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Solubilization Inclusion Bodies from Synthetic Recombinant PGA Gene Expressed in *E. coli* BL21(DE3) by Denaturing and Non-denaturing Agents

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Abstract

Background*: With the rise in green chemistry, the synthesis of antibiotic compounds through enzymatic processes is a preferred option. Penicillin-G acylase (PGA) is an important enzyme for producing important antibiotics, such as penicillin and its derivatives. Therefore, studies on PGA have been conducted worldwide. In the penicillin biosynthetic pathway, PGA catalyzes the conversion of penicillin G into 6-amino penicillanic acid (6-APA), a precursor for the enzymatic synthesis of penicillin derivatives. Unfortunately, bacteria naturally produce PGA in small quantities.* ***Objective:*** *One strategy for producing this enzyme in large quantities is DNA recombination, which is expressed in Escherichia coli. The formation of inclusion bodies (IBs) is a common obstacle to protein overexpression in Escherichia coli. In this study, we discuss IBs solubilization methods for recombinant PGA derived from E. coli (rPGAEc) expressed in E. coli BL21 (DE3). Recombinant E. coli BL21 (DE3) cells harboring rPGAEc were induced with IPTG for enzyme expression. Induction was performed at 16 °C for 4 h and 24 h. The PGA enzyme expressed in the IBs form was then incubated in two solutions containing 8 M urea and 0.2% sarcosine to obtain a soluble enzyme.* ***Results:*** *Based on protein analysis by SDS-PAGE, a solution containing 8 M urea solubilized PGA more abundantly than 0,2% sarcosine.* ***Conclusion:*** *The solubilization technique of PGA expressed by E. coli proposed in this study is an alternative solution that can be considered for this purpose.*

Keywords*: amoxicillin, isopropyl-beta-D-thiogalactopyranoside (IPTG), recombinant PGA, solubilization*

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INTRODUCTION

The manufacturing process to produce penicillin derivatives, which are important in first-line therapy for many infectious diseases, can be carried out using synthetic or enzymatic methods. The synthetic method has many disadvantages, such as the requirement of toxic reagents, complicated reaction conditions, and the production of non-specific products. These problems can be avoided using enzymatic methods. In the production of semisynthetic antibiotics, PGA, an industrially significant enzyme, converts penicillin G into 6-aminopenicillanic acid (6-APA). *Escherichia coli* PGA is a periplasmic heterodimeric enzyme composed of two subunits: the α-subunit with a molecular weight of 23.8 kD and the β-subunit with a molecular weight of 62.2 kD. A single-polypeptide cytoplasmic precursor comprising a 26-amino acid signal peptide and a 54-amino acid connector peptide that connects the α- and β-chains was used to manufacture the mature periplasmic protein. Within the bacterial periplasm, the PGA precursor undergoes autocatalytic processing to yield a mature enzyme and eliminate the spacer peptide (Flores et al., 2004).

PGA naturally produced by bacteria, such as *Escherichia coli* and *Bacillus megaterium* (Chiang & Bennett, 1967; Cole, 1969; Illanes & Valencia, 2017). The problem with PGA production by its natural host is a low level of expression that can be overcome through overexpression using a recombinant DNA approach, where the gene encoding PGA (*PGA*) is cloned into the vector and then reproduced and expressed in certain hosts, generally *E. coli* (Gomes et al., 2020; Karthikeyan et al., 2011).

E. coli is the most popular host in DNA recombination techniques. However, protein overexpression in *E. coli* often poses an obstacle, that is, the formation of inclusion bodies (IBs) (Bhatwa et al., 2021). IBs are usually formed when bacterial cells fail to perform the quality control functions of the expressed protein. Consequently, a protein that fails to fold properly at the post-translational stage forms an aggregate. IBs formation is a major obstacle in the production and purification of bioactive proteins such as PGA enzymes (Bhatwa et al., 2021; Burgess, 2009). Several strategies can be used to obtain active PGA from IBs, including solubilization and refolding techniques using denaturing or non-denaturing agents. Examples of denaturing agents include urea and GdnCl, while examples of non-denaturing agents include sarcosine, 5% DMSO, and 5% n-propanol (Singh et al., 2015; Ventura & Villaverde, 2006).

In this study, the gene encoding the PGA enzyme from *Escherichia coli* (*PGAEc*) was optimized to contain only *E. coli*-favored codons. Synthetic *PGAEc* was ligated to pET22b (*rPGAEc*) and transformed into *E. coli* BL21 (DE3) for expression. PGA enzymes expressed as IBs were solubilized in two types of solutions containing a denaturing agent (8 M urea) and a non-denaturing agent (0.2% sarcosine). Thus, we aimed to evaluate whether the two methods can solubilize PGA IBs, which have better capabilities.

