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Research Article

A Comparative Study of Penicillin G Acylase Expression in Two *Escherichia coli* Strains: BL21 (DE3) and Arctic Express

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ABSTRACT

The growing demand for semisynthetic beta-lactams has directed attention towards enzymes, specifically Penicillin G Acylases (PGAs), for their potential in synthesizing these antibiotics. This study delves into the expression of *Achromobacter xylosoxidans* PGA (AxPGA) in *Escherichia coli*, with a focus on enhancing the yield of active PGA, often constrained by a complex maturation process. The optimization of PGA expression included variations in IPTG concentration and the addition of $CaCl_2$. Furthermore, the study compared PGA expression in *E. coli* BL21 (DE3) with that in *E. coli* Arctic Express (DE3), capable of co-expressing chaperones (chaperonin Cpn60 and Cpn10). Induction with 0.5 mM IPTG resulted in the highest hydrolytic activity in both strains, with Arctic Express (AE) exhibiting significantly higher activity due to improved folding facilitated by cold-adapted chaperonins. Alongside optimal IPTG induction, the addition of 10 mM $CaCl_2$ in the culture media significantly increased PGA activity in both strains, highlighting that Ca^{2+} supplementation is an effective strategy to enhance the yield of functional PGA. Subcellular fractionation demonstrates that the periplasmic fraction yielded higher volumetric and specific activities compared to the cytoplasmic fractions in both *E. coli* strains, highlighting the importance of periplasmic processing for PGA maturation. This suggests that extracting the periplasmic fraction is an effective strategy for recovering active PGA while avoiding or reducing contamination either from co-expressed cytoplasmic chaperones or other intracellular proteins. These findings emphasize that induction strategy, ionic stabilization, and host strain selection play synergistic roles in increasing active recombinant PGA expression.

1. Introduction

Penicillin, a member of the β-lactam antibiotic group, holds a distinguished position within the global market, achieving substantial annual sales of approximately $15 billion (Parmar *et al.* 2000; Chandel *et al.* 2008; Srirangan *et al.* 2013). This covers approximately 65% of global market for antibiotics as it is widely used in the treatment of several bacterial infections (Rodriguez-Herrera *et al.* 2019). Nevertheless, the extensive use of penicillin antibiotics has led to the emergence of antimicrobial resistance as a consequential outcome. In response to this challenge, the pursuit of novel semisynthetic antibiotics has garnered importance (Davies and Davies 2010; Srirangan *et al.* 2013; Illanes and Valencia 2017).

Having higher clinical efficacy, lower toxicity, and a broader spectrum of bactericidal activity semisynthetic beta-lactam antibiotics have greater advantages compared to the natural ones. The increasing demand for semisynthetic beta-lactams, particularly amoxicillin, has led to a focus on enzymes that can potentially synthesize

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these antibiotics. Through enzymatic acylation of the nucleus with various side chains in the beta-lactam structure, novel antibiotics can be generated, offering an altered antibacterial spectrum as shown in Figure 1.

Penicillin G acylase (PGA), also known as penicillin amidohydrolase (EC 3.5.1.11) is one of the most relevant enzymes in the pharmaceutical industry. The enzymes have broad substrate specificity and catalyze the hydrolysis of the amidic bond of penicillin G and cephalosporin G to produce 6-APA and 7-ADCA, key components in the chemical production of new β-lactam antibiotics with greater effectiveness (Chandel *et al.* 2008; Torres *et al.* 2012; Srirangan *et al.* 2013; Sambyal and Singh 2021). Recently, the PGA has been reported to be used to synthesize many valuable semi-synthetic antibiotics such as amoxicillin, cefadroxil, and cefazolin (Liu *et al.* 2017; Pan *et al.* 2018; Wang *et al.* 2021).

PGA is a member of the N-terminal nucleophilic hydrolase structural superfamily, and its constituents undergo a complex maturation process. Synthesized initially as an inactive preprotein, it comprises a leader peptide guiding the protein to its destination and a spacer peptide that separates the α and β subunits. The activation of PGA involves a sequence of post-translational steps, encompassing translocation and periplasmic processing and folding, which stand out as atypical for prokaryotic proteins (Hewit *et al.* 2000; Ignatova *et al.* 2003).

In the present work, we optimized expression of axPGA by varying IPTG concentration as inducer and supplementation of $CaCl_2$ in *E. coli* BL21 (DE3) and *E. coli* Arctic Express (DE3). The effect of inducer concentration and $CaCl_2$ supplementation on the level of soluble protein expression was determined. We also evaluated the effects of the molecular chaperones Cpn60 and Cpn10 on expression of soluble axPGA.

