



ORIGINAL ARTICLE

Optimization of expression JTAT protein with emphasis on transformation efficiency and IPTG concentration



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Abstract One of small accessory genes between *pol* and *env* is *tat* gene encoding TAT protein. This research was aimed to optimize the expression of Jembrana TAT (JTAT) protein with preparing *Escherichia coli* (*E. coli*) in advance using adopted methods of M1 (MgCl₂ + CaCl₂) and M2 (CaCl₂ + Glycerol). The best transformation efficiency resulting from a better transformation method was used to subsequent expression of JTAT protein. A synthetic *tat* gene encoding protein JTAT was previously cloned into pBT-*hisC*. Concentration of 200; 400; 600 μM IPTG was induced to a small volume culture (200 ml; OD₆₀₀ = 4), incubated for 3 h. Pellets were harvested by centrifugation (4000 rpm; 4 °C; 15 min). Buffer B (10 mM Imidazole) was added into pellets, lysed by freeze-thaw followed by sonication. Supernatant was collected by centrifugation (10,000 rpm; 4 °C; 20 min) and purified using Ni-NTA Agarose resin, released by elution buffer (E) containing 400 mM Imidazole to collect purified protein twice (E1, E2). The protein was characterized by SDS-PAGE and Western Blot (WB), quantified (at 2595 nm) with BSA standard method in prior. The result showed that transformation efficiency was better in M2 (2.53 × 10⁶) than M1 (3.10 × 10⁵). The JTAT protein was expressed at a right size of 11.8 kDa. Concentration of 200 μM IPTG produced a significantly better protein yield (1.500 ± 0.089 mg/ml; *P* < 0.05) than 600 μM IPTG (0.896 ± 0.052 mg/ml) and not different to 400 μM IPTG (1.298 ± 0.080 mg/ml). This research indicated that transformation efficiency needs to be taken account in prior of optimization of the protein expression.

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1. Introduction

Transactivator of transcriptions (TAT) is one protein encoded by an accessory gene of *tat* which locates between *gag* and

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envelop (env) genes of jembrana viral genome. This viral jembrana belongs to bovine lentivirus family [26] which causes a jembrana disease virus (JDV) in Bali cattle (*Bos javanicus*). The JDV contains three genes *gag*, *pol* and *env*. This TAT protein is the most important for viral replication [1,5]. As reported that Jembrana TAT (JTAT) contributes largely to rapid viral replication and causes an acute jembrana disease [23,3,21]. The recent synthetic *tat* gene was constructed and successfully transformed into *Escherichia coli* (*E. coli*) in our laboratory (unpublished research report, Margawati et al., 2016) and stored in 80% glycerol and kept at -80°C . Maintenance of the construct in *E. coli* is therefore needed monthly to keep the construct bearing synthetic *tat* still alive and stable expressing the protein.

In the protein recombinant technology, transformation is commonly conducted into host cell of *E. coli* in regard to maintenance of the construct (clone containing foreign DNA). There are many methods of bacterial transformation. It was demonstrated several optimization methods and factor affecting transformation of *Escherichia coli* (*E. coli*) in many strains of DH5 α , XL-1 Blue, SCS110, JM109, TOP10, and BL21-(DE3)-PLysS [2]. They found that a method of the calcium chloride (CaCl_2) followed by a glance heat shock was the best for SCS110, TOP10 and BL21 *E. coli* strains ($P < 0.05$). This method was an original CaCl_2 method used to increase transformation efficiency [25,14]. Competent cells need to be prepared before transformation. There are several methods for preparing bacterial competency. Those methods were from complicated [9,8] up to the simple methods of using DMSO and PEG (polyethylene glycol with replacing Ca^{2+} ions) and a short incubation of both bacteria and DNA on ice (replacing the heat shock) [2]. Those methods are intended to increase transformation efficiency of *E. coli* subsequently facilitating further step in expression of recombinant protein.

