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Research article

Expression of Synthetic *pac* Gene Encoding Penicillin G Acylase (PGA) Enzyme in *E. coli* BL21(DE3) and HB101

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Abstract

High bacterial infection cases in Indonesia cause a high need for antibiotic drugs. Unfortunately, most of the raw materials used for antibiotic production in Indonesia are still imported. For this reason, the government is eager to find better ways to produce penicillin and its derivatives, which are widely used in society. The production of penicillin-derivative requires penicillin G acylase (PGA) as a catalyst. In previous studies, the expression of the syn-*pac* gene in *E. coli* BL21(DE3) to produce a recombinant PGA enzyme was performed, but the enzyme activity was low (0.01754 U/mg). Thus, the expression was carried out in different hosts and inducers. The purpose of this research was to obtain the production of PGA with higher enzyme activity. The transformation was carried out in the pET22b-*pac*Ec in *E. coli* BL21(DE3) and HB101. For enzyme expression, the recombinant hosts were induced by 0.05 mM IPTG, 176 mM lactose, and 1998 mM arabinose at a temperature of 20°C and 150 rpm of shaking for 17 h. Protein isolation was performed by sonication and freeze-thawing to recover biologically active PGA. Verification of PGA was performed by SDS-PAGE and the enzyme activity was tested by pDAB. *E. coli* HB101 produced PGA with higher activity (10.17 U/mg) than BL21(DE3) (6.67 U/mg), and arabinose was the strongest inducer for enzyme expression.

Keywords

expression;

PGA;

E. coli BL21(DE3);

E. coli HB101

1. Introduction

The skyrocketing rate of bacterial infections in Indonesia needs to be balanced by the independence production of raw materials for antibiotic drugs. One of the widely used antibiotics is penicillin and

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its derivatives. The production of this antibiotic requires a catalyst in the form of the enzyme penicillin G acylase (PGA), and it is crucial that PGA enzymes are to be produced on an industrial scale. For the purpose of industrial scale, the PGA enzyme requirement was mostly provided by recombination technique which uses *E. coli* as the host expression system.

In a previous study, the recombinant plasmid vector pET22b-*pac*Ec which was expressed in *E. coli* BL21(DE3) with 0.05 mM IPTG as the inducer (incubation 16°C and 180 rpm of shaking for 4 h), produced a high PGA expression result but only low PGA enzyme activity (0.0175 U/mg) [1]. Therefore, in this investigation, three distinct inducers—0.05 mM IPTG, 176 mM lactose, and 1998 mM arabinose—were used to optimize expression in two different hosts, *E. coli* BL21(DE3) and HB101. The objective of the study was to identify the most effective host expression system and inducer for PGA enzyme synthesis.

Tapia-Pastrana *et al.* [2] used the *E. coli* HB101 strain optimally used for VirK periplasmic protein expression. This strain did not have OmpT and OmpP proteases [3] and had a recA13 mutation genotype that functioned to minimize unwanted recombination and stabilize insertion of the gene of interest, restriction minus hsdS20 (rB-mB-), which functions to prevent cleavage of cloned DNA by endogenous restriction enzymes. While *E. coli* BL21(DE3) was easy to overexpress heterologous CGTase protein [4] and did not have lon and OmpT proteases [5, 6]. *Escherichia coli* BL21 (DE3) also has a restriction genotype of minus hsdB (rB-mB-) which allows DNA cloning without cleavage by endogenous restriction endonucleases.

The selection of IPTG inducers was based on a report from Briand *et al*. [7] who observed that IPTG could induce high levels of GSTA1 expression and retain biological activity. However, IPTG, which is a strong inducer, can induce host metabolic stress leading to the expression of less biologically active proteins [8]. Contrary to IPTG, lactose inducer is a medium strength inducer which can decrease the host metabolic stress. As the result, this lactose's property may enhance the expression of biologically active recombinant proteins [8]. Shahzadi *et al*. [9] reported that lactose inducer can be used to scale up endoglucanase with high activity. Pan *et al*. [10] reported that arabinose inducer is a superior inducer for the expression of PrPGA, AfPGA, and AxPGA. In this research, we discuss the effect of different enzyme inducers on PGA enzyme expression. Moreover, because recombinant PGA is a periplasmic protein, in this research we also would like to analyze the expression of the intended enzyme not only in *E. coli* BL21 (DE3) but also in *E. coli* HB101, since this *E. coli* is a good host for periplasmic proteins.