2. Materials and Methods

2.1. Plasmid Construction and Bacterial Hosts

The gene encoding *Achromobacter xylosoxidans* Penicillin G Acylase (axPGA, Genebank accession number CP025774) was synthesized and cloned into pET28b (+) vector between NdeI and HindIII restriction sites by GenScript, USA. Two strains of *Escherichia coli* BL21 (DE3) and Arctic Express (DE3), were used as the host for expressing the recombinant enzymes. The *E. coli* Arctic Express was employed as an attempt to enhance the recombinant axPGA expression since it contains a recombinant pACYC-based plasmid capable of constitutively expressing the *Oleispira antarctica* chaperonins Cpn10 and Cpn60 (Agilent Technologies 2015).

2.2. Medium and Culture Conditions

A seed culture was prepared by inoculating a 1% (v/v) glycerol stock culture into 5 mL of fresh LB media, supplemented with kanamycin (50 µg/mL) or with an additional gentamycin (20 µg/mL) when culturing *E. coli* ArcticExpress (AE). The seed culture was then incubated at 37°C with 150 rpm agitation overnight. One

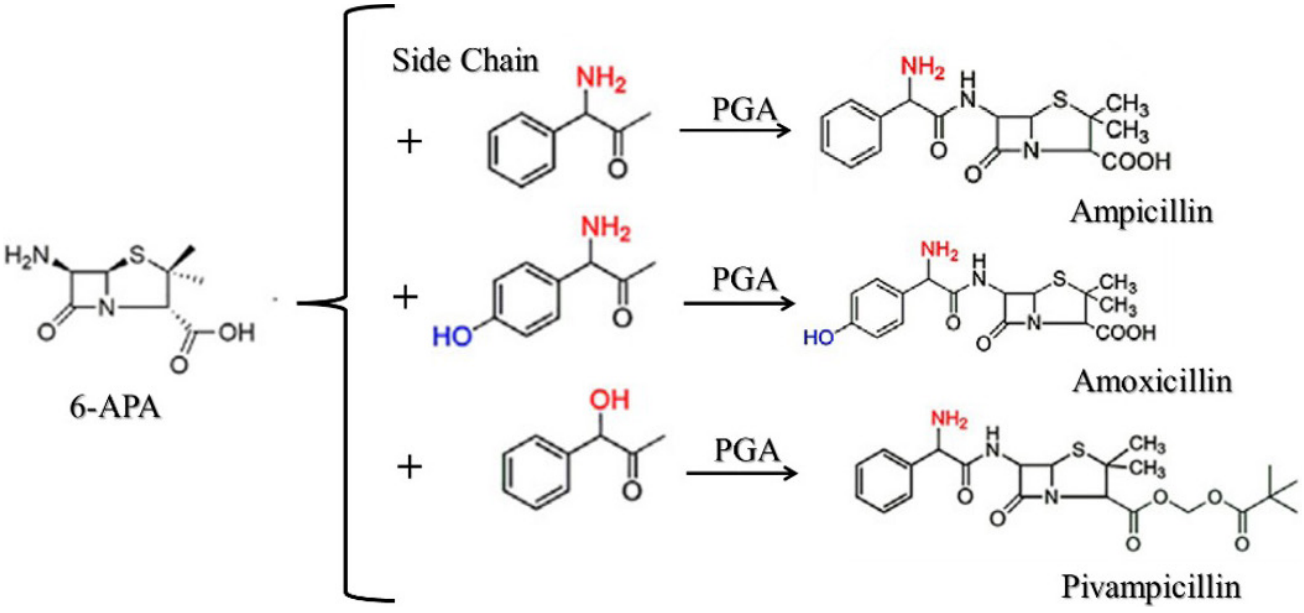

Figure 1. Penicillin G acylase-catalyzed synthesis of semisynthetic penicillins

percent (v/v) of the seed culture was inoculated into LB media containing appropriate antibiotics, supplemented with or without 10 mM $CaCl_2$, and grown at 37°C in a rotary shaking incubator until the cell density at λ600 nm reached approximately 0.6. The recombinant protein expression was induced by adding IPTG to a final concentration ranging from 0 to 1 mM IPTG and continued to grow overnight at 20°C for *E. coli* BL21 or at 10°C for *E. coli* AE, respectively. Cells were harvested by centrifugation at 6,000 rpm, 4°C, for 10 minutes. The supernatant was discarded, and the pellet was stored at -80°C until further protein analysis. Unless otherwise specified, all cultivation was conducted in at least a duplicate of 10 mL culture volume.