MATERIALS AND METHODS

Materials

A recombinant, synthetic gene encoding PGA from *E. coli*/*rPGAEc* (Genscript®), TEMED (Liofilchem, Himedia), sodium ampicillin, $CaCl_2$, acrylamide/bisacrylamide, bromophenol blue, 6-APA, benzylpenicillin G, urea, sarcosine (Sigma-Aldrich), glycerol, isopropyl β-D-1-thiogalactopyranoside/IPTG, Na_2HPO_4, NaH_2PO_4, NaOH, HCl, β-mercapto ethanol (Merck), Tris-base (Biobasic), CH_3COOH, methanol absolute, ammonium persulphate/APS, glycine (Smartlab), sodium dodecyl sulphate/SDS (Bio-Rad Laboratories). The pET22b plasmid, the pET series vector, was used as the vector. The pET22b plasmid was equipped with a strong promoter system, namely, the T7 promoter and ampicillin resistance gene (*AmpR*). This strong promoter supports PGA overexpression. *E. coli* BL21 (DE3) was chosen as the host for PGA enza expression because of its ability to express T7 RNA polymerase, which supports the function of the T7 promoter in plasmid pET22b.

Methods

Competent *E. coli* BL21 (DE3) cells were prepared using the $CaCl_2$ method (Sambrook and Russel, 2001). In this study, genes encoding PGA was codon-optimized, synthesized, and inserted into pET22b to generate recombinant plasmid *rPGAEc* (Genscript®). E. *coli* BL21 (DE3) was grown in Luria Bertani (LB) medium (containing 1 g tryptone, 0.5 g yeast extract, and 1 g NaCl in 1 L). Colonies from glycerol stocks were grown on LB agar medium containing 15 g/L bacteriological agar (Oxoid). PGA expression was measured in an LB medium containing 50 µg/mL ampicillin (LB-amp).

Transformation

The recombinant plasmid *rPGAEc* was transformed into competent *E. coli* BL21 (DE3) cells using the heat shock method (Sambrook and Russel, 2001). Bivalent ions such as Ca^{2+} in $CaCl_2$ provide high transformation efficiency. Colony PCR was performed to analyze

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positive colonies harboring *rPGAEc* (Bergkessel & Guthrie, 2013).

Expression of PGA enzyme

The expression method was adapted from that described by Purwanto et al. (2017). Positive colonies harboring *rPGAEc* were further confirmed by PCR, using orientation-specific primers. The confirmed colonies were expressed in the presence of an IPTG inducer. IPTG with final concentrations of 0,05 mM and IPTG (0.1 mM) were added to the fermented culture when the OD600 values reached 0.5 and 0.7. Fermentation was continued for 4 h and 24 h at 16 °C with shaking at 180 rpm. Cells were separated from the culture media by centrifugation at 3500 rcf, 20-30 minutes at 4^{o}C for further isolation of the PGA *enzyme.*

Isolation of PGA enzyme

Cell disruption was performed using the homogenization technique to release intracellular PGA. Homogenization was performed for four cycles. Each cycle consisted of 10 s with an 80% amplitude, followed by 40 s off. The mixture of cytosolic and periplasmic fractions (referred to as the soluble fraction) was separated from cell debris and IBs (referred to as the insoluble fraction) by centrifugation (8000 rpm, 15 min) (Purwanto et al., 2017). IBs contained within insoluble fractions were solubilized with two kinds of solubilization mixtures, solutions A and B. Solution A contained 50 mM sodium phosphate buffer (pH 8.0), 300 mM NaCl, and 8 M urea. Solution B contained 5% dimethyl sulfoxide (DMSO), 5% n-propanol, and 0.2% sarcosine. Subsequently, it was centrifuged at 8000 rpm for 15 min to obtain a solubilized supernatant that was expected to contain soluble PGA enzyme and pellets that were a mixture of IBs and cell debris remnants. All processes in this step were performed at 4 °C, as temperature is the most important factor affecting protein conformation and activity (Bhat et al., 2018). The total protein content was measured using the UV 280 nm spectrophotometric method (Simonian, 2002).

RESULTS AND DISCUSSION

Recombinant gene design and transformation

Wild-type *PGAEc* naturally expressed in *E. coli* (GenBank Accession: M17609.1) is 2538 bp in length. To optimize PGA expression, we attempted codon optimization of the wild-type *PGAEc* gene. The optimization results showed an increase in codon usage from 0,46 0,96 (Supp. 1), an adjustment in the GC load from 48,30 57,99 (Supp. 2). The usage frequency of 10% was reduced from 12% to zero, usage frequency of 10–20% was reduced from 6% to zero, usage frequency of 31–40% was reduced from 12% to zero, usage frequency of 51–60% was reduced from 4% to zero, usage frequency of 61–70% was reduced from 1% to zero, usage frequency of 71–80% was reduced from 10% to 8%, usage frequency of 81–90% was reduced from 7% to 6%, and usage frequency of 91–100% was increased from 43% to 85% (Supp. 3). Gene optimization changed the DNA base sequence of *PGAEc*, leaving only approximately 77%, similar to the wild-type (Supp. 4). Codon optimization did not change the amino acid sequence of *PGAEc*.

