**JURNAL**

# EFISIENSI TRANSFORMASI PLASMID pTA7002-*AtRKD4* PADA *Escherichia coli* BL21(DE3) DENGAN METODE KEJUTAN PANAS

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# HEAT-SHOCK TRANSFORMATION EFFICIENCY OF pTA7002-*AtRKD4* INTO *Escherichia coli* BL21 (DE3)

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## ABSTRACT

Plasmid transformation into a host cell is one of an important method in gene cloning and usually used heat-shock metho, which can be affected by heat-shock time and temperature. This research main goal is to know how these two factors affected heat-shock transformation of pTA7002-*AtRKD4* using *E. coli* BL21(DE3) as a host cell. This research have the following step, such as pTA7002-*AtRKD4* isolation from *Agrobacterium tumefaciens* EHA105, plasmid purity and quantity test, detection of *HPT* and *AtRKD4* gene, competent cell, plasmid heat-shock transformation using randomized factorial design with two variables (heat-shock temperature (42$^{o}$C, 44$^{o}$C, and 46$^{o}$C) and heat-shock time (30s, 60s, 90s, 120s, and 240s), triplicate), bacterial selection with kanamycin and hygromycin, and determination and statistical analysis of pTA7002-*AtRKD4* transformation efficiencies. Based on transformation efficiency calculation, the optimum heat-shock transformation is 44$^{o}$C for 30 seconds.

Keywords: *Escherichia coli*, heat-shock transformation, transformation efficiency

## INTRODUCTION

A plasmid is a double stranded, circular, and small DNA molecule. A plasmid can take genes apart from the bacterial chromosome. It is used as a cloning vector for making many copies of gene and producing protein in big scale (Reece et al., 2011).

One of the steps for gene cloning is transformation (Brown, 2010). Heat-shock transformation is using a principle of high-temperature shock in seconds at bacterial cell to make plasmid DNA enter the cell. This method is usually done at 42$^{o}$C for 90 seconds (Hanahan, 1983) or 2 minutes (Sambrook and Russel, 2001). On the other hand, the transformation efficiency of this method can be affected by the modification of heat-shock time for 30 seconds (Inoue et al., 1990), 45 seconds (Froger and Hall, 2007), 60 seconds (Hanahan, 1983), or 240 seconds (Yoo, 2010) and heat-shock temperature at 44$^{o}$C, 46$^{o}$C, 48$^{o}$C, 50$^{o}$C or 52$^{o}$C (Bergès and Barreau, 1989). Because of that, there are still no optimum heat-shock time and temperature for plasmid transformation.

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pTA7002-*AtRKD4* is a modified natural plasmid of *Agrobacterium tumefaciens* that carried *AtRKD4* gene. This plasmid has resistance to kanamycin and hygromycin (Zuo et al., 1999). Meanwhile, *AtRKD4* is a gene that induces the production of somatic embryo on seeds (Nakajima et al., 2010). This research will clone pTA7002-*AtRKD4* using *Escherichia coli* BL21(DE3).

*Escherichia coli* is used as plasmid transformation's host cell because it can replicate itself quickly and can grow on simple or complex medium (Casali and Preston, 2003). *E. coli* BL21(DE3) is used for transformation because it transformation efficiency is quite high, which is $1{,}9 \times 10^5$ CFU/ng/mL for pBR322 (Liu et al., 2014). This bacteria strain is also used for another plasmid transformation, such as pRSETB (Saraniya et al., 2012), pET-32b(+)-IFN α2a (Kusumawati et al., 2013), and pGEM-T (Retnoningrum et al., 2010).

## RESEARCH METHODOLOGY

### Materials

The main material that is used in this research is pTA7002-*AtRKD4* extracted from *Agrobacterium tumefaciens* EHA105 and *Escherichia coli* BL21(DE3). The bacteria is grown on Luria-Bertani media with rifampicin, kanamycin, or hygromycin. A competent cell is made with $CaCl_2$ solution. PCR step is done using KappaTaq *Ready Mix* 2X and KOD FX Neo. Meanwhile, the primer sequence used in this research is on Table 1.