2.3. Preparation of Protein Fractions

Soluble axPGA was analyzed in intracellular, periplasmic, and cytoplasmic fractions. Intracellular protein extract was obtained from the whole cell pellet lysate by ultrasonication, performed in an ice bath for 10 minutes (5 seconds on and 20 seconds off) at 80% amplitude. The clear supernatant containing soluble protein was recovered by centrifugation at 13,000 x g at 4°C for 15 minutes.

The periplasmic protein fraction was extracted using a cold-osmotic shock procedure as described by Alvandi *et al.* (2011). Frozen whole cell pellets were resuspended in ice-cold buffer A (0.03 M Tris-HCl, pH 7.3) at a ratio of 100 mg wet weight of cell pellets per 4 mL of buffer. The cell pellets were harvested by centrifugation at 5,000 rpm, 4°C, for 10 minutes, followed by resuspension in 4 mL of buffer A and 4 mL of ice-cold buffer B (0.3 M Tris-HCl, pH 8, 1.5 mM EDTA, 40% (w/v) sucrose). The cell suspension was shaken in a rotary shaker for 30 minutes on ice and then re-centrifuged. The resulting pellets were rapidly resuspended in 4 mL of cold water as a hypotonic solution and shaken in a rotary flask for 30 minutes on ice. After centrifugation, the supernatant was collected as the periplasmic fraction, and the pellet was stored in a deep freezer for further cytoplasmic extraction.

The cytoplasmic protein fraction was carried out by the sonication method with mercaptoethanol lysis buffer. The cell pellets were resuspended in the 1 mM mercaptoethanol lysis buffer and centrifuged at 2,935 rpm, 4°C, for 10 minutes. The pellet was dried from the supernatant and resuspended in lysis buffer for sonication at the ice bath. The cell suspension was sonicated for 10 minutes using 80% amplitude, followed by centrifugation at 13,000 rpm, 4°C, for 20 minutes. The supernatant (lysate) and pellet (debris) were separated as soluble and insoluble fraction samples. Lysate and debris samples were stored at -4°C and -80°C respectively, for the next analysis or quantified by the Bradford method for assaying the protein concentration. All the samples were also visualized by 10% polyacrylamide gel electrophoresis (SDS-PAGE) using a constant voltage of 60 V for approximately 1.5 hours to identify the protein by its molecular mass.

2.4. Assay of PGA Activity

The hydrolytic activity of PGA was determined by reacting enzyme samples with penicillin G (20 mg/mL^{-1} in 0.05 M phosphate buffer pH 7) at 37°C. The generated product, 6-aminopenicillanic acid (6-APA), was measured spectrophotometrically using p-dimethylaminobenzaldehyde as the chromogenic reagent, yielding a yellowish-green solution in acidic medium with a maximum absorption peak at 415 nm (Balasingham 1972, 1984). One unit (U) of enzyme activity is defined as the amount of enzyme required to produce 1 µmol of 6-APA per minute at 37°C. Specific productivity of the cells was calculated as the ratio of total PGA activity to the value of biomass represented by cell density at λ = 600 nm (Sharipova *et al.* 2008).

2.5. Statistical Analysis

Statistical analysis was performed using Analysis ToolPak–VAB available in MS Excel to conduct analysis of variance ($p<0.05$) and post hoc test Tukey's Honest Significant Difference (HSD) to asses' significant differences within samples. All enzyme assays were conducted in triplicate, and the data are represented as mean ± standard deviation.

3. Results

3.1. Effect of IPTG Concentration and Supplementation of $CaCl_2$

To determine the optimum IPTG concentration, various concentrations (0.0, 0.1, 0.5, and 1.0 mM) were tested. The IPTG induction experiments demonstrate that IPTG concentration strongly influences expression of active PGA in both strains, with *E. coli* AE consistently surpassing BL21. As shown in Figure 2A, AE exhibited significantly higher PGA hydrolytic activity at all concentrations tested (0-1.0 mM) with maximum activity achieved at 0.5 mM IPTG (13.57 U/mg, denoted by g). On the other hand, *E. coli* BL21 showed relatively low activity, reaching a maximum of 3.44 U/mg at 0.5 mM IPTG. Statistically grouping