2. Materials and Methods

2.1 Competent cell development and transformation

Competent cells were made using the $CaCl_2$ method [11]. Plasmid recombinant pET22b-*pac*Ec was taken from the transformants in previous studies [1]. The transformation was carried out by the heat shock method [12] with modification from protocol in Biochemistry Laboratory, Research Center for Biotechnology, Universitas Gadjah Mada, which involves heat shock at 42°C for 45 s (*E. coli* BL21(DE3)) and 55°C for 60 s (*E. coli* HB101) followed by immediate incubation on ice for 2 min. The amount of 100 µL of transformant cells was cultured on an LB agar medium containing 50 µg/mL ampicillin.

2.2. Syn-*pac* gene sequence analysis

Before transforming into host cells expression system, we analyzed the sequence of the *pac* gene which was inserted in plasmid pET 22b (+), duplicated by *E. coli* DH5α, and expressed by *E. coli*

BL21(DE3) and HB101. A recombinant plasmid construct (pET22b) with codon optimization of *Syn-pac* gene on host *E. coli* (*pac*Ec) was synthesized by GenScript Express Gene (Figure 1). The isolation of plasmid recombinant from *E. coli* DH5α, BL21(DE3), and HB101 was performed by FavorPrep^{TM} Plasmid Extraction Mini Kit (Favorgen). *Syn-pac* gene amplification with Forward T7 Prom primer : 5'-TAA TAC GAC TCA CTA TAG GG-3'; Reverse T7 Term primer: 5'-GCT AGT TAT TGC TCA GCG G-3'. PCR was performed with the GoTaq® Green Master Mix (Promega) kit using the Applied Biosystems-2720 thermal cycle. PCR results were visualized by 1% agarose gel electrophoresis. Gene sequencing was carried out at the Integrated Research and Testing Laboratory Universitas Gadjah Mada. The sequencing results were analyzed by BioEdit v.2.7.5. Software.