Table 1. Primer types and sequences

| Primer | Sequences | Amplified gene fragment | References |
|---|---|---|---|
| 22,4F | 5'-AGGTAATCGAAATCCCAGGGAGC-3' | *22,4* | Bauer et al. (2007) |
| 22,4R | 5'-ATCTACGTTTAGTTAACGAGGTTAGC-3' | | |
| HPT *F* | 5'-ACGACGGTCTCATTTCCAAC-3' | *HPT* | Mursyanti et al. (2015) |
| HPT *R* | 5'-CTCTTCCATTCCAACATTCTTGAG-3' | | |
| AtRKD4 *F* | 5'-TCGGACGATTGAGTCGCATC-3' | *AtRKD4* | Mursyanti et al. (2015) |
| AtRKD4 *R* | 5'-AGGCTATGGATGCGATCGACTG-3') | | |

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**Research method**

pTA7002-*AtRKD4* is extracted from *Agrobacterium tumefaciens* EHA105 using Presto™ Mini Plasmid Ki and verified with electrophoresis. pTA7002-*AtRKD4* purity and quantity are calculated by measuring its absorbance at 260 nm and 280 nm (Casali and Preston, 2003). After that, the existence of *HPT* and *AtRKD4* gene on pTA7002-*AtRKD4* is detected using colony PCR with Kappa Taq *Ready Mix* 1x, 10 µM primer F, 10 µM primer R, and dd$H_2O$ for each gene (Sambrook and Russel, 2001).

*E. coli* BL21(DE3) competent cell is made with 3% of starter at $OD_{600}$ 0,4 with agitation on 220 rpm (Sezonov et al., 2007) using $CaCl_2$ solution. The next step is plasmid transformation into competent cell using heat-shock with temperature (42°C, 44°C, and 46°C) and time treatment (30 seconds, 60 seconds, 90 seconds, 120 seconds, and 240 seconds), triplicate (Sambrook and Russel, 2001). Negative control (no plasmid transformed) is used for each temperature treatment for 240 seconds. After that, transformant is selected using kanamycin and hygromycin and detected the existence of *22,4* and *HPT* gene (Casali and Preston, 2003). The single colony grown from antibiotic selection is used to calculate the transformation efficiency using the formula

$$\text{Transformation efficiency (CFU/µg)} = \frac{\text{Sum.of growth single colony(CFU)}}{\text{plasmid mass that has been spread on the plate (µg)}}$$

The following step is to analyze the transformation efficiency with ANAVA and DMRT test using SPSS 22.0 to determine real difference between heat-shock temperature and time treatment (Lim et al., 2015).

**RESULT AND DISCUSSION**

A. pTA7002-*AtRKD4* plasmid isolation

pTA7002-*AtRKD4* isolated from *Agrobacterium tumefaciens* is verified with electrophoresis (Figure 1). The isolated plasmid form two band with size more than 10000 bp. Das and Dash (2015) state that plasmid isolation will be separated based on its molecular weight. An isolated plasmid with supercoil form has the biggest molecular weight so it migrates the slowest. Meanwhile,

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bacterial genome with linear form has lighter molecular weight so it migrates faster.

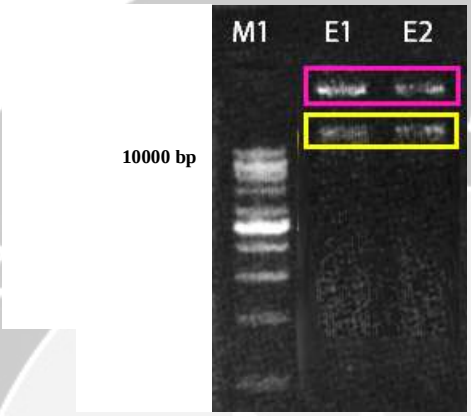


Figure 1. Isolated pTA7002-*AtRKD4* electrophoresis result
M1 = DNA *Ladder* 1 Kb; E1= Isolated plasmid 1; E2 = Isolated plasmid 2; Red box signify pTA7002-*AtRKD4*; Yellow box signify bacterial genome

Isolated pTA7002-*AtRKD4* is measured at 260 nm ($A_{260}$) and 280 nm ($A_{280}$) using spectrophotometer. pTA7002-*AtRKD4* purity ($A_{260}/A_{280}$) and concentration is at Table 2. The plasmid purity is lower than 1,8. This result indicates that the isolated plasmid is not pure because it has protein contamination (Das and Dash, 2015). On the other hand, it still can be used for transformation because it has supercoil form that stable and unaffected from protein contamination (Sinden, 2012).

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Table 2. Absorbance, purity, and concentration of isolated plasmid

| | |
|---|---|
| $A_{260}$ | 1,837 |
| $A_{280}$ | 1,561 |
| $A_{260}/A_{280}$ | 1,177 |
| pTA7002-*AtRKD4* concentration (µg/mL) | 2.755,5 |

Molecular detection of isolated plasmid is done for *HPT* and *AtRKD4* gene. Amplification result shows that the isolated plasmid has *HPT* gene at 500 bp and *AtRKD4* gene at 382 bp (Figure 2). The results indicate that the isolated plasmid is pTA7002-*AtRKD4*.

