500 µl. The solution was incubated at 65^{o}C for 10 minutes. Immediately after the incubation, the solution was added with 3 µl biotinylated oligo (dT) probe and 13 µl 20XSSC. The solution was mixed and incubated at room temperature for 10 minutes.

2) Wash streptavidin-paramagnetic particles (SA-PMPs). Streptavidin-paramagnetic particles (SA-PMPs) resuspended to solution, then captured by placing a magnetic stand for 30 seconds until the SA-PMPs collected on the wall (the solution becomes clear). Streptavidin-paramagnetic particles were washed three times with 0.5x SSC (each wash as much as 0.3 ml) in a manner as was done previously and then resuspended in 0.1 ml of 0.5x SSC.

3) The arrest and washing hybrid mRNA with oligo (dT). The reaction solution annealing inserted into the tube containing the SA-PMPs, then mixed and incubated at room temperature for 10 minutes. Complex SA-PMPs with hybrid mRNA and oligo (dT) captured by placing a magnetic stand until the solution becomes clear. Supernatant was discarded carefully no damaging pellets SA-PMPs. Streptavidin-paramagnetic particles were washed four times with 0.1x SSC (each wash as much as 0.3 ml) as shown above, by placing a magnetic stand until the solution becomes clear.

4) Elution of mRNA. SA-PMPs pellet were resuspended in 0.1 ml RNAse free water, then captured by the magnetic stand. Aquaeus phase is taken and put in a new sterile RNase-free tubes. Washing was repeated by adding 0.15 ml of RNase-free water, then SA-PMPs captured using a magnetic stand. Phase aquaeus taken with caution and put in a new sterile tube which had been washed with distilled DEPC.

5) Precipitation and concentration of mRNA. Two hundred and fifty microliters of mRNA elution process results coupled with 25 µl Na-acetate and 250 µl isopropanol, then blended until smooth and incubated on -20^{o}C overnight. The reaction mixture was then centrifuged at 12,000 rpm at 4^{o}C for 25 minutes. Pellet resuspended in 1 ml of ethanol; 75% cold, then centrifuged at 12,000 rpm at 4^{o}C for 25 minutes. The supernatant was discarded and the pellets were dried at room temperature. Pellets resuspended with 10 µl RNase free water and stored at a temperature -70^{o}C. mRNA concentrations seen by spectrophotometer at OD_{260}.

2.3. cDNA Synthesis

Single strand cDNA synthesis. Ten microliters of solution containing 2 µg mRNA, plus 1 µl oligo (dT) primer and 4 RNase-free water. The reaction mixture was incubated at 70^{o}C for 5 minutes and centrifuged for 5 seconds at a speed of 12,000 rpm for lowering all solution. The reaction mixture was added with 5 µl single strand 5X buffer, 25 U rRNasin ribonuclease inhibitor, 2.5 µl 40 mM sodium pyrophosphate, 15 U AMV reverse transcriptase RNA and RNA free water to a final volume of 25 µl. The reaction solution was mixed well and incubated at 37^{o}C for 60 minutes. After incubation, the solution is stored in ice to be used a double strand synthesis.

Synthesis of double-stranded cDNA.Twenty microliters of the reaction mixture on the synthesis of single-stranded, plus an additional 10 µl 10X second strand buffer, 23 U

of DNA polymerase I, 0.8 U RNaseH and nuclease free water to obtain a final volume of 100 µl. The reaction solution was mixed well and incubated at 14°C temperature for 3 hours. The polymerization reaction was stopped by heating at 70°C for 10 minutes. The tube containing the reaction mixture was centrifuged at 12,000 rpm for 5 seconds to bring down all liquid and then taken in the ice. The reaction mixture was further added with 2 U T4 DNA polymerase and incubated at 37°C for 10 minutes. Reaction stopped by adding 10 µl 200 mM EDTA and placed on ice. DNA extraction was done by added phenol; isoamylalcohol chloroform 1: 1. Solution mixed well and centrifuged at 12,000 rpm for 2 minutes at room temperature. Aquaeus phase was transferred into a new tube, then added half times the volume of 7.5 M ammonium acetate and two and a half times the volume of cold absolute ethanol. The solution is mixed with gentle, then incubated at -20°C overnight. After incubation, the reaction mixture was centrifuged at 12,000 rpm at 4°C temperature for 25 minutes. Pellet resuspended with cold 75% alcohol and centrifuged at the same speed. The supernatant was discarded and the pellets were dried at room temperature until totally dry. Pellet resuspended with 10-50 µl TE buffer. DNA concentration was measured with a spectrophotometer at OD_{260}.

2.4. Addition of EcoRI Adapter in cDNA

The addition of the adapter *Eco*RI use Riboclone®*Eco*RI Adaptor Ligation System. A total of 2.5 µl DNA (100 ng/µl) added 3 µl T4 DNA ligase buffer, 3 µl BSA (1 mg/ml), 1 µl *Eco*R I adapter, 2.5 U T4 DNA ligase and nuclease free water to a final volume of 30 µl. The reaction mixture was mixed well and incubated at 15°C temperature overnight. To stop the reaction, the reaction mixture was incubated at 70°C for 10 minutes.

2.5. Reaction of Phosphorylation

Thirty microliters of DNA has been ligated with *Eco*RI adapter were added to 4 µl T4 polynucleotide kinase buffer 10X, 2 µl 0.1 mM ATP, 1 µl polynucleotide T4 kinase 10 U and nuclease free water to obtain a final volume of 40 µl. The reaction mixture was mixed well and incubated at 37°C for 30 minutes. The reaction mixture was extracted with phenol chloroform adding as much and blended well with the manner here in after sacker for 30 seconds at a speed of 12,000 rpm centrifuged for 3 minutes. Aquaeus phase was transferred into a new tube and re extraction to obtain maximum results by adding 20 µl TE buffer. Phase aquaeus then added with a half times volume 7.5 M ammonium acetate and two volumes of cold absolute ethanol. The solution was mixed well and incubated at -20°C overnight. To obtain a pellet, a solution was then centrifuged at 3,000 rpm at 4°C for 25 minutes. The supernatant was discarded and the pellet was washed with cold 75% ethanol. Pellets were dried at room temperature until completely dry and resuspensed with 10 µl TE buffer.