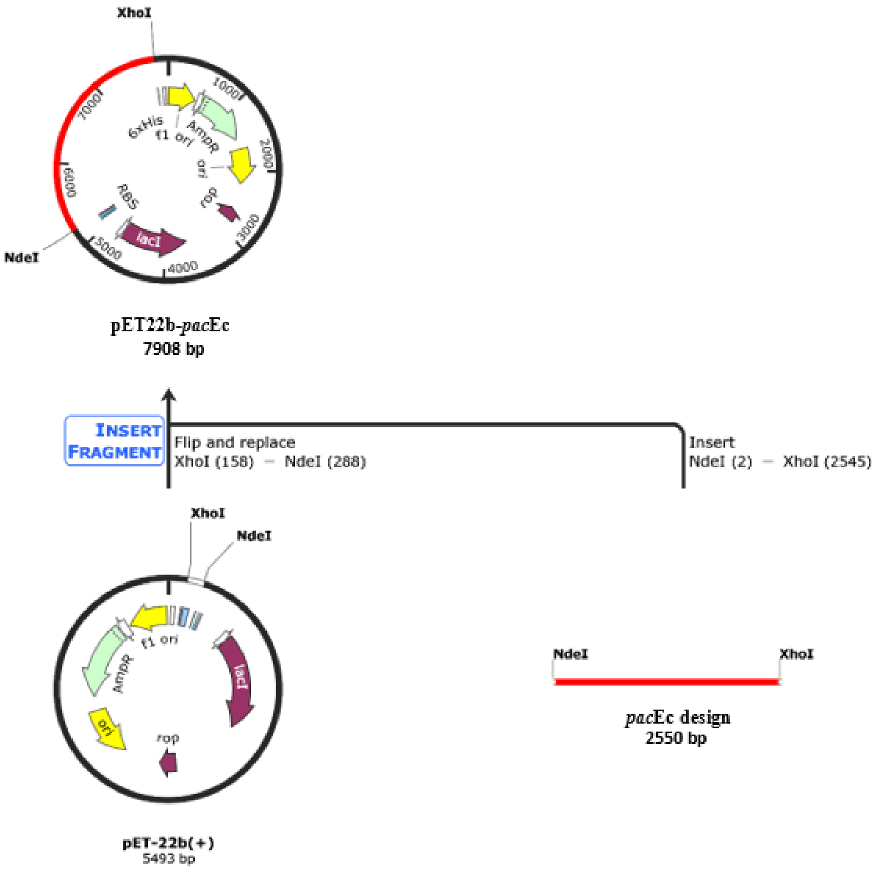

Figure 1. The assembly of the pET22b-*pac*Ec recombinant plasmid

2.3. Induction of PGA recombinant protein expression

An aliquot of 1 mL of preculture suspension was inoculated in 50 mL of LB broth medium containing ampicillin 50 µg/mL and incubated at 37°C and 150 rpm of shaking to get OD_{600}: 0.5-0.7. Furthermore, the final concentration of 0.05 mM IPTG, 176 mM lactose, and 1998 mM arabinose was added when OD_{600} met the requirement and then incubated at 20°C (150 rpm of shaking) for 17 h.

2.4 Cell harvesting and isolation of PGA recombinant proteins

Transformant cells were harvested by centrifugation at 3500 rpm for 15 min at 4°C. The pellet was resuspended with extraction buffer pH 7.8 (10 mL 1 M potassium phosphate buffer pH 7.8; 20 mL 20% glycerol ; 5 mL 100 mM Na_2EDTA; and 65 ml dH_2O) with a volume of 1/10 the volume of the fermentation culture. Cells were disrupted by sonication of 180 amplitude for 4 cycles @ 30 s. The sonicated pellets were subjected to solubilization treatment with 8 M urea solution, then incubated for 4 h at 4°C, and finally incubated at -20°C overnight (freeze-thawing method). The solubilized sample was filtered through a filtration membrane (Amicon Ultra 15 mL centrifugal filters) with a washing solution in the form of an added extraction buffer. In this filtration, 5 mL of a solution containing 8 M urea was filtered (concentrated) into 1 mL. To decrease the concentration of urea, a number of 15 mL extraction buffer was added to the concentrated protein solution. Based on the dilution formula, the final urea concentration after Amicon filtering was 0.53 M. To re-concentrate the protein solution, a centrifugation at 10000 rpm for 15 min at 4ºC was performed. As the next step, the supernatant fraction containing recombinant protein was measured for its protein concentration by direct absorbance at 280 nm with a nanodrop spectrophotometer with standard Bovine Serum Albumin (BSA). The sample then was stored at -80°C before use.

2.5 Protein profiles and PGA enzyme activity

Protein profiles were analyzed by SDS PAGE with 12% resolving gel and 5% stacking gel. Protein bands were visualized by the Coomassie Blue staining method. The SDS PAGE sample was a mixture of crude protein and 5X SDS sample buffer (4:1), which was denatured at 95°C for 5 min, then put on ice for 30 min. The sample was ready for loading and running for 2.5 h at 70 volts. The enzyme activity of PGA was determined by the spectrophotometric method [13]. The enzyme activity test used was the pDAB method as described by Balasingham *et al.* [13]. The enzyme activity test works by converting the penicillin G substrate to 6-aminopenicillanic acid (6-APA) under alkaline conditions. The interaction of the 6-APA molecule's free amino group with the reactant chemical in the form of p-dimethylaminobenzaldehyde (pDAB) produces a yellow Schiff base (chromogen), which is read at 415 nm. A 6-aminopenicillanic acid (6-APA) standard curve was used to assess PGA enzyme activity. For U/mL calculations, a standard factor of 100 g/mL (6 APA) was used. One international unit of PGA enzyme activity is defined as the amount of enzyme required to produce 1 mol 6-APA per minute.