Recombinant plasmid r*PGAEc* (Figure 1) was transformed into *E. coli* BL21 (DE3) cells. Transformed colonies grown on selection media (LB agar + ampicillin 50 µg/mL) were estimated to carry the ampicillin resistance gene/*AmpR* from the pET22b plasmid. The efficiency of this transformation was 4×10^5 transformant/µg DNA, which means that each microgram of the transformed plasmid resulted in 4×10^5 colonies of transformant cells. This efficiency value is common in pUC and pBR plasmid, around 4.8×10^4 and 1.8×10^4 (Lim et al., 2015).

The *AmpR* gene is part of the pET22b plasmid, so the presence of the *AmpR* gene in *E. coli* BL21 colonies (DE3) has not proven that *PGAEc* has been transformed into *E. coli* BL21 (DE3) cells and replicated correctly. Therefore, polymerase chain reaction (PCR) using orientation-specific primers is necessary.

Orientation-specific primers were designed to ensure that the *PGAEc* gene was replicated properly in *E. coli* BL21 (DE3), so that the PCR product would cover the area where the pET22b plasmid section meets the *PGAEc* gene section. Primers were designed using the PrimerBlast website, where the forward primer was predetermined, namely the T7 promoter sequence representing the plasmid pET22b. Using this website, a reverse primer was obtained, namely 5'-GTACCAACAAAGATCATCG-3, ', which represents the *PGAEc* gene section. This primer pair produced PCR products with a length of 588 bp, which covered the area where the pET22b plasmid section met the *PGAEc* gene section. The plasmid section was 92 bp long and the upstream *PGAEc* gene section was 496 bp long (Figure 2a). PCR analysis results from several samples confirmed that resistant ampicillin colonies harbored the *PGAEc* recombinant clone gene with the correct orientation, indicated by the appearance of a single band at the appropriate base pair size (Figure 2b).

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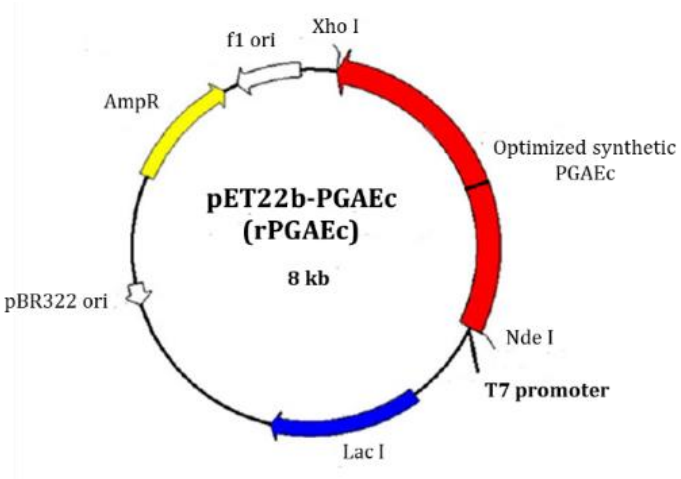

Figure 1. Map of recombinant plasmid *rPGAEc*. Optimized synthetic *PGAEc* was inserted in the multiple cloning site area between Nde I and Xho I restriction enzyme cutting sites (shown in red)

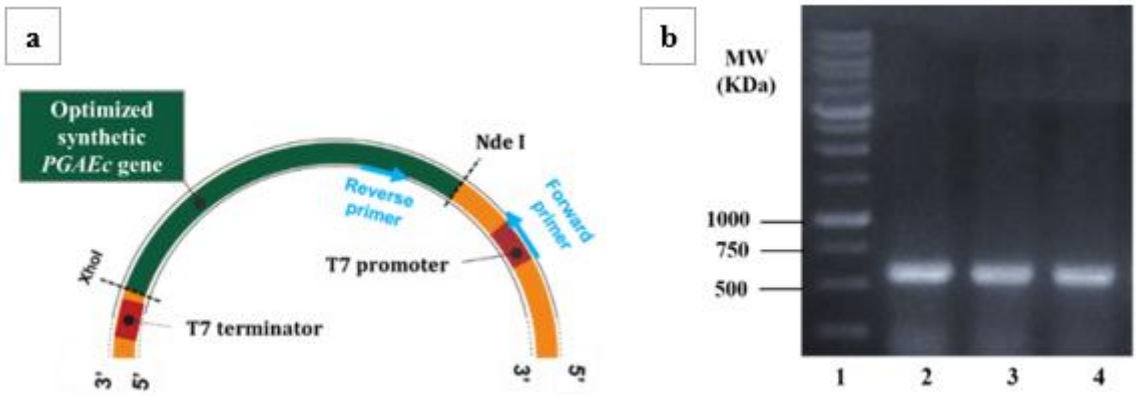

Figure 2. PCR Analysis result of *E. coli* BL21 (DE3) colony were confirmed to harbor *PGAEc* gene a) Specific primer map positions in the *rPGAEc.* Forward primer using T7 promoter sequence while reverse primer using specific sequence: 5'- GTACCAACAAAGATCATCG-3'. b) The PCR product yielded a band of approximately 588 bp. Lane 1 was a DNA marker, and lanes 2-4 were the PCR results of *E. coli* BL21 (DE3) colonies, which were picked up as the PCR template. MW = molecular weight