2.6. Elimination of Excess Adapters

Sephacryl S-400 which has been thoroughly mixed in a tube is inserted into the column and allowed a few minutes for all solvent buffer down to the bottom. Column tube inserted into the tube and centrifuged at 2500 rpm washing with rotor SW for 5 minutes. Centrifugation is repeated when a column of Sephacryl still not perfectly dry. Column tube is taken and DNA samples inserted into the column using a

micropipette. The addition of DNA samples is done right at the top of the column and in each column maximum volume of 60 µl. Column tube inserted into the collector tube and centrifuged at 2500 rpm for 5 minutes. Supernatant are accommodated in the collector tubes ready for ligation reactions with the vector.The addition of the adapter *Eco*RI use Riboclone®*Eco*R I Adaptor Ligation System. A total of 2.5 µl DNA (100 ng/µl) added 3 µl T4 DNA ligase buffer, 3 µl BSA (1 mg/ml), 1 µl *Eco*R I adapter, 2.5 U T4 DNA ligase and nuclease free water to a final volume of 30 µl. The reaction mixture was mixed well and incubated at 15^{o}C temperature overnight. To stop the reaction, the reaction mixture was incubated at 70^{o}C for 10 minutes.

2.7. Preparation pUC19

Escherichia coli colonies containing pUC19 on the plate so as propagated by culturing in 10 ml of LB media which already added with ampicillin, and incubated at 37°C overnight. After the bacteria grew, coupled with 100 ml of new LB media was added ampicillin, and incubated at 37°C for 2-3 hours to obtain the logarithmic growth phase (OD_{600} ranging from 0.3 to 0.6). Bacteria are harvested by means of centrifugation at 3000 rpm at 4°C for 20 minutes. The supernatant was discarded and the pellet resuspended with 4 ml of lysing solution I, and mixed by means of tossing and turning the tube, then incubated on ice for 5 minutes. A mixture of bacteria and then added with 6 ml of lysing solution II, and mixed as the above and incubated on ice for 15 minutes. After incubation, add 4.5 ml of lysing solution III, and mixed in the same manner as before. The reaction mixture was then incubated on ice for 15 minutes, and centrifuged at 3000 rpm at 4°C for 20 minutes. Aquaeus phase was transferred into a new tube and added phenol CIAA 1: 1, mixed well and centrifuged at 4°C for 20 minutes. Aquaeus phase was transferred into a new tube and added voleme Na acetate 0.1x, 2.5x the volume of cold absolute ethanol. The reaction mixture was incubated at -20°C overnight, and centrifuged at 3000 rpm at 4°C for 25 minutes. The supernatant was discarded, and the pellet was washed by means resuspended with 75% ethanol and centrifuged at 3000 rpm at 4°C for 25 minutes. Pellets were dried at room temperature and diluted with 100 mL of TE buffer. Concentration measured with a spectrophotometer at OD_{260}[15].

2.8. pUC19 digestion with *Eco*RI

Four microliters of pUC19 with a concentration of 1 mg / mL, 2 mL buffer coupled with *Eco*RI, 4 ml *Eco*RI and nuclease free water so that the total volume of the final 20 ml and mixed well. The reaction mixture was then incubated at 37^{o}C for 2 hours. Cutting reaction results was electrophoresed in a 1% agarose gel at 100 Volts.

2.9. Ligation of cDNA Expression Vector

A total of 1 ml pUC19 (200 ng/ml) plus 2 kinase cDNA mL, 1 ml ATP, 1 ml of 10X ligase buffer and 4 µl nuclease T4DNA free water so that the final total volume 10 ml. The reaction mixture was incubated at 15^{o}C for 4 hours. Results of the reaction are stored at -20°C and is ready to be transformed in host cells.

2.10. Transformation and Analysis of Transformants Results

Preparation of competent cells. A single colony of *E. coli* DH5α was cultured into 5 ml LB medium and incubated overnight at 37°C with agitation speed of 200 rpm. Once the bacteria grow, coupled with 25 ml of new LB and incubated for 2-3 hours until the

OD_{260} obtained between from 0.3 to 0.6A total of 3 ml of media containing the bacteria, then harvested in a way centrifuged at 12,000 rpm for 5 seconds, at room temperature. Pellets were washed with sterile distilled water 5 times, 1.5 ml respectively. Pellet resuspended with 200 mL of distilled water and is ready for the process of transformation.

*Transformation by electroporation technique.*Two hundred microliters of solution containing 5 mL of bacterial cells with a plasmid that has been ligated with cDNA sample, and then mixed well. Electroforating done with Gene PulserTM at 2.5 kV. Control the transformation consists of the positive control (*E. coli* with pUC19) and negative control (*E. coli* without pUC19), ligation control and work control (sterility). If the transformation shown in the tool Gene PulserTM under number 13 or above 16, the transformation process is repeated until around 14. Samples were then added new LB media, and mixed well. Samples were incubated at 37°C for 1 hour. After incubation, the samples were centrifuged at 12,000 rpm for 5 seconds, and the supernatant was discarded. Pellet was resuspended in 200 mL LB media new and ready to be planted on the plate in order.

Planting on the plate. Sample results of the transformation process and then grown on LB plates that are already coupled with X-gal, IPTG and ampicillin. Especially for sterility control is not coupled with the substances mentioned above. The number of samples that were planted done in stages ranging from 5 mL, 25 ml, 50 ml and 100 ml, to get the best colonies. Planting is done evenly on all the surface of the plate in order. LB plates that were then incubated at 37°C overnight. Results of bacterial growth in the form of colonies of blue and white, then recombinant colonies (white) were analyzed further.