Host cell of *Escherichia coli* (*E. coli*) is commonly used for recombinant protein expression. The *E. coli* is one organism of choices for the production of recombinant proteins [19]. As a cell manufacturer, the *E. coli* host cell is well-established and has become the most popular expression media. Due to those reason, *E. coli* is often used as a bio-manufacture of protein expression and even for the high-level production of heterologous protein. There are many strains of *E. coli* in which one of them is NiCo strain. This NiCo strain has some advantageous compared to other strains in terms of increasing expression and purity of protein (13).

E. coli strain NiCo is a result of modified BL21 strain of *E. coli* by substituting *GLMs* gene with *GLSs6Ala* gene and adding genes of *SlyD*, *can* and *ArnsA* at ORF chitin binding domain [18]. By addition of those genes into NiCo strain it would be the advantage of NiCo in increasing the production of recombinant protein target and minimize non targeting recombinant protein [7,17].

The protein encoded by an interest gene would express their protein after inducing with IPTG [22]. Optimization of protein expression is always expected by researchers with focus on the strategy to reduce time and expenses. Different IPTG concentrations might be the manner to find which is more effective and efficient in protein production.

Combination of transformation efficiency for host cell preparation in prior protein expression and applying different IPTG concentrations were to be interested in this study in order to optimize the protein yield. In previous study, a viral

tat gene was constructed conventionally into pET his-tag and successfully expressed recombinant protein JTAT through *E. coli* BL21 strain [15]. Strain of *E. coli* NiCo was also compared to the BL21 strain in expression of JTAT [16]. It was reported that the NiCo expressed a higher protein yield than BL21 strain.

This recent study was therefore emphasized on both transformation efficiency and different IPTG concentrations in dealing to optimize JTAT protein expression. The clone of Jtat used in this study was a synthetic *tat* gene as mentioned in early above statement. The protein yield was subsequently calculated based on the eluted protein after purification and characterization.

2. Material and methods

2.1. Competent cell

Two methods of M1 ($\text{MgCl}_2 + \text{CaCl}_2$) [20] and M2 ($\text{CaCl}_2 + \text{Glycerol}$) [14] were applied for cell competency. Amount of 20 μl glycerol stock of *E. coli* NiCo was cultured in a 5 ml Luria Bertani (LB) medium (non-Ampicillin/Amp), incubated in a shaker (150 rpm; 37°C) for overnight (O/N) culture. Amount of 2 ml O/N culture was transferred into flask containing 50 mL LB (non-Amp), and prepared 3 flasks for further IPTG treatments (200; 400; 600 μM). The flasks were incubated with a shaker (37°C ; 150 rpm; 1 h) to achieve $\text{OD}_{600} = 0.4$. Pellets of *E. coli* NiCo were harvested by centrifugation (4000 rpm; 4°C ; 10 min). The pellets were made competent cells by immersed either into M1 or into M2 and homogenized. The suspension was centrifuged (4000 rpm; 4°C ; 10 min), and the collected pellets are competent NiCo cells.

2.2. Transformation

A ratio of 1:50 (plasmid: NiCo) with heat-Shock method [10] was applied for transformation. A 400 ml fresh LB medium was added into suspension of transformed cells and then incubated at 37°C , 150 rpm for 1 h. The suspension was then spread on LB agar plate (+100 $\mu\text{g}/\text{ml}$ Amp, 0.80 $\mu\text{g}/\text{ml}$ X-Gal and 0.1 μM IPTG). Transformation of efficiency was calculated with concerns on colony forming unit (CFU) per μg [25].

2.3. Protein expression

2.3.1. Overnight culture

Amount of 20 μl *E. coli* NiCo culture bearing the Jtat clone was cultured into a total 5 mL LB media (+100 $\mu\text{g}/\text{ml}$ Amp) and incubated at 37°C with 150 rpm for O/N.