3. Results and Discussion

3.1 Characterization of the *Syn-pac* gene

Before enzyme expression is performed, a colony PCR should be done on the host bacteria cells containing the recombinant plasmid to ensure the correct transformant is present. The recombinant plasmid in this research was prepared by a previous study and was ensured to be of true orientation [1]. The primers for PCR to amplify the *syn-pac* gene from transformant *E. coli* BL21(DE3) and *E. coli* HB101 were designed by Primer3 (https://primer3.ut.ee/). Those primers set covered the complete open reading frame of the gene; therefore, the whole part of the sequence's gene was expected as a product of amplification. The PCR amplification successfully amplified a single fragment with a size of approximately 2.600 bp (Figure 2).

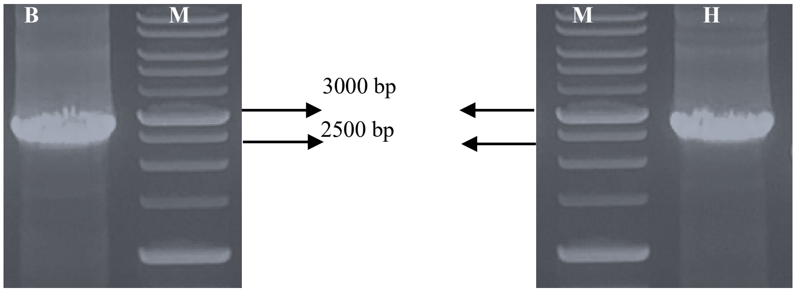

Figure 2. Gel agarose electrophoresis of PCR amplification of *syn-pac* gene by T7 promoter as forward primer and T7 terminator as reverse primer. M= DNA ladder 1kB; B=PCR product of BL21 (DE3); H=PCR product of HB101

Besides the length of *syn-pac* gene analysis, the sequence of the *syn-pac* gene needed to be analyzed. The sequencing results showed that the sequence of the expected gene revealed sequence similarity (100%) with the reference optimization of the syn-*pac* gene, and so did the amino acid sequence encoded by the gene. Blasting UniProt resulted in high similarity to the PGA of *Escherichia coli* (99.8%) (P06875).

3.2 Expression of PGA recombinant protein and enzyme activity test

In this research, we expressed the PGA enzyme by a host expression system, *E. coli* BL21 (DE3). This bacterium is suitable for expressing various kinds of protein due to the absence of protease OmpT gene, which is known to be a factor that causes degradation of an expressed recombinant protein. In addition, the presence of the T7 RNA-pol gene overexpresses the target protein when paired with the pET expression vector [14, 15]. The pET expression system has a *pel*B secretion signal at the N-terminus, which directs the results of polypeptide synthesis to the periplasm of *E. coli* [16]. Host *E. coli* HB101 is also used for target protein expression because this strain excels in periplasmic recombinant protein expression [2, 17].

An inducer is necessary for protein expression and the use of *Isopropyl-D-1-thiogalactopyranoside* (IPTG), a lactose analogue inducer molecule that is not metabolized and has a very high affinity level, resulted in extremely effective gene expression induction [18]. Lactose, a sugar molecule with a lesser affinity for repressors than IPTG, is the natural inducer employed for gene expression induction [19]. Lactose stimulates gene expression in 'intermediates' and optimizes protein synthesis by lowering the stress load of cell metabolism. Furthermore, mild inducers such as arabinose can be utilized to stimulate gene expression. Arabinose is a natural pentose with a very low affinity that is not actively absorbed [20, 21]. Based on a survey of the literature and preliminary testing (*personally documented*), the results indicated that 0.05 mM IPTG, 176 mM lactose, and 1998 mM arabinose were the most attractive inducers to be observed in this work in order to discover the most optimal inducer to generate PGA protein synthesis.

PGA is a type of periplasmic protein that has a molecular mass of 86.1 kDa with α subunits of 23.8 kDa and β subunits of 62.3 kDa. Induction of expression on BL21(DE3) with 0.05 mM IPTG and 176 mM lactose produced a high protein expression in the 86.1 kDa band (Figures 3A and 3B-lines 4, 5, 6). Meanwhile, 1998 mM arabinose produced a low degree of protein expression in the 86.1 kDa band (Figure 3C - line 4, 5, 6). Concurrently, HB101 with 0.05 mM IPTG, 176 mM lactose, and 1998 mM arabinose showed no clear protein expression in the 86.1 kDa band (Figures 3D, E, F - line 4, 5, 6).

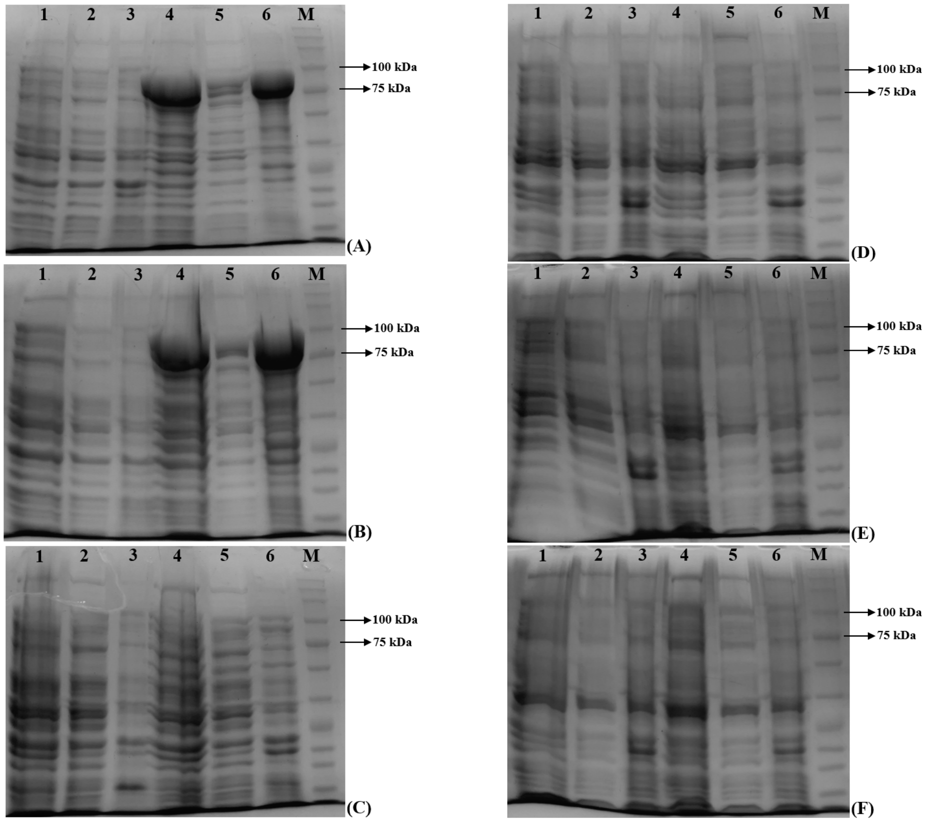

Figure 3. SDS-PAGE analysis of recombinant PGA protein in the different inducers.
E. coli BL21(DE3): (A) 0.05 mM IPTG (B) 176 mM lactose (C) 1998 mM arabinose;
E. coli HB101: (D) 0.05 mM IPTG (E) 176 mM lactose (F) 1998 mM arabinose;
Lane 1. Crude debris cells after sonication of control, 2. Supernatant after sonication and centrifugation of control, 3. Inclusion body of control, 4. Crude debris cells after sonication of sample 5. Supernatant after sonication and centrifugation of sample 6. Inclusion body of sample, M. Protein marker

The relationship between protein concentration and enzyme activity is shown in Figure 4. Samples of *E. coli* BL21(DE3) with 0.05 mM IPTG inducer resulted in activity values of 0.77 U/mg (in the supernatant after sonication and centrifugation (Sup)) and 2.84 U/mg (in inclusion body (IB)); whereas in the treatment with 176 mM lactose as inducer, the activity values were 1.07 U/mg (Sup) and 6.13 U/mg (IB). Meanwhile, the addition of 1998 mM arabinose as the inducer resulted in the highest activity values of 3.17 U/mg (Sup) and 6.67 U/mg (IB) (Figure 4B). Low activity values but high levels of protein expression were observed in BL21(DE3) samples with IPTG and lactose inducers, while low protein expression but high levels of activity were observed in samples with arabinose inducers (Figures 3A, 3B, 3C).

Interestingly, samples of *E. coli* HB101 with 0.05 mM IPTG inducer showed activity values of 1.32 U/mg (Sup) and 8.07 U/mg (IB); whereas in the treatment with 176 mM lactose inducer resulted in activity values of 0.97 U/mg (Sup) and 7.04 U/mg (IB). Meanwhile, the addition of a 1998 mM arabinose inducer resulted in activity values of 1.78 U/mg (Sup) and 10.17 U/mg (IB)

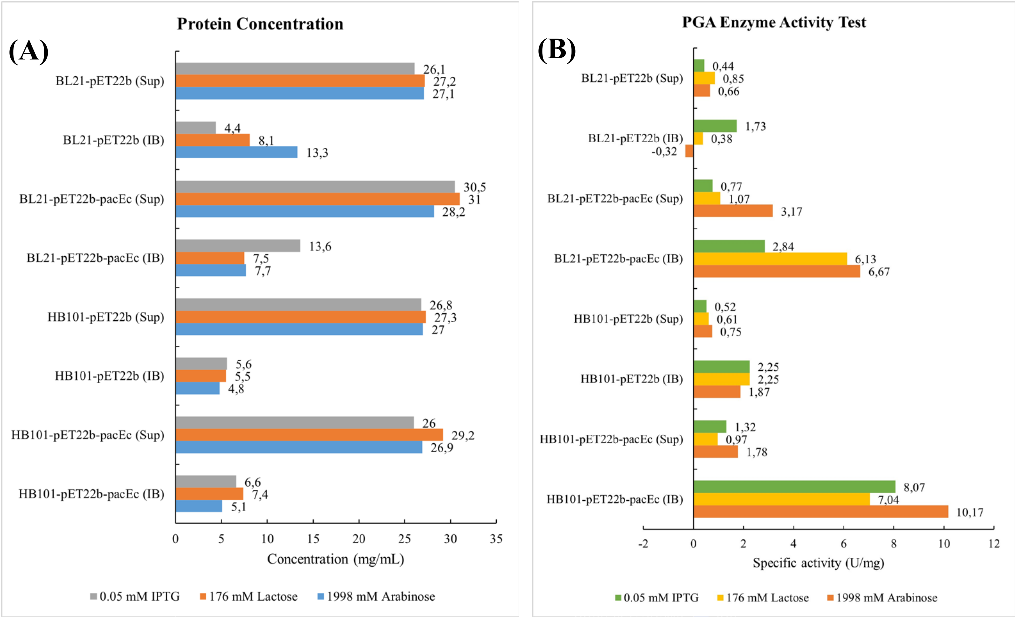

Figure 4. Expression of PGA recombinant protein.
(A) PGA recombinant protein concentration (mg/mL); (B) PGA enzyme activity test (U/mg)

(Figure 4B). For all types of inducers, protein expression could not be observed clearly on SDS-PAGE analysis, but high activity values were observed in samples with 1998 mM arabinose inducer (Figures 3D, 3E, 3F).

E. coli BL21(DE3) was able to produce high protein bands due to the strong inducer IPTG and lactose-induced overexpression of the PGA recombinant protein regulated by the T7 promoter in the pET expression system. Unfortunately, the formation of an inclusion body was a possibility. According to Stargardt *et al*. [22], inclusion body proteins experience a lot of misfolding and are biologically nonfunctional. That is why for this *E. coli* strain, PGA protein was expressed in high amounts, but its activity value was low.