Analysis of recombinant pUC19. Single colonies were brought pUC19 recombinant cultured into 25 ml LB medium containing ampicillin, and incubated at 37°C overnight with agitation speed of 200 rpm. Plasmid isolation is done in the same manner as in the preparation of plasmid pUC19. Four microliters of recombinant pUC19 with a concentration of 1 µg/ml, coupled with with 2 ml of 10X *Eco*RI buffer, 4 ml *Eco*RI and nuclease free water so that the total volume of the final 20 ml and mixed well. The reaction mixture was incubated at 37°C for 2 hours. Results of cutting reaction electrophoresed on a 1% agarose gel at 100 Volts.The addition of the adapter *Eco*R I use Riboclone®*Eco*R I Adaptor Ligation System. A total of 2.5 µl DNA (100 ng/µl) added 3 µl T4 DNA ligase buffer, 3 µl BSA (1 mg/ml), 1 µl *Eco*R I adapter, 2.5 U T4 DNA ligase and nuclease free water to a final volume of 30 µl. The reaction mixture was mixed well and incubated at 15^{o}C temperature overnight. To stop the reaction, the reaction mixture was incubated at 70^{o}C for 10 minutes.

2.11 Total Protein Isolation

A total of 30 ml culture of recombinant bacteria carrying pUC19 in LB media supplemented with ampicillin, centrifuged at 3000 rpm at a temperature of 4^{o}C for 10 minutes. The pellet was washed with PBSI three times by centrifugation at 3000 rpm at temperature of 4^{o}C for 10 minutes. Pellet diluted with 1 ml PBSI and broken by sonication for 30 seconds 5 times, at intervals of 1 minute. Supernatant obtained by centrifugation of 3500 rpm at temperature of 4^{o}C for 10 minutes. Samples were obtained used for immunoblotting after electrophoresis on SDS-PAGE.

2.12 Immunoblotting

Basic bloter moistened with bloting buffer, and coated stacked with Whatman paper that has been moistened with bloting buffer. Nitrocellulose membrane that has been moistened with bloting buffer placed on Whatman paper stack. SDS gel electrophoresis results placed on nitrocellulose membrane, and labeled with a 20-gauge needle as a sample layout orientation. Gel was closed as much as 4 papers Whatman layer that has been moistened with bloting buffer. Bloter closed with a cover bloter. Transfer of proteins performed on current red indicates the number 500, for 1-2 hours nitrocellulose membrane was removed and washed with TTBS 0.5%, for 30 minutes. Niroselulose membrane was blocked with 1% BSA, and incubated for 1 h with agitation at room temperature. Mouse monoclonal antibodies against protein membrane of *Toxoplasma gondii* local isolates (1: 500) was added and incubated for 1 h with agitation at room temperature. Washing performed 5 times with TTBS 0.05%, respectively washing for 10 minutes with agitation. Nitrocellulose membrane added with goat antimouse IgG alkaline phosphatase conjugate (1: 4000), and incubated for 1 hour at room temperature with agitation. Washing is done 5 times with 0.05% TTBS, each wash for 10 minutes with agitation. Nitrocellulose membrane coupled with a substrate solution containing NBT and BCIP in the dark room. The reaction was stopped by entering a nitrocellulose membrane in distilled water when it happens the formation of color. Nitroseluluse membrane was dried at room temperature and analyzed.

3. RESULTS AND DISCUSSION

The results of transformation use the pUC19 vector by electroporation, two clones of recombinant obtained as shown in Figure 1.

Figure 1. Result of pUC19 transformation in *E. coli* DH5a by electroporation (White colony is recombinant, blue colony is no recombinant)

pUC19 plasmid is one that is often used in genetic engineering. This plasmid has a size of about 2,686 kb, with the restriction to various endonuclease restriction, and the genes that encode markers of ampicillin resistance. The genes express the enzyme β-lactamase to the outside of the host cell. The enzyme catalyzes the hydrolysis reaction of the β-lactam ring causing bacteria transformed with pUC19 become resistant to ampicillin. Its fragments in *E. coli* lacZ genes that produce β-

galactosidase, the pUC19 can be identified by means of screening hystochemical or alpha complementation[15]. Genetically pUC19 lacZ operon having the nucleotide sequence derived from *E. coli*. LacZ operon into 14 first amino origin of β-galactosidase enzyme. If β-galactosidase is active it will hydrolyze a substrate X-gal and chromogenic dye is released to form blue colonies. Insertion of DNA in pUC19 polycloning site will inactivate β-galactosidase and eliminates the ability to complement on bacteria which carry recombinant pUC19 will form white colonies due to not being able to hydrolyze a substrate due to the formation of β-galactosidase enzyme active[15].

Low number of recombinant due to several factors such as the amount of cDNA that is too little. In just a few percent cDNA synthesis mRNA is synthesized into cDNA. Comparison between the cDNA plasmid that is not balanced will affect the outcome of the transformation, as well as some other factors in the transformation process.

From the results obtained recombinant bacterial multiplication recombinant pUC19 which can be seen in Figure 2.

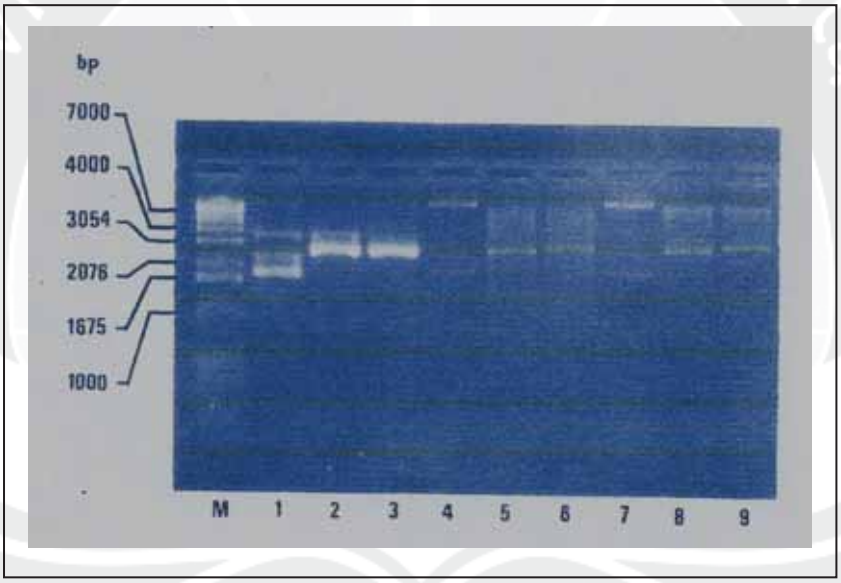

Figure 2. Results of electrophoresis of pUC19 recombinant with cutting endonucleases restriction of *Eco*RI and *Hind*III