Table 1. Protein content (mg/mL) of supernatant resulted from solubilization unsoluble fraction of *E. coli* BL21 harboring rPGAEc cell using solution A (measured by UV 280 nm)

Fermentation Time	Without IPTG	IPTG 0,1 mM
4 hours	11.70	37.70
24 hours	38.03	116.59

Effect of fermentation time on protein and PGA expression

By studying the length of fermentation time, the optimum time for protein expression can be obtained while considering the efficiency aspects. The effect of fermentation time on protein expression was observed by comparing protein expression at 4 and 24 h after IPTG induction. IPTG mimics lactose, a natural lac operon inducer contained in pET22b. However, IPTG is preferred for protein expression inducers because bacterial cells cannot possess IPTG as a carbon source for cell growth. Therefore, the concentration of IPTG in the fermentation medium was more stable (Fernandez-Castane et al., 2012).

Protein expression level was estimated from solubilization results of unsoluble fraction using solution A. The results of protein content measurement showed that fermentation for 24 h resulted in three times greater protein expression than fermentation for 4 h, both with and without induction IPTG 0,1 mM (Table 1).

The measured protein content was the concentration of large-sized proteins precipitated during post-homogenization centrifugation. Therefore, protein content did not directly represent the effect of

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fermentation time on PGA enzyme expression. More representative data can be discerned from the protein separation results using SDS-PAGE (Figure 3).

SDS-PAGE results showed that the expression of PGA enzymes in IBs formed in the presence of 0.1 mM IPTG was not significantly different between 4 h and 24 h of of fermentation. This is presumably the reason why protein content in 24-hours fermentation time is three times higher than that in 4-hours. Thus, 4-hours fermentation was considered more appropriate for PGA enzyme expression in this study. PGA enzymes are well expressed in a shorter time; hence, they are more efficient.

Effect of IPTG concentration on PGA expression

It has previously been reported that high inducer concentrations can lead to IB formation (Bhatwa et al., 2021; Singh et al., 2015). In this research, fermentation was carried out with various IPTG concentrations to determine their effect on PGA enzyme expression and IBs formation, namely: 0; 0,05; and 0,1 mM. Protein content measurement results of the soluble fraction and solubilization of the unsoluble fraction with solution B (Table 2) showed that higher IPTG concentration caused lower protein expression in the soluble fraction. Higher IPTG concentrations caused higher protein expression in the insoluble fractions. This phenomenon might be due to an increase in the expression of large proteins in the cells, which specifically refers to IBs. This is apparently due to the higher IPTG concentration leading to higher PGA enzyme expression, so more resources in the cells are directed to produce PGA enzymes. As a result, other proteins not associated with the *PGAEc* gene, especially those expressed in the cytosol and periplasm, showed lower expression levels.

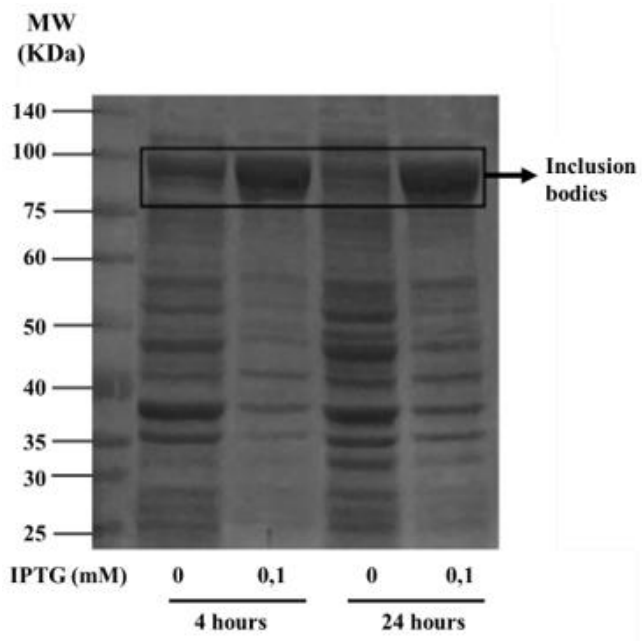

Figure 3. SDS-PAGE results to see the effect of fermentation time on PGA enzyme expression
SDS-PAGE sample was the supernatant resulting from solubilization of the insoluble fraction by solution A. Cultures were treated with and without (IPTG 0.1 mM IPTG induction. The fermentation volume was 50 mL in the LB + amp medium. Fermentation was performed for 4 and 24 h (after IPTG induction) at 16 °C and 180 rpm. The DNA ladder is located in the first lane from the left. Inclusion bodies are shown in the black box.