2.3.2. IPTG induction

Five (5) ml O/N culture was added into a total of 200 ml LB media (+100 $\mu\text{g}/\text{ml}$ Amp) and incubated (37°C ; 150 rpm) to achieve $\text{OD}_{600} = 0.4$, prepared 3 culture of 200 ml each as repetition of each treatment. Three treatments of 200, 400 and 600 μM final IPTG concentrations were applied. Those treated cultures with IPTG containing the *E. coli* NiCo bearing Jtat were again incubated (37°C ; 150 rpm; 3 h). Pellets were

harvested by centrifugation (4000 rpm; 4 °C; 15 min). Buffer B (10 mM Imidazole) was added into the pellets and homogenized. The *E. coli* cells were lysed by freeze-thaw method followed by sonication (3 cycles; 15 s per cycle; 1 min interval).

2.4. Protein purification

Suspension of *E. coli* lysates was centrifuged (10,000 rpm; at 4 °C; 20 min). Purification was embarked using Ni-NTA Agarose resin. Elution (E) was conducted twice as E1 and E2 using elution buffer (400 mM Imidazole) and stored at a freezer (−20 °C) before used for SDS-PAGE and Western Blot characterizations.

2.5. Protein characterization

2.5.1. SDS PAGE

SDS-PAGE of 12% resolving and 3.9% stacking gels [20] was prepared for protein characterization with a protein marker ladder (BIO-RAD). Denaturation of protein sample in a ratio of 2:1 (sample: loading buffer) was conducted at 95 °C for 15 min. The SDS-PAGE electrophoresis was at 100 V for 2.5 h and then soaked in *Staining Coomassie Blue* (1 h) and de-stained (1 h) twice. The SDS-PAGE was documented by a camera.

Western Blot. Western blot (WB) characterization was conducted using a primer antibody of *Anti-His-probe* (H-15, Millipore) and secondary antibody of Goat anti-Mouse HRP Conjugate SC2005 (St. Cruz) both were in ratio of 1:3000. TBS-Tween-20 was used for washing the WB membrane and developed by 10 ml developing color (HRP Color Development).

2.6. Protein quantification

The collected purified protein of each E1 and E2 of each IPTG concentration was quantified by a GeneQuant machine at 2595 nm with BSA standard and was conducted in prior.

2.7. Statistical analysis

A completely randomized design was arranged with 3 treatments of IPTG concentrations and repeated 6 times of each treatment. All observed data of protein quantification were analyzed in one way ANOVA using software of SPSS version 17.0. Significant differences among treatments were examined by Duncan Test at 95% significant level ($P < 0.05$).

3. Results and discussion

3.1. Transformation efficiency and competent cell

There are many methods for preparing competent cells. This study applied *Escherichia coli* (*E. coli*) strain NiCo for cell transformation. Two methods termed as M1 [20] and M2 [14] were applied for preparing competent cells of *E. coli* strain NiCo. Those competent cells were examined for their transformation efficiency [25].

Based on the applied competent cell methods, it resulted that the M2 method [14] found a better transformation

efficiency (2.53×10^6 CFU/ μ g) compared to M1 method (3.1×10^5 CFU/ μ g) [20] (Table 1).

Even though both competency methods containing CaCl_2 are intended to impart antibiotic resistance into the competent *E. coli* cells [2], the content of glycerol in M2 [14] might be a better chemical to increase the CFU in this study. This finding CFU (M2 = $2.53 \times 10^6/\mu$ g) was lower than the previous finding ($1-5 \times 10^8$ CFU/ μ g) across many *E. coli* strains [9,8]. As reported in previous study that the CaCl_2 chemical is the best effective factor for competent cells in all BL21 strains [2]. More detail was reported that bivalent ions (Ba^{2+} , Ca^{2+} , Mn^{2+} , Mg^{2+} , Sr^{2+}) have capacity transformation and the ion Ca^{2+} was found to be the best bivalent ion in cell transformation.

The better CFU result of competent cells (M2) was therefore used for transformation of plasmid containing *tat* gene of this study. Subsequently the transformed cells were used for further expression process in order to optimize yield of the JTAT recombinant protein.