(M, marker; 1, pUC19; 2, *Eco*RI cut pUC19; 3, pUC19 cut with *Eco*RI; 4, pUC19 recombinant 1; 5, pUC19 recombinant 1 cut with *Eco*RI; 6, pUC19 recombinant 1 cut with *Hind*III; 7, pUC19 recombinant 2; 8, pUC19 recombinant 2 cut with *Eco*RI; 9, pUC19 recombinant 2 cut with *Hind*III)

After electrophoresed on 1% agarose gel, the recombinant plasmid 1 and recombinant 2 shows the same banding pattern, but greater than the control pUC19. The big difference plasmid ribbon before being cut with restriction endonucleases enzyme, can be clarified with the recombinant plasmid cutting results with certain restriction endonucleases. Insertion of cDNA done on the restriction *Eco*RI know, so expect to cutting with the same enzymes obtained a piece or some pieces on fragment inserts. Results of electrophoresis on agarose gel 1% is not visible any fragments cutting inserts. The possibility of invisibility of long pieces of DNA fragments as small insertions, or DNA cut up into small fragments on a place known

by *Eco*RI, because the cloned DNA fragment contains the base sequence is unknown constituent. To prove the existence of inserts, used cutting with restriction endonucleases *Hind*III which recognize one specific side of the polycloning site on pUC19. Results showed that the obtained ribbon cutting linear larger than the control pUC19, thus allegedly carrying two recombinants cDNA having the same length, but not necessarily have the same base sequences.

Results recombinant protein immunoblotting 1 and 2 with several monoclonal antibodies can be seen in Figures 3 and 4.

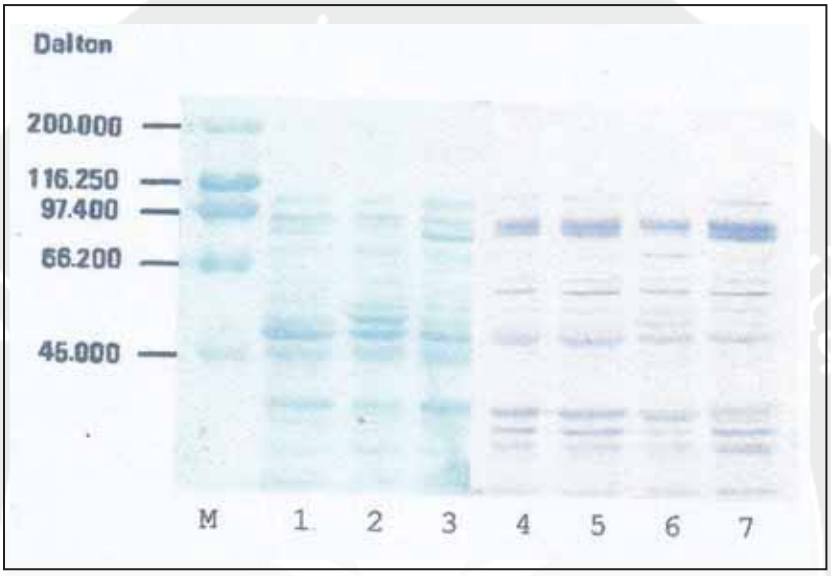

Figure 3. Result of SDS-PAGE and Immunoblotting of recombinant 1 proteins
(M, 1,2,3 Amidoblack staining; 3,5,6,7 the addition of monoclonal antibodies P55,1F6, D1, D9; M, protein marker; 1,4 protein of *E. coli* DH5α; 2,5 protein of *E. coli* DH5α with pUC19; 3,6,7 protein of *E. coli* DH5α with pUC19 recombinant).

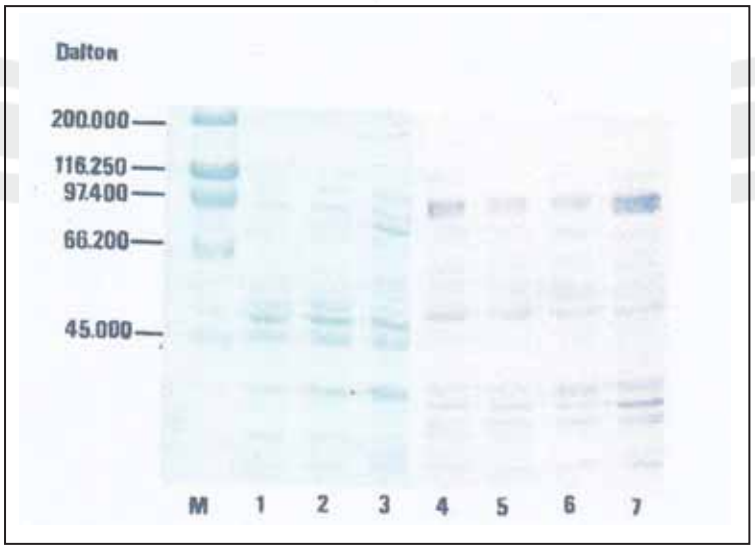

Figure 4. Result of SDS-PAGE and Immunoblotting of recombinant 2 proteins
(M, 1,2,3 Amidoblack staining; 3,5,6,7 the addition of monoclonal antibodies P55,1F6, D1, D9; M, protein marker; 1,4 protein of *E. coli* DH5α; 2,5 protein of *E. coli* DH5α with pUC19; 3,6,7 protein of *E. coli* DH5α with pUC19 recombinant)

Both recombinant proteins produced after reacted with monoclonal antibodies showed several bands with variations of a few bands in accordance with the monoclonal antibody used. These bands are also found in the control of bacterial proteins that carry pUC19 and who do not carry pUC19. This reaction occurs because monoclonal antibodies recognize specific epitopes on proteins. A minimal epitope consisting of amino acids making up 4-6, so there is the possibility of being used for screening antibodies recognize similar epitopes on proteins from bacteria. Monoclonal antibodies are antibodies produced by one type of cells (B lymphocytes) which are specific and homogeneous in nature, class, affinity and specificity, being a polyclonal antibody is an antibody that is heterogeneous who knows some epitope of an antigen.