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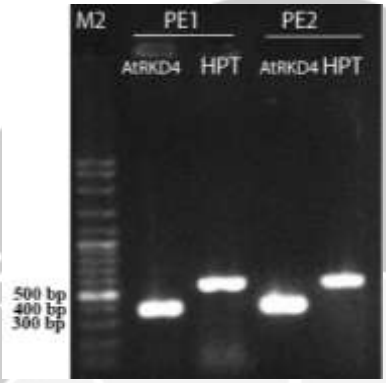


Figure 1. Molecular detection of isolated PTA7002-*AtRKD4*
M2 = DNA *Ladder* 100 bp plus; PE1 = PCR of isolated plasmid 1; PE2 = PCR of isolated plasmid 2; *HPT* gene at 500 bp; *AtRKD4* gene at 380 bp

B. pTA7002-*AtRKD4* transformation into *Escherichia coli* BL21(DE3)

Plasmid transformation needs a competent cell. *E. coli* BL21(DE3) competent cell is made using $CaCl_2$. This solution will increase bacterial cell wall permeability and bind plasmid into cell wall. On the other hand, competent cell is made on cold condition to maintain its competency. After that, heat-shock treatment will make plasmid entering the bacterial cell and close its cell wall (Das and Dash, 2015).

The transformed *E. coli* BL21(DE3) is selected using LB/Kan and LB/Hyg. All treatment has single colonies growth on LB/Kan (Figure 3a) and LB/Hyg (Figure 3b) so it shows that the transformation is successfully done. There is no single colony growth on negative control medium (Figure 3c.). Single colonies then used to calculate transformation efficiency.

Single colonies from every treatment are used to detect the existence of *22,4* gene on *E coli* BL21(DE3) with colony PCR by mix one single colony into PCR mix. The amplification result shows that each transformant has *22,4* gene at 232 bp (Figure 3). It indicates that the transformants are *E. coli* BL21(DE3). *22,4* gene is a specific gene on *E. coli* BL21(DE3) genome (Bauer et al., 2007).

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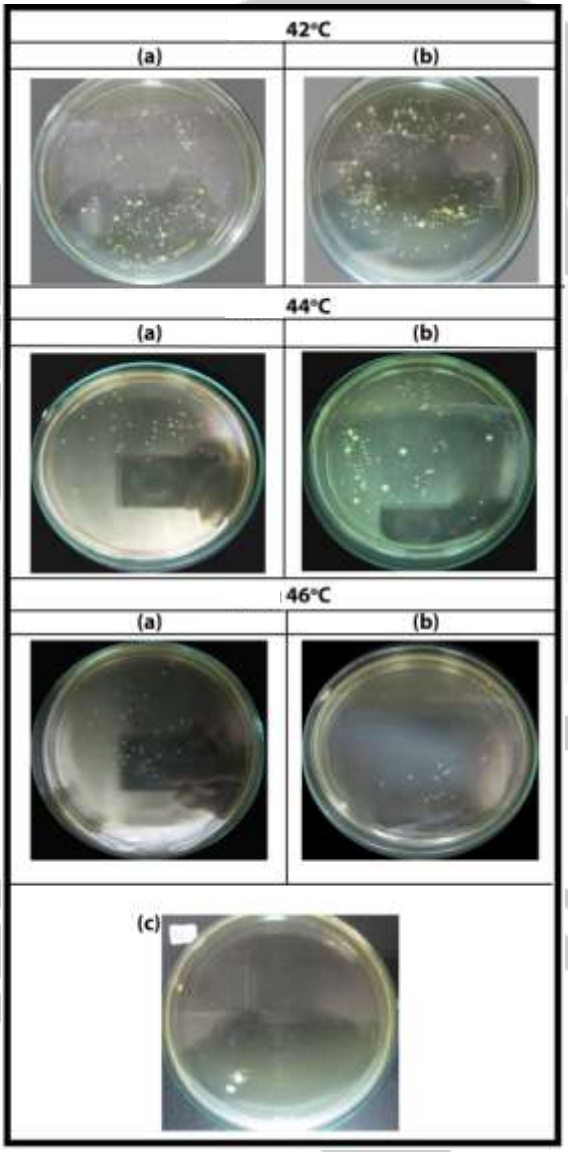