Table 2. Protein content (mg/mL) of soluble fraction and supernatant resulting from solubilization unsoluble fraction of *E. coli* BL21 harboring *rPGAEc* cell using solution B (measured by UV 280 nm)

Sample	Protein Content in Certain IPTG Concentrations (mg/mL)		
	0 mM	0,05 mM	0,1 mM
Soluble fraction	137.3	131.2	104.9
Solubilization unsoluble fraction*)	18.7	32.8	42.6

*) SDS-PAGE result is not shown

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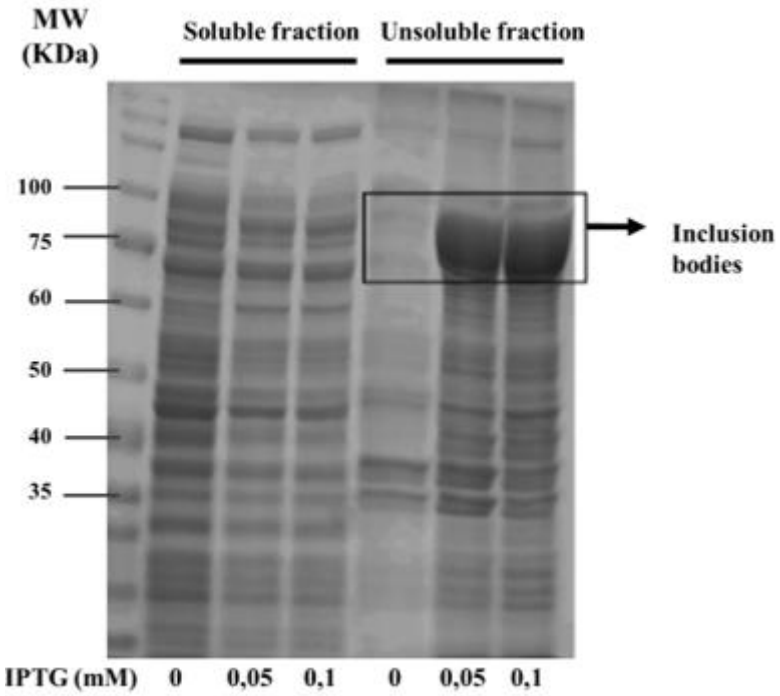

Figure 4. SDS-PAGE results showed the effect of IPTG concentration on PGA enzyme expression
SDS-PAGE samples were soluble and insoluble fractions of *E. coli* BL21 (DE3) harboring rPGAEc cell lysate, which were separated through homogenization and centrifugation. Cultures were treated with various IPTG concentrations (0; 0,05, and 0,1 mM. The fermentation volume was 100 mL in the LB + amp medium. Fermentation was performed for 4 h after IPTG induction at 16 °C and 180 rpm. A smaller amount of inducer IPTG (0,05 mM) can express PGA enzyme as much as a higher amount (0,1 mM), which is shown as an inclusion bodies protein band (in the black box)

Protein separation using the SDS-PAGE method (Figure 4) showed similar results. The separation results of the unsoluble fraction indicated that IBs formed in the 0.1 mM IPTG induction treatment were slightly more than those induced by IPTG 0.05 mM. Therefore, the separation result of the soluble fraction indicated that the protein bands became thicker at lower IPTG concentrations. Thus, the higher the IPTG concentration, the higher the number of IBs expressed. Theoretically, a higher expression of IBs leads to a lower expression of active PGA enzymes. Previous studies have shown that at low IPTG concentrations between 0.025 and 0.1 mM, enzyme activity increased with increasing IPTG concentrations. However, enzyme activity declined progressively at higher IPTG concentrations between 0.2 and 0.5 mM (Sriubolmas et al., 1997). IPTG is a strong inducer that often drives IB formation. To reduce IBs expression expression, low IPTG concentrations are required (Rinas et al., 2017).

Comparison of Solubilization Methods

pET22b vector belongs to a class of plasmids with strong promoters, as the desired gene can only be expressed with strong inducers, such as IPTG. However, using a strong inducer in an *E. coli* host will always lead to IB formation. Therefore, finding the correct solubilization method is important to obtain active PGA enzymes.

Two solubilization methods were used in this study. The first method uses a denaturing agent, urea. The second method uses a non-denaturing agent, sarcosine. Urea causes denaturation of proteins, whereas sarcosine does not (Singh et al. 2015). If solubilization is performed using protein-denaturing agents, further refolding is required to obtain an active PGA enzymes. This study aimed to determine the difference in the ability of urea and sarcosine to dissolve IBs and to determine whether successfully dissolved IBs are in an active state. Therefore, refolding was not performed in the present study. Based on the results of protein separation using SDS-PAGE, it can be seen that IBs dissolved in urea have a significant decrease in the number of aggregates compared to sarcosine (Figure 5). The remaining aggregates appeared in the pellet lane (P) after the solubilization process, while the successfully solubilized IBs appeared in the supernatant lane (S). This indicates that urea is more soluble than sarcosine. Because of this ability, a previous study reported the use of urea to solubilize the IBs form of cephalosporin acylase enzyme expressed in *E. coli* BL21 (Hardianto et al., 2018). On the other hand, although the solubilization

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ability of sarcosine is not as good as urea, its ability to solubilize proteins without denaturing them is quite attractive. Several studies have reported the use of sarcosine to solubilize Ibs (Chung et al., 2017; Manissorn et al., 2023; Nagy et al., 2021).