The amino acid sequence of a protein in a species or other species in certain parts of the same possibilities. The existence of common epitopes on the protein will be known monoclonal antibodies that recognize the same epitope. [16]SAG1 and SRS2 on *Toxoplasma gondii* has a sequence that is similar to the NCP 29 and NCP 35 in *Neospora caninum*. Both protein in *Toxoplasma gondii* and *Neospora caninum* both have 12 amino acid cysteine residues. The use of monoclonal antibodies specific for clone selection purposes must have a high specification against epitopes on the protein expected but on the other epitopes. Another constraint that the foreign DNA proteins expressed in *E. coli* has a specific conformation or less different from the proteins that directly expressed by *Toxoplasma gondii* tachyzoite. Protein conformational epitopes will determine if the conformational change will lead to changes in the epitope of a protein that monoclonal antibodies do not recognize the epitope. [17]Antibodies against P30 P30 only recognize native nonreduced expressed *E. coli*. One of the obstacles the use of the expression vector pUC19 was not issued cell protein expression in sufficient quantities, and screening of bacterial colonies can not be done, so as to obtain the expected protein must break up the cells. Screening cDNA clones that carry *Toxoplasma gondii* monoclonal antibodies that are not specific to membrane protein of *Toxoplasma gondii* can not detect clones were expected. Another alternative to determine the clones which carry cDNA encoding a membrane protein of *Toxoplasma gondii* is using a vector that can express the protein out of the host cell and the antibody used for the screening does not react with the host cell expressed proteins.

4. CONCLUSION

Cloning of the gene encoding a membrane protein takizoit *T. gondii* using pUC19 in *E. coli* DH5a host got two recombinants.

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Accumulation of Mercury in Legume Cover Crops Inoculated with Rhizosphere Microbes

*Hanna Artuti Ekamawanti and Wiwik Ekyastuti
Department of Forestry, Faculty of Forestry, Tanjungpura University, Pontianak, Indonesia
Email: hanna.artuti@gmail.com

Abstract

Legume cover crops (LCC), such as *Pueraria javanica* (kacang ruji) and *Centrosema pubescens* (sentro) and rhizosphere microbes, such as arbuscular mycorrhizal fungi (AM fungi) and mercury-resistant bacteria (MR bacteria) have an ecological role in degraded land and mercury (Hg) contaminated soil. An experimental study to evaluate the ability of Hg accumulation in LCC inoculated with rhizosphere microbes had been conducted. AM fungi and BRM isolates were inoculated on *P. javanica* and *C. pubescens* seedlings grown on media of tailings and compost mixture (1:1) and treated with $HgCl_2$ (concentration of 2.5 ppm, 5 ppm, and 10 ppm) for 8 weeks. The growth of both LCC non-inoculated and inoculated with rhizosphere microbes were not inhibited by Hg exposure (the tolerance index >100%). Mercury was accumulated in the aerial part (0.07-0.89 mg kg^{-1}) higher than in the roots of non-inoculated *P. javanica*. Instead, *P. javanica* did not accumulated Hg in the plant tissue when inoculated with rhizosphere microbes (as shown by bioconcentration factor of shoots and roots were 0.0). On the other hand, *C. pubescens* tended to accumulate Hg in the aerial part (0.79-0.89 mg kg^{-1}) when inoculated with rhizosphere microbes (arbuscular mycorrhizal fungi/AM fungi and mercury-resistant bacteria/MR bacteria). Therefore, both species of LCC, with or without rhizosphere microbes association, have potency as phytoremediation agents of Hg-polluted soils.

Keywords: arbuscular mycorrhizal fungi, *Centrosema pubescens*, mercury-resistant bacteria, phytoremediation, *Pueraria javanica*

1. INTRODUCTION

Mercury (Hg) is a toxic heavy metal that is dangerous because of its toxicity, mobility, metilation, and its bioaccumulation and biomagnification in food chain of living cells (Rodríguez *et al*., 2003). There are numerous options for the remediation of polluted sites, such as Hg-polluted soils. One approach is phytoremediation technology (Raskin dan Ensley 2000) that involves a variety of biological processes using plants and rhizosphere microbes (such as fungi and bacteria) (Salt *et al*., 1998). In polluted soils, Hg is a non-degradable toxic heavy metal pollutant when it is accumulated by plants (Ling *et al*., 2010). Identification of tolerant plants, which could clean up toxic metals-polluted soils, is the first step necessary for the development of phytoremediation technology. Basic of phytoremediation is using the plant rhizosphere microbial communities to support and facilitate the absorption of some metals (Henry, 2000), included mercury.

Arbuscular mycorrhizal fungi (AM fungi) as potential mycosymbiont could be used synergistically with plants as phytosymbiont in soil remediation of heavy metal-

polluted soil (Gaur dan Adholeya, 2004). Vosatka (2001) stated that the AM fungi is considered as a vital component of the phytoremediation system because it could be actively or passively involved in phytoextration, phytodegradation, phytostabilisation or phytoexclusion and could stimulate the activity of other microbes in the rhizosphere. Schiering *et al.,* (1991) proved that a group of rhizosphere bacteria can detoxify organic-Hg and Hg-salts through organomercury lyase enzyme activity (MerB) and mercuric ion reductase (MerA). The mercury-resistant bacteria (MR bacteria) can be resistant to Hg because it has a detoxification mechanism by reducing the toxic organic-Hg into Hg^{2+} then into Hg^{0} which then becomes volatile and non-toxic through a series of enzymatic reactions (Andrea *et al.,* 2003; Barkay *et al.,* 2003).