Figure 2. Selection of transformants on LB/Kan and LB/Hyg
(a) transformant on LB/an; (b) transformant on LB/Hyg; (c) negative control

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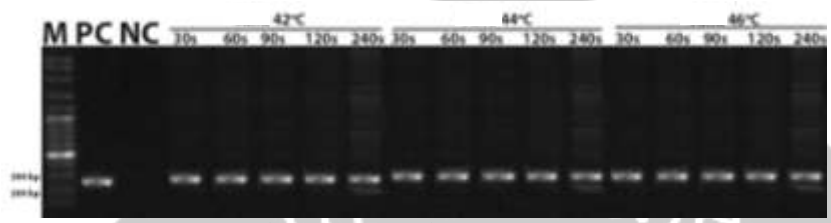


Figure 4. Amplification result of *22,4* gene with colony PCR
M = DNA *Ladder* 100 bp plus; PC = *22,4* gene positive control; NC = negative control (does not contain bacterial colony sample). *22,4* gene is at 232 bp

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Molecular detection is also done with *HPT* gene. Each successful treatment is taken for one single colony. The amplification results show that every heat-shock treatment has *HPT* gene at 500 bp (Figure 5). It indicates the existence of hygromycin resistance on pTA7002-*AtRKD4* (Mursyanti et al., 2015)

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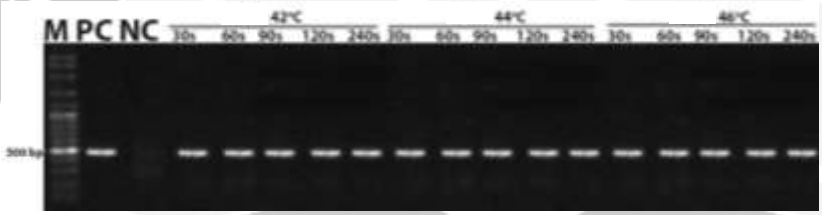


Figure 5. Amplification results of *HPT* gene with colony PCR
M = DNA *Ladder* 100 bp plus; PC = *HPT* gene positive control; NC = negative control (does not contain bacterial colony sample). *HPT* gene is at 500 bp

C. Transformation efficiencies pTA7002-*AtRKD4* using *Escherichia coli* BL21(DE3) as host cell

Transformation efficiency is calculated using the formula. pTA7002-*AtRKD4* mass used for transformation is 0,0511 µg in 450 µL of bacterial culture. In addition, pTA7002-*AtRKD4* mass in 10 µL of *spread plate* culture is 0,000122 µg.

Transformation efficiency then is analyzed using Analysis of Variance (ANOVA) test. The results signify that there is real difference between heat-shock temperature treatment and there is no real difference between heat-shock time treatment on 95% confidence level. After that, the statistical analysis is continued with *Duncan's Multiple Range Test* (DMRT) to determine the

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optimum heat-shock temperature and time for pTA7002-*AtRKD4* transformation (Table 3)

Tabel 3. pTA7002-*AtRKD4* transformation efficiency using *Escherichia coli* BL21(DE3) with variance of heat-shock temperature and time (x$10^4$ CFU/µg)

| Time (seconds) | Temperature | | |
|---|---|---|---|
| | 42°C | 44°C | 46°C |
| 30 | 15,513 [a,b,x] | 81,927 [a,b,x] | 12,52 [a,b,y] |
| 60 | 54,163 [a,x] | 57,16 [a,x] | 24,77 [a,y] |
| 90 | 35,930 [a,x] | 31,3 [a,x] | 30,757 [a,y] |
| 120 | 37,017 [a,x] | 13,637 [a,x] | 30,213 [a,y] |
| 240 | 112,957 [b,x] | 116,770 [b,x] | 10,883 [b,y] |

[1]Numbers followed by same alphabet do not show any real difference at 95% confidence level

Transformation efficiency is used to notice the effect of temperature on pTA7002-*AtRKD4* heat-shock transformation. Figure 6 shows that transformation efficiency trend is lower along with the higher heat-shock temperature for each time treatment. Temperature 42°C and 44°C present no real difference at DMRT test therefore the suitable heat-shock temperature for pTA7002-*AtRKD4* transformation is between 42° and 44°C.