3.2. The effect of IPTG induction on JTAT expression

Induced and without induced IPTG were conducted in the culture for protein expression. Without inducing IPTG in the culture was intended to check whether or not protein leakage in the cells. In this study there was no found leakage of recombinant protein JTAT in the cell culture of uninduced IPTG culture on the SDS-PAGE characterization. While crude protein recombinant JTAT derived from induced IPTG was expressed in all IPTG concentrations (200, 400 and 600 μ M) at right size of 11.8 kDa. Both SDS-PAGE characterizations of uninduced and induced IPTG were presented (Fig. 1).

Those crude protein recombinant induced with IPTG (Fig. 1) was purified and characterized with SDS-PAGE (Fig. 2) and Western Blot (WB), (Fig. 3). Recombinant protein of JTAT target was emerged in all IPTG concentrations both in SDS-PAGE and Western Blot with right sizes of 11.8 kDa either in Elution 1 (E1) or in Elution 2 (E2).

After purification, the recombinant protein of JTAT was still expressed at the right size both in the SDS-PAGE (Fig. 2) and Western Blot (Fig. 3). It seems that both Elutions (E1 and E2) expressed in the similar thick bands of 11.8 kDa on each concentration of IPTG. In the production of recombinant protein IPTG has function in inducing of protein expression under Lac operon control in triggering the transcription of genes [27]. In other hand, the use of IPTG needs to be considered economically more over in a large scale production due to the cost reason. In this research, cell cultivation was conducted at 37 °C which it is as an optimal temperatures for *E. Coli* growth [13]. A lower temperature (28 °C) of cell

Table 1 Average of transformation efficiency (CFU^a/ μ g).

Agar Plate (P)	Method	
	M1 [20]	M2 [14]
P1	1.80×10^4	2.59×10^6
P2	7.80×10^4	2.86×10^6
P3	8.50×10^5	2.15×10^6
Average	3.10×10^5	2.53×10^6

Note: Sambrook and Russel [20]; Li et al. Li et al. [14].

^a Colony Forming Unit per microgram DNA plasmid.

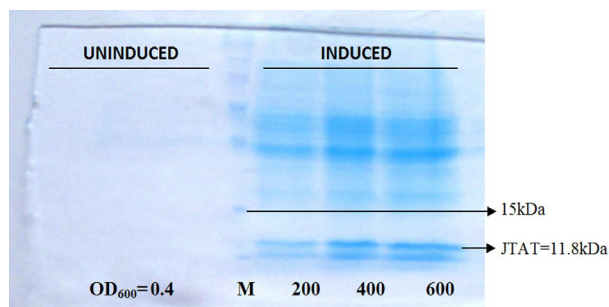


Figure 1 SDS-PAGE of recombinant protein JTAT without induced and induced IPTG (200, 400, 600 μ M) (E = Elution; M = Protein Ladder Dual XTra Prestained, BIO-RAD).

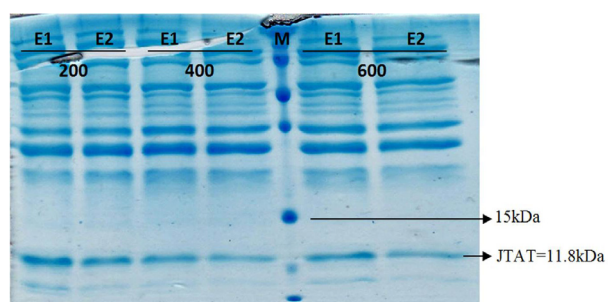


Figure 2 SDS-PAGE of purified recombinant protein JTAT at different IPTG concentrations (200, 400 and 600 μ M) (E = Elution; M = Protein Ladder Dual XTra Prestained, BIO-RAD).