Freidland (1990) recommended that suitable plant species for phytoremediation should have one of the following charactetistics: (a) a low biomass plant with a very high metal accumulation capacity or (b) a high biomass plant with enhanced metal uptake potential. Metal tolerant plants with lower metal accumulation are preferred for phytostabilization and heavy metal hyperaccumulators are the best choice for phytoextraction (Cobbet 2000; Clemens 2001). Based on their high biomass, the use of legume cover crop, such as *Pueraria javanica* Benth. (kacang ruji) and *Centrosema pubescens* Benth. (sentro), could be suggested. *P. javanica* is belong to sub-family Papilionaceae of plant Leguminosae (GRIN 2015). *C. pubescens* was developed in the area of ex-mining for revegetation program of Pomalaa project, Center Sulawesi, relatively resistant to drought due to deep root system, easy to grow on various types of soil (from sand to clay), poor drainage, as well as grows well in a pH range from 4.9 to 5.5 (https://greenmining.wordpress.com/2009/07/04/centrosema-pubescens-purple-butterfly/ 2015). The usefulness of these plants is as forage, weed control, and in soil conservation, such as erosion control. However, if these crops are being grown in the Hg-contaminated area, we need to investigate their capability to accumulate Hg in their tissues. This experiment was established to determine whether mercury ($HgCl_2$) treatment and rhizosphere microbes inoculation had a marked effect on Hg accumulation in *P. javanica* and *C. pubescens* plant tissues.

2. METHODS

2.1. Materials

Seeds of *P. javanica* and *C. pubescens* from Bogor. Indigenous AM fungi inoculum, that was propagated using maize as host plant in river sand media. Isolate of MR bacteria, i.e. *Pseudomonas* sp.1 (HgTL2). KOH 10%, HCl 2%, trypan blue (0.05%) in lactoglycerol for root samples staining; $HgCl_2$; river sands (diameter 1-2 mm) for culture media of AM fungi, tailing and compost for growth media.

2.2. Procedures

Growth media preparation. Sterilized tailing (autoclaved at 121 C°, 1 atm. for 15 min) was mixed with compost (ratio 1:1). This mixture was used as growth media. For Hg treatment, the tailing-compost mixture was added with $HgCl_2$ solution according to the treatments (2.5; 5; and 10 ppm) and incubated at room temperature (at about 20ºC) for 4 weeks allowing Hg to distribute into various fractions. During the period, soil moisture content was carefully monitored. Furthermore, this media was filled into polyethylene bag (15 cm diameter x 20 cm diameter).

Seed germination

Seeds of *P. javanica* and *C. pubescens* were purchased from a local seed company, Bogor, West Java, Indonesia. Seeds surface were sterilized for 5 min in NaOCl l5% (v/v), then soaked in water at 80°C for 1-2 minutes. Seeds were sown and germinated in germination container containing AM inoculated and non-inoculated river sand for 2 weeks.

Rhizosphere microbes inoculum preparation and inoculation

The microbes (AM fungi and MR bacteria) were isolated from rhizosphere of pioneer plants in ex-gold mining area Mandor, West Kalimantan. *Zea mays* (maize) was cultivated in river sands media to propagate the AM fungi under screen house condition for 90 days. The roots, river sand, and spores were used as the AM fungi inoculum. First inoculation of AM fungi was accomplished by layering technique in germination container when seeds were germinated. Second inoculation was accomplished by adding 5 g/polyethylene bag of the AM inoculum to seedling when was transplanted. MR bacteria were cultured in Canstein media (Canstein et al. 2002) by agar plate culture method (Anas 1989). Calculation of the bacterial population was done by pour plate count method (Somasegaran and Hoben 1994). MR bacteria isolate was inoculated as much as 10 mL of the number colonies 226 x 10^6 cfu/mL to seedling when was transplanted.

Experiments

Each legume species was a split experimental unit using factorial experiment with basic pattern of completely randomized design, with treatment combinations of 1) four $HgCl_2$ concentration (0 ppm; 2.5 ppm; 5 ppm; and 10 ppm); 2) two rhizosphere microbes inoculation (non-inoculated and inoculated with AM fungi and MR bacteria). Each treatment combinations had 3 replications and give total 24 pots for each species. NPK fertilizer was given as a basis fertilizer in each polyethylene bag, one week after planting. All the seedlings were placed and maintained in screen house. Aerial parts and roots were separately harvested 8 weeks after transplanting.

Variables measurement

Variables measurement was fresh weight (g/plant) of aerial part and root, and Hg concentration (mg/kg) in aerial part, root and growth media (tailing and compost mixture). Total fresh weights of seedlings were used for tolerance index (%) calculation, i.e. comparison of total fresh weight of seedlings treated with Hg and total fresh weight of control seedlings (untreated Hg) (Rabie 2005). Some variables were calculated to study the accumulation and mobility of Hg in plant tissue, such as bioconcentration factor (BF) and translocation factor (TF). The bioaccumulation factor (BF) refers to metal accumulation in plant tissue from soil/media. BF in root or aerial part was calculated as the ratio of Hg concentration in root/aerial plant and Hg concentration in media (Moreno-Jiménez *et al*., 2007). BF >1 indicates that metal accumulation in plant tissue is higher than in soil/media. The translocation factor (TF) for metal within plant expresses metal mobility in plant, which shows metal translocation properties from roots to aerial part shoots (Stoltz and Greger 2002). TF was calculated as the ratio of Hg concentration in aerial part and root. TF >1 indicates that metal translocation was high to aerial part.

Analytical methods

Root and aerial part dried samples were mashed with mortar and pestle. Then, Hg concentration was measured using a reference to the SM Ed. 20, 1998 (AOAC). Analyses were performed in the Sucofindo laboratory, Pontianak.

Assessment of AM colonisation

The roots were cleared in 10% KOH (100 g L^{-1}) at 80ºC for 15 minutes, acidified with 2% HCl for 24 hours, and stained with 0.05% trypan blue in lactoglycerol (Koske and Gemma 1989). Percentage of AM colonization was examined by the slide method (McGonigle *et al*., 1990) under a compound microscope at 200x magnification.