Solubilization with method A succeeded in dissolving almost all the IBs in the cell debris and yielded only a few IBs that remained in the pellets. For comparison, we used method B, which contained organic solvents (5% DMSO and 5% n-propanol) and non-denaturing detergents at low concentrations (0.2% sarcosine). This method does not cause denaturation of the proteins composing the IBs. However, the disadvantage is their low solubilization capacity (Figure 5b). Solubilization with solution B only slightly dissolved IBs (S after solubilization), leaving many IBs in the insoluble fraction (P before solubilization). Despite its relatively low solubilization ability, sarcosine has been reported to have the best ability to solubilize IBs compared to other non-denaturing agents such as Triton X-100, NP-40, and Tween-20 (Chung et al., 2017).

Urea is commonly used to solubilize IBs in E. coli (Singh et al., 2015). However, there have been no reports on its use in PGA enzymes, so this research can contribute to our knowledge. After the IBs of the PGA enzyme were successfully solubilized, the next step was the refolding process to obtain the active enzyme. The active enzyme was purified using a Ni-NTA resin in an affinity chromatography system. Purified enzymes can be used to convert penicillin G to 6-APA, a precursor for the synthesis of penicillin-derived antibiotics. A roadmap for further research is presented in Figure 6.

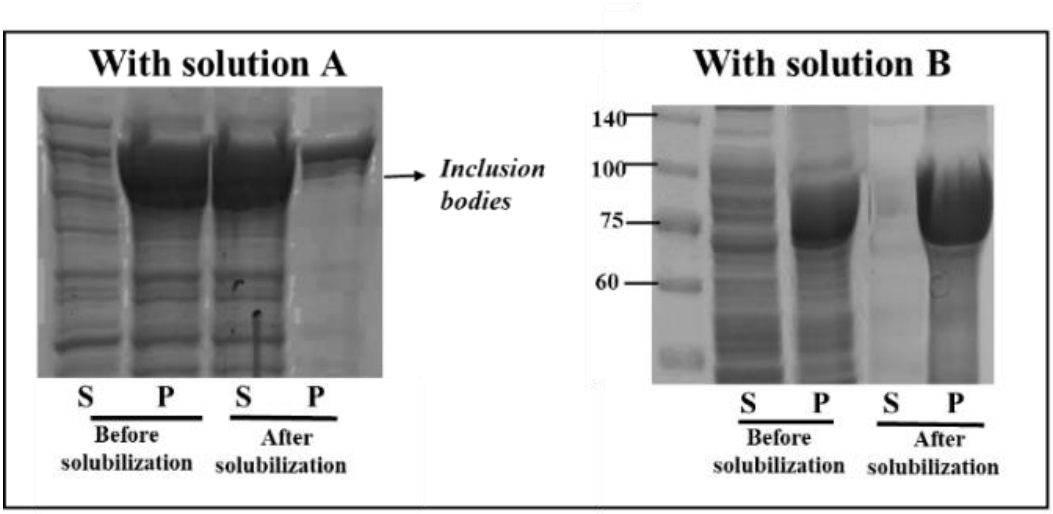

Figure 5. SDS-PAGE results show differences in the ability of urea and sarcosine to dissolve IBs
The soluble fraction (supernatant/S before solubilization) and an unsoluble fraction (pellet/P before solubilization) of *E. coli* BL21(DE3) harboring rPGAEc were separated by homogenization and centrifugation. The insoluble fractions were solubilized using solutions A and B, and and then centrifuged to separate IBs that successfully dissolved (supernatant/S after solubilization) and failed to dissolve (pellet/P after solubilization). Fermentation was carried out in LB + amp medium for 4 hours after 0.1 mM IPTG induction at 16 °C and 180 rpm. Based on data of area under the curve, which was analyzed by ImageJ software (in triplicate and p = 0.05), solution A (containing urea) is significantly better as a solubilizing agent for rPGAEc than solution B (containing sarcosine)