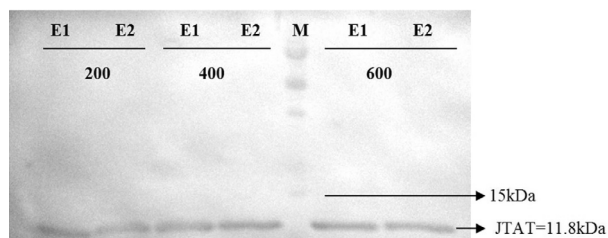


Figure 3 Western Blot of purified recombinant protein JTAT at different IPTG concentrations (200, 400 and 600 μ M) (E = Elution; M = Protein Ladder Dual XTra Prestained, BIO-RAD).

cultivation could decrease over 60% in the bacteria growth rate after induction with IPTG [27]. Those conditions of appropriate cultivation temperature and concentration of IPTG induction contributed to the merge of thick band of JTAT protein both in SDS-PAGE and Western blot characterizations.

3.3. Protein yield

Average concentration of purified recombinant protein JTAT induced with different IPTG concentrations was presented in Table 2. Yield of the JTAT protein was calculated based on Elution1 and 2 (E1 and E2) and a total of 6 repetitions (E1 and E2) of each IPTG treatment were calculated for mean \pm SEM (Standard Error for the Mean).

Table 2 Purified recombinant protein JTAT (Mean \pm SEM) at different IPTG concentrations.

Treatment IPTG conc (μ M)	N	Protein concentration (mg/ml)
200	6	1.500 \pm 0.089 ^a
400	6	1.298 \pm 0.080 ^a
600	6	0.896 \pm 0.052 ^b

Difference superscripts in the same column are significantly different ($P < 0.05$).

Concomitant with the result of SDS-PAGE and Western Blot characterizations (Figs. 2 and 3), all IPTG concentrations expressed the higher yield of recombinant protein JTAT. Table 2 showed that a concentration of 200 μ M IPTG was in fact yielded averagely the best protein concentration of 1.500 \pm 0.089 mg/ml compared to other IPTG concentrations. Those two other IPTG concentrations yielded 1.298 \pm 0.080 and 0.896 \pm 0.052 mg/ml, respectively for 400 μ M and 600 μ M IPTG (Table 2). It was proved that 200 μ M IPTG concentration could optimize effectively the JTAT expression with competent cells prepared in prior by a method of CaCl₂ with glycerol supplementation [14]. This finding proved that induction of 200 μ M IPTG in cultivation could reduce cost production of JTAT yield. The induction of IPTG relates to the growth of the *E. coli* cells. It might be higher IPTG concentrations (400 and 600 μ M) in cell cultivation affecting protein toxicity. Subsequently this condition caused metabolic burden which imposed on the cells due to heterologous gene expression or protein toxicity [6,12].

This TAT protein was originated from a JTAT clone tagged with 6 his-tag. It is important in choosing an affinity tag at the beginning of constructing the target gene into a plasmid. Another strategy is taking to the account the size of small size tags are useful for protein detection and antibody production. It was stated that small size tags are not immunogenic as large tags [24]. A recent report stated that Fh8 fusion system can be used for soluble protein production, purification, and immunogenicity in *E. coli* [4].

The clone of JTAT used in this study was transformed into NiCo strain of *E. coli*. The advantage of NiCo was explained in previous of this article [18]. In addition the NiCo strain is a unique complement and could improve the purity of recombinant his-tagged. It was reviewed that there have been numerous new strains, vectors and tags developed to overcome the limitations of recombinant protein expression through *E. coli* system [11].

4. Conclusions

Several findings can be extracted from this research. Transformation efficiency of *E. coli* strain NiCo was better delivered from competent cell prepared with addition of glycerol. The CaCl₂ solution of competent cell was the best selected solution for cell transformation. Expression of recombinant protein was perfect since there was no leakage protein expression on un-induced culture. Recombinant protein JTAT was expressed at right size of 11.8 kDa both in SDS-PAGE and Western Blot at all induced IPTG of 200, 400 or 600 μ M. Yield of the recombinant protein JTAT was optimized from cell prepared with addition of glycerol in competency cells and then induced with 200 μ M IPTG in its production.

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