Statistical analysis

Statistical significance of treatments combination was analysed using CoStat 6.4 (CoHort Software, Monterey, CA, USA) software for analysis of variance (ANOVA), after doing the variance homegenity test (Bartlett's test). Further analysis was performed using the Duncan's test ($p < 0.05$). Data transformation was done using Minitab 16 software.

3. RESULTS AND DISCUSSION

Observations during the study and at the harvest revealed that visually, there were no symptoms of toxicity or deficiency, such chlorosis and necrosis, on tested plants grown in Hg-polluted media. The results obtained during this research had shown that the fresh weight of *P. javanica* and *C. pubescens* plants, either aerial part or root, were not significantly affected by the Hg treatments and microbes inoculation (Table 1). This is an early indication that the two legume cover crops species are tolerant to Hg up to 10 ppm for 8 weeks of Hg exposure time. The high tolerance of *P. javanica* and *C. pubescens*, either inoculated or non-inoculated plants, to Hg exposure up to 10 ppm were shown by tolerance index >100% (Table 1).

However, there was a different response to Hg between non-inoculated and inoculated plants of each species. The tolerance index of inoculated *P. javanica* was 115-177% higher than the tolerance index of non-inoculated one. In contrast, the tolerance index of non-inoculated *C. pubescens* plant was 108-136% higher than the tolerance index of inoculated one with the increase of $HgCl_2$ treatment. Hg influence on the growth and metabolism of plants varies depending on the concentration and the status of Hg in the plant tissue (Patra and Sharma 2000), either by the presence or absence of microbes in rhizosphere.

The development of AM symbiosis in *P. javanica* and *C. pubescens* root was not inhibited by Hg exposure, as shown by the percentage of mycorrhizal colonization in root plant treated with $HgCl_2$ was not different with non-treated one (Table 1). However, mycorrhizal colonisation in *P. javanica* and *C. pubescens* were categorized into low class (<50%). This might be caused the AM fungi have not been optimally developed for 8 weeks, either with or without Hg exposure.

Table 1. The effect of mercury and rhizosphere microbes on fresh-weight yield of *P. javanica* and *C. pubescens*, tolerance index, and mycorrhizal colonization (8 weeks after planting)

$HgCl_2$ (ppm)	Rhrizosphere microbes	Fresh weight (g/plant)		Tolerance index (%)	Mycorrhizal colonisation (%)
		Aerial part	Root		
P. javanica					
0	Without	6.67	0.001	-	0
	With	6.70	0.001	-	29
2.5	Without	6.93	0.001	105.5	0
	With	8.07	0.001	120.8	32
5	Without	5.17	0.001	77.7	0
	With	9.20	0.001	137.9	38
10	Without	8.40	0.001	126.1	0
	With	8.30	0.001	124.5	27
Significance [a] of:					
$[HgCl_2]$		ns	ns		
Microbes		ns	ns		
$[HgCl_2]$ x Microbes		ns	ns		
C. pubescens					
0	Without	7.56	0.97	-	0
	With	8.00	0.47	-	6
2.5	Without	10.70	1.17	139.1	0
	With	8.07	0.60	102.4	28
5	Without	9.57	0.67	119.9	0
	With	7.30	0.67	94.1	20
10	Without	9.87	0.90	126.2	0
	With	8.93	0.97	116.9	41
Significance [a] of:					
$[HgCl_2]$		ns	ns		
Microbes		ns	*		
$[HgCl_2]$ x Microbes		ns	ns		

[a]Analysis of variance showed signicant difference at: * p<0,05; ns, non significant.

Hg concentration in aerial part of *P. javanica* was affected significantly by $HgCl_2$ treatments and rhizosphere microbes inoculation (Table 2). *P. javanica* did not accumulate Hg in plant tissue, either in non-inoculated or inoculated plants, as shown by the BF value <1 or BF = 0.0. Hg was not accumulated in AM plant root of *C. pubescens* (the BF of root <1) when exposed to Hg up to 5 ppm. It indicated that the rhizosphere microbes inhibited Hg accumulation in plant tissues (aerial part and root) of *P. javanica* and *C. pubescens*. In *P. javanica* plant inoculated with rhizosphere microbes, the Hg concentration in aerial part treated with 2.5 to 10 ppm $HgCl_2$ was not significant difference compared to control plant (without $HgCl_2$ treatment) (Table 3). In addition, the Hg concentration in aerial part of non-inoculated plant was higher than in aerial part of inoculated plant when the plants were treated with $HgCl_2$ up to 5 ppm. These results showed that the presence of rhizosphere microbes inhibited Hg accumulation in the aerial part, even though the plants were exposed to Hg in media.

Table 2. Effect of mercury and rhizosphere microbes on Hg concentration in plant tissues of *P. javanica* and *C. pubescens*, bioconcentration factor, and translocation factor (8 weeks after planting)

$HgCl_2$ (ppm)	Rhizosphere microbes	[Hg] (mg/kg)		Bioconcentration factor		Translocation factor
		Aerial part	Root	Aerial part	Root	
P. javanica						
0	Without	0.872	0.001	0.9	0.0	0.1
	With	0.001	0.001	0.0	0.0	1.0
2.5	Without	0.877	0.001	0.7	0.0	0.3
	With	0.001	0.001	0.0	0.0	1.0
5	Without	0.886	0.001	0.6	0.0	3.3
	With	0.001	0.001	0.0	0.0	0.0
10	Without	0.067	0.001	0.0	0.0	0.3
	With	0.001	0.001	0.0	0.0	0.0
Significance [a] of:						
$[HgCl_2]$		**	-			
Microbes		**	-			
$[HgCl_2]$ x Microbes		**	-			
C. pubescens						
0	Without	0.876	5.818	17.9	118.7	0.2
	With	0.892	0.001	2.5	0.0	892.0
2.5	Without	0.880	3.443	13.3	52.2	0.5
	With	0.843	0.001	2.6	0.0	842.7
5	Without	0.843	0.266	843	266.0	298.6
	With	0.823	0.053	4.3	0.3	527.9
10	Without	0.881	0.250	6.8	1.9	295.9
	With	0.788	1.999	3.5	8.8	264.4
Significance [a] of:						
$[HgCl_2]$		ns	*			
Microbes		ns	**			
$[HgCl_2]$ x Microbes		ns	**			

[a]Analysis of variance showed signicant difference at: ** p<0.01; * p<0.05; ns, non significant.