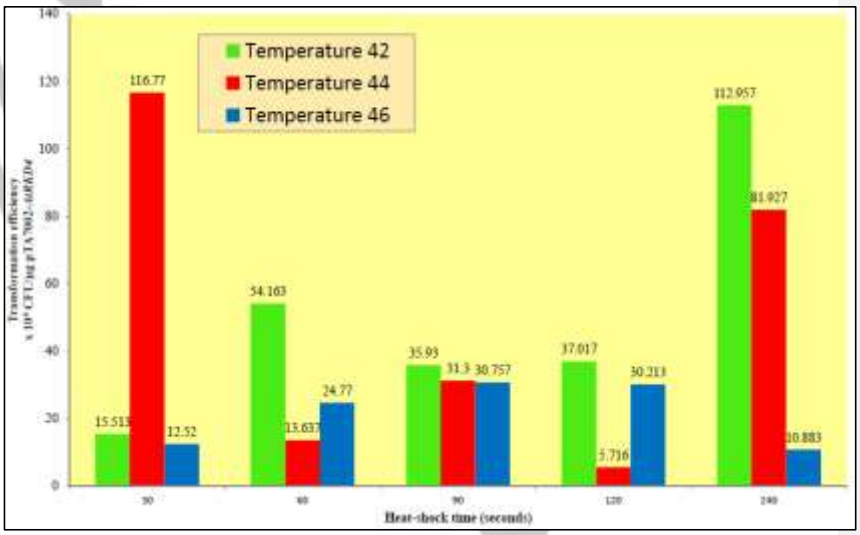


Figure 6. Heat-shock temperature (°C) and time (seconds) effects on transformation efficiency

Based on transformation efficiency on Figure 6, 42$^{o}$C treatment has the highest transformation efficiency for 60 seconds, 90 seconds, 120 seconds, and 240 seconds. 44$^{o}$C treatment has the highest transformation efficiency for 30 seconds and the lowest for 120 seconds. Meanwhile, 46$^{o}$C has the lowest transformation efficiency for 30 seconds, 60 seconds, 90 seconds, and 240 seconds.

Heat-shock transformation on 42$^{o}$C temperature has transformation efficiency as the increase of heat-shock time duration. This is because of heat-shock temperature is given at heat-shock protein optimum temperature, which is 40$^{o}$-45$^{o}$C based on Urban-Chmiel et al. (2013). In addition, the cell that has temperature stress will express *σ-32* gene six hundred times more than normal condition based Yoo (2010) research. This gene is a promoter for heat-shock protein expression in *E. coli* BL21(DE3). Therefore, the longer the heat-shock time, the more heat-shock protein is expressed so the cell functional protein is protected from heat-shock and the survived cell is increased.

Transformation efficiency on 44$^{o}$C reaches the highest on 30 seconds. This is caused by the degradation of heat-shock protein after 30 seconds based on Rithcer et al. (2010) research. As the result, some functional protein undergoes protein misfolding and can not form back into its regular form after growing the bacteria at normal growth temperature (37$^{o}$C) so the bacteria colony grow at 60 seconds, 90 seconds, 120 seconds is lower than 30 seconds.

Transformation efficiency tends to lower along with the longer of heat-shock time duration at 46$^{o}$C. This result is consistent with Thomas and Baneyx (1998) result whereas heat-shock protein expressed by *E. coli* BL21(DE3) starting to denature at 46$^{o}$C. In addition, bacterial cell can not tend its functional proteins structure so they more easily damaged by heat-shock based on Urban-Chmiel et al. (2013) research. As the result, the longer the heat-shock time duration, the more cell proteins are damaged and the amount of single colony growth is low.

Transformation efficiencies of pTA7002-*AtRKD4* are quite high, whereas between 5,716 and 116,770 x $10^4$ CFU/µg for 0,000122 µg pTA7002-

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*AtRKD4*. This is caused by the host cell characteristic. *E. coli* BL21(DE3) as the host cell is a commercial strain for plasmid cloning, such as pRSETB (Saraniya et al., 2012), pET-32b(+)-IFN α2a (Kusumawati et al., 2013), pGEM-T (Retnoningrum et al., 2010), pBR322, and pUC19 (Liu et al, 2014). In addition, *E. coli* BL21(DE3) has good transformation efficiency, which is 190 x $10^4$ CFU/µg for 0,015 µg pBR322 and 200 x $10^4$ CFU/µg for 1 µg pUC19 based on Liu et al. (2014).

## CONCLUSION

This research on pTA7002-*AtRKD4* transformation efficiency using *E. coli* BL21(DE3) conclude that 44$^o$C for 30 seconds at pTA7002-*AtRKD4* heat-shock transformation increase plasmid transformation efficiency.

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