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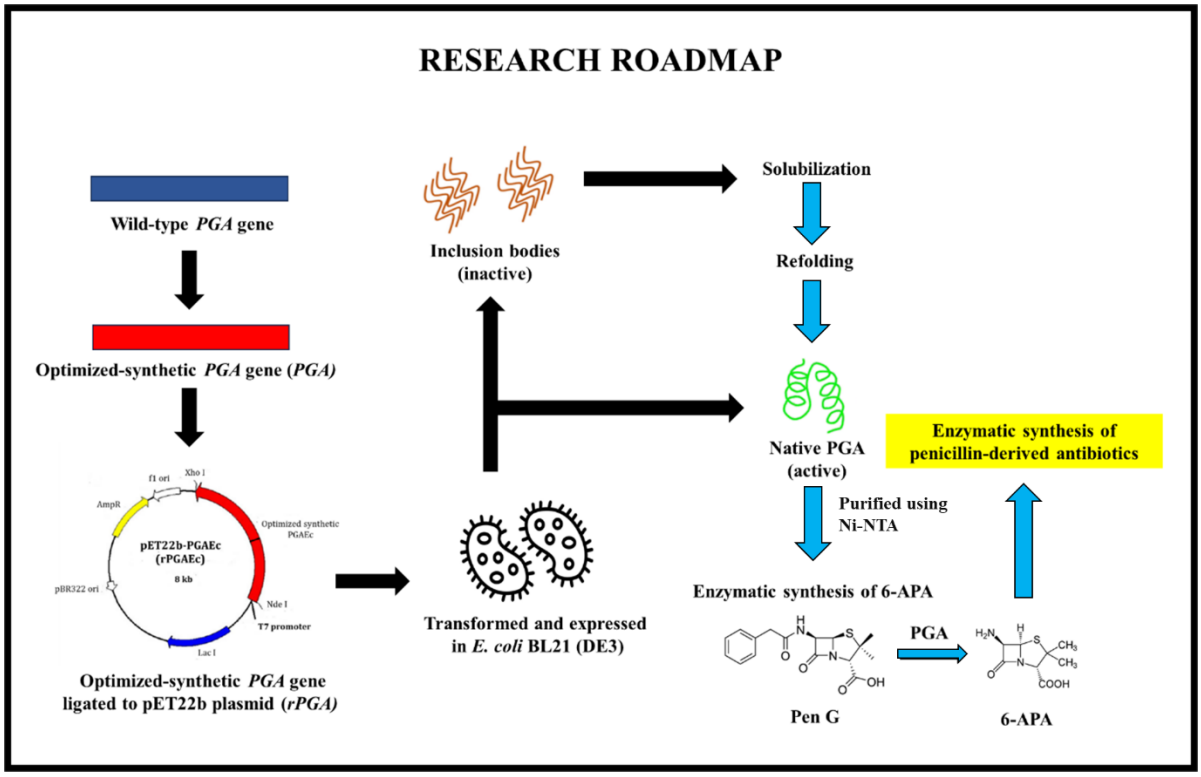

Figure 6. Research roadmap
Black arrows indicate the stages of the research conducted in this study. While the blue arrows show further research plans

CONCLUSION

The expression of PGA enzymes using strong promoter vectors in *E. coli* hosts has encountered obstacles in the formation of IBs. The solubilization technique of IBs from PGA expressed in *E. coli* BL21 (DE3) proposed in this study may be an alternative method that can be considered for this purpose. However, further research is needed to determine the reliability of this method is for producing active PGA enzymes from IBs.

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AUTHOR CONTRIBUTIONS

Conceptualization, P., S., I.P., R.; Methodology, P., S., I.P., R.; Validationon, P., S., I.P., R., M.R., M.A.M.; Formal analysis, P., S., M.A.M.; Investigation, P., M.R., M.A.M.; Resources, P., S.; Data Curration, P., S., M. R., M.A.M.; Writing - Original Draft, P., S., M.R.; Writing - Review and Editing, P., S., I.P., R., M.R., M.A.M.; Visualization, P. M.R.; Supervision, P. S.; Project Administration, P., S.; Funding Acquisition, P.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

REFERENCES

Bergkessel, M., & Guthrie, C. (2013). Colony PCR. *Methods in Enzymology*, *529*, 299–309. https://doi.org/https://doi.org/10.1016/B978-0-12-418687-3.00025-2

Bhatwa, A., Wang, W., Hassan, Y. I., Abraham, N., Li, X. Z., & Zhou, T. (2021). Challenges associated with the formation of recombinant protein inclusion bodies in Escherichia coli and strategies to address them for industrial applications. *Frontiers in Bioengineering and Biotechnology*, *9*. https://doi.org/https://www.frontiersin.org/articles/10.3389/fbioe.2021.630551

Burgess, R. R. (2009). Refolding solubilized inclusion body proteins. *Methods in Enzymology*, *463*, 259–282. https://doi.org/https://doi.org/10.1016/S0076-6879(09)63017-2

P-ISSN: 2406-9388
E-ISSN: 2580-8303

Chiang, C., & Bennett, R. E. (1967). Purification and properties of penicillin amidase from Bacillus megaterium. *Journal of Bacteriology*, *93*(1), 302–308. https://doi.org/10.1128/jb.93.1.302-308.1967

Chung, J. M., Lee, S., & Jung, H. S. (2017). Effective non-denaturing purification method for improving the solubility of recombinant actin-binding proteins produced by bacterial expression. *Protein Expression and Purification*, *133*, 193–198. https://doi.org/http://dx.doi.org/10.1016/j.pep.2016.06.010

Cole, M. (1969). Hydrolysis of penicillins and related compounds by the cell-bound penicillin acylase of Escherichia coli. *The Biochemical Journal*, *115*(4), 733–739. https://doi.org/10.1042/bj1150733