On the other hand, *E. coli* HB101 showed low expression properties. This *E. coli* strain is widely used for the expression of recombinant proteins whose expression is concentrated in the periplasm [23]. When *E. coli* HB101 was induced by IPTG and lactose, the strain was not able to overexpress the target protein so the PGA level was quite low, but it showed quite high activity results when compared to the *E. coli* BL21(DE3) sample (Figure 4B). In this study, the results of the solubilization treatment of inclusion bodies from both hosts were recovered by the freeze-thawing method, and then an enzyme activity test was performed using pDAB, which revealed a satisfactory PGA enzyme activity value.

The host *E. coli* HB101 has poor expression characteristics and can be employed to generate recombinant proteins that are concentrated in the periplasm [23]. When fermenting at low temperatures, the induction of recombinant protein expression is lower, allowing many target proteins to be expressed with proper folding and maturation [24]. Fermentation conditions at low temperatures can cause changes in the periplasmic function of the housekeeping protease DegP, causing it to become a chaperon protein that aids in the folding of periplasmic proteins, allowing the formation of biologically active proteins [2, 3, 25]. Arabinose induction has a greater 'superpower' than IPTG and lactose in that it can improve PGA activity [10, 26].

According to Vélez *et al*. [24], a temperature of 20°C is optimal for the generation of recombinant PGA protein from recombinant *E. coli*. Low temperatures can slow the production of recombinant proteins, which means that it also slows down the process of appropriately folding biomolecules. Thus, determining the best temperature is critical because it relates to the balance of protein flux formed during the stages of protein synthesis (transcription) and maturation (translocation and periplasmic processing) [27, 28]. The optimization of the agitation speed from 180 to 150 rpm is related to the length of the fermentation process, which was also changed from 4 to 17 h, which means that the cell density does not increase significantly quickly and maintains the availability of carbon sources required for host energy to grow and express recombinant protein.

In both hosts, when they were induced by 1998 mM arabinose, the highest activity values were achieved (6.67 U/mg in *E. coli* BL21(DE3) and 10.17 U/mg in *E. coli* HB101), but the protein level could not be observed as clear protein bands when analyzed by SDS-PAGE. Previous reports stated that the induction of arabinose is ‘stronger’ than IPTG and lactose because it can increase PGA activity [10, 17]. Arabinose is thought to be able to influence the induction of recombinant protein expression in slow-processing conditions. As a result, the transcription and translocation stages from cytoplasm to periplasmic processing will run more efficiently, which means that there will be an increase in the amount of mature recombinant protein expression. In other words, it also reduces the formation of inclusion body in the *E. coli* expression system [10, 23, 29].

The enzyme activity analysis of the *E. coli* BL21(DE3) sample with 0.05 mM IPTG had a value of 2.84 U/mg (IB), which was greater than the activity reported by Purwanto and Purwantini [1], which was 0.0175 U/mg (IB). When viewed from the optimization of host cells, *E. coli* HB101 with 1998 mM arabinose had the highest PGA enzyme activity. Thus, it can be interpreted that *E. coli* HB101 with 1998 mM arabinose is more suitable for the expression of PGA recombinant protein because it displays the highest activity compared to the enzyme activity of the host *E. coli* BL21 (DE3).

In a previous study, Mönster *et al*. [30] reported that PGA enzyme activity from *E. coli* was 4.97 U/mg. It was only around half the value of this study (10.17 U/mg). Hassan [31] showed that the specific activity of PGA was 3.22 U/mg and Kafshnochi *et al*. [32] reported that PGA activity isolated from *E. coli* BL21(DE3) was 1.50 U/mg, while a report from Balci *et al*. [33] obtained a PGA enzyme activity test result of 5.51 U/mg. Some of these previous reports indicated that the PGA enzyme activity value was lower when compared to the activity test results obtained from the PGA enzyme activity in this study, which was 10.17 U/mg

4. Conclusions

The results of the *syn-pac* gene sequencing showed no mutations after being transformed into *E. coli* HB101 and BL21(DE3). PGA recombinant protein was expressed by both host expression systems. In terms of enzyme activity, *E. coli* HB101 outperformed *E. coli* BL21 (DE3) and 1998 mM arabinose was the most effective inducer which yielded the highest PGA enzyme activity in both *E. coli* BL21(DE3) (6.67 U/mg) and HB101 (10.17 U/mg).

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