Previous result of research by Yu *et al*., (2010) showed the role of AMF (*Glomus mosseae*) was in decreasing of Hg concentration in maize (*Zea mays*) root when Hg was applied at the rates of 2.0 to 4.0 mg/kg. We predicted that AM fungi adsorbed Hg on the surface of external hyphae, stored Hg in vacuola of spore, and chelating by glomalin (glycoprotein) (Smith and Read 2008) thus Hg absorption by root was restricted. In addition, reducing Hg^{2+} to Hg^0 by MR bacteria caused Hg in soil could be evaporated into the atmosphere allowed Hg accumulated in plants was also limited (Gadd 1990).

In contrast with *P. javanica*, the Hg concentration in aerial part of *C. pubescens* was not affected by $HgCl_2$ treatments and microbes inoculation (Table 2), but affected significantly to Hg concentration in root. The BF of aerial part >1 indicated that *C. pubescens*, either inoculated or non-inoculated plants, absorbed and accumulated Hg in aerial part (Table 2). At the absence of rhizosphere microbes, *C. pubescens* accumulated Hg in root. It indicated that *C. pubescens* tend to accumulate Hg when the AM fungi and MR bacteria, was absence in the rhizosphere. The response of plant to Hg exposure varies in each plant species and the presence or absence of

microbe activities in the rhizosphere (Patra and Sharma, 2000). Rhizosphere microbes inoculation decreased Hg concentration when root of *C. pubescens* was exposed to Hg up to 5 ppm (Table 3). On the contrary, the Hg concentration in inoculated root was higher than in non-inoculated root when plants were exposed to 10 ppm $HgCl_2$. The Hg concentration in non-inoculated root was decreased with the increase of $HgCl_2$ concentration, whereas the Hg concentration in inoculated root tended to increase with the increase of $HgCl_2$. This result show that up to 5 ppm $HgCl_2$, the presence of rhizosphere microbes inhibited Hg absorption by plant root.

Table 3. Hg concentration in aerial part of *P. javanica* and in root of *C. pubescens* inoculated with rhizosphere microbes and treated with mercury (8 weeks after planting)

$HgCl_2$ (ppm)	Rhizosphere microbes	
	Without	With
P. javanica	..... Hg concentration in aerial part (mg/kg)	
0	0.872 a (A)	0.001 a (B)
2.5	0.877 a (A)	0.001 a (B)
5	0.886 a (A)	0.001 a (B)
10	0.067 b (A)	0.001 a (A)
C. pubescens	..... Hg concentration in root (mg/kg)	
0	5.818 a (A)	0.001 a (B)
2.5	3.443 ab (A)	0.001 a (B)
5	0.266 c (A)	0.053 a (B)
10	0.250 c (A)	1.999 b (B)

Significant differences among $HgCl_2$ treatments are indicated by small different letter and among microbes inoculation treatments with different capital letters (mean ± SD, n = 3; Duncan's test, $p < 0.05$).

The mobility of Hg in *P. javanica*, both non-inoculated and inoculated plant, was very restricted, as shown by the TF value <1 or TF value = 1 (Table 2). Even though Hg was accumulated in non-inoculated roots, the translocation of Hg to aerial part from root was very limited. The accumulation of Hg in a larger amount in the roots than in the aerial part also found in several other plant species such as *Enterolobium cyclocarpum* (sengon buto) seedlings (Ekamawanti *et al*., 2013), *Sesbania drummondii* seedlings (Israr *et al*., 2006), and the tree mastic and tamarisk (Moreno-Jimenez *et al*., 2009). The restrictions of Hg translocation to the aerial part from root showed that the plant using a strategy of exclusion (Baker, 1981) to be tolerant to Hg. This result showed that *P. javanica* was not an accumulator of Hg but an excluder plant with presence or absence of rhizosphere microbes. As an excluder plant, *P. javanica*, either inoculated or non-inoculated with rhizosphere microbes, could be used in Hg phytostabilisation in the Hg-polluted soil.

In contrast with *P. javanica*, Hg accumulation by *C. pubescens* plant was largely translocated to shoot as shown by general TF values > 1 (Table 2). It showed that microbes inoculation increased Hg translocation from root to aerial part of plant. Exception was occurred on non-inoculated plants, both treated with 2.5 ppm $HgCl_2$ and non treated plants (TF value < 1). The most of Hg which distributed to the aerial part of *C. pubescens*, both not-inoculated and inoculated plant, indicated that

accumulation strategy was used by plant to be tolerant to Hg. Baker (1981) stated that accumulation is one of the plant strategies to be a tolerant to heavy metals.

4. CONCLUSIONS

In conclusion, this study showed *P. javanica* was an Hg excluder plant, whereas *C. pubescens* was an Hg accumulator plant. As an excluder plant, *P. javanica* did not accumulate Hg in the aerial part of plant, whereas as an accumulator plant, *C. pubescens* accumulate Hg more in aerial part than in root. The presence of rhizosphere microbes in Hg-polluted media will inhibit the translocation and accumulation of Hg into the aerial part of plant. These results indicate that the both types of legume cover crops, with or without rhizosphere microbes, are potential to become candidate phytoremediation agents of Hg-polluted soil, such as in ex-gold mining area in Indonesia. Considering the different physiological response of each species inoculated with rhizosphere microbes under Hg exposure, more works need to be done to develop the potency of legume cover crop and rhizosphere microbes (AM fungi and MR bacteria) as agents of phytoremediation of Hg-polluted soil.

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ISSN: 2460-8238

Universitas Atma Jaya Yogyakarta
Jl. Babarsari No. 44, Yogyakarta 55281, Indonesia
Telp.: +62-274-487711 (hunting), Fax. +62-274-487748