Fernandez-Castane, A., Caminal, G., & Lopez-Santin, J. (2012). Direct measurements of IPTG enable analysis of the induction behavior of E. coli in high cell density cultures. *Microbial Cell Factories*, *11*(58). https://doi.org/https://doi.org/10.1186/1475-2859-11-58

Flores, G., Soberon, X., & OSUNA, J. (2004). Production of a fully functional, permuted single-chain penicillin G acylase. *Protein Science*, *13*, 1677–1683. https://doi.org/http://www.proteinscience.org/cgi/doi/10.1110/ps.03436604

Gomes, L., Monteiro, G., & Mergulhao, F. (2020). The impact of IPTG induction on plasmid stability and heterologous protein expression by Escherichia coli biofilms. *Inter. J. Mol. Sci.*, *21*(576), 1–14. https://doi.org/https:// doi:10.3390/ijms21020576

Hardianto, D., Isdiyono, B. W., & Royani, J. I. (2018). Solubilization of cephalosporin acylase from Escherichia coli BL-21 (DE3) inclusion bodies. *The 2nd International Conference on Biosciences*, *197*. https://doi.org/https:// doi :10.1088/1755-1315/197/1/012040

Illanes, A., & Valencia, P. (2017). Industrial and therapeutic enzymes: Penicillin Acylase. in A. Pandey, S. Negi, & C. R. Soccol (Eds.). *Current Developments in Biotechnology and Bioengineering*, *13*, 267–305. https://doi.org/https://doi.org/10.1016/B978-0-444-63662-1.00013-0

Karthikeyan, R., Surianarayanan, M., Sudharshan, S., Gunasekaran, P., & Asit Baran, M. (2011). Biocalorimetric and respirometric studies on production of Penicillin G acylase from Bacillus badius pac in E. coli DH5α. *Biochemical Engineering Journal*, *55*(3), 223–229. https://doi.org/10.1016/j.bej.2011.05.003

Lim, G., Lum, D., Ng, B., & Sam, C. (2015). Differential transformation efficiencies observed for pUC19 and pBR322 in E. coli may be related to calcium chloride concentration. *Journal of Experimental Microbiology and Immunology*, 1–6. https://doi.org/https://jemi.microbiology.ubc.ca/sites/default/files/Lim%20et%20al.pdf

Manissorn, J., Tonsomboon, K., Wangkanont, K., & Thongnuek, P. (2023). Effects of chemical additives in refolding buffer on recombinant human BMP-2 dimerization and the bioactivity on SaOS-2 osteoblasts. *ACS Omega*, *8*, 2065–2076. https://doi.org/https://doi.org/10.1021/acsomega.2c05802

Nagy, K., Kovacs, Z., Miklossy, I., Salamon, P., Orban, C., Albert, B., & Lanyi, S. (2021). Detergent aided refolding and purification of recombinant xiap from inclusion bodies. *Studia UBB Chemia*, *4*, 355–368. https://doi.org/https://doi:10.24193/subbchem.2021.4.26

Purwanto, R., Hori, K., Yamada, Y., & Sato, F. (2017). Unraveling additional o-methylation steps in benzylisoquinoline alkaloid biosynthesis in California poppy (eschscholzia californica). *Plant and Cell Physiology*, *58*(9), 1528–1540. https://doi.org/10.1093/pcp/pcx093

Rinas, U., Garcia-Fruitos, E., Corchero, J. L., Vazquez, E., Seras-Franzoso, J., & Villaverde, A. (2017). Bacterial inclusion bodies : discovering their better half. *Trends Biochem. Sci.*, *42*(9), 716–737. https://doi.org/https :// doi: 10.1016/j.tibs.2017.01.005

Sambrook, J., & Russel, D. W. (2001). *Molecular cloning: A laboratory manual* (3rd ed.). Cold Spring Harbor Laboratory Press.

Simonian, M. H. (2002). No Title. *Curr. Protoc. Cell Biol.* https://doi.org/doi: 10.1002/0471143030.cba03bs15

Singh, A., Upadhyay, V., Upadhyay, A. K., Singh, S. M., & Panda, A. K. (2015). Protein recovery from inclusion bodies of Escherichia coli using mild solubilization process. *Microbial Cell Factories*, *14*(1), 1–10. https://doi.org/10.1186/s12934-015-0222-8

P-ISSN: 2406-9388
E-ISSN: 2580-8303

Sriubolmas, N., Panbangred, W., Sriurairatana, S., & Meevootisom, V. (1997). Localization and characterization of inclusion bodies in recombinant Escherichia coli cells overproducing penicillin G acylase. *Applied Microbiology and Biotechnology*, *47*(4), 373–378. https://doi.org/https://doi.org/10.1007/s002530050943

Ventura, S., & Villaverde, A. (2006). Protein quality in bacterial inclusion bodies. *Trends in Biotechnology*, *24*(4), 179–185. https://doi.org/https://doi.org/10.1016/j.tibtech.2006.02.007

P-ISSN: 2406-9388
E-ISSN: 2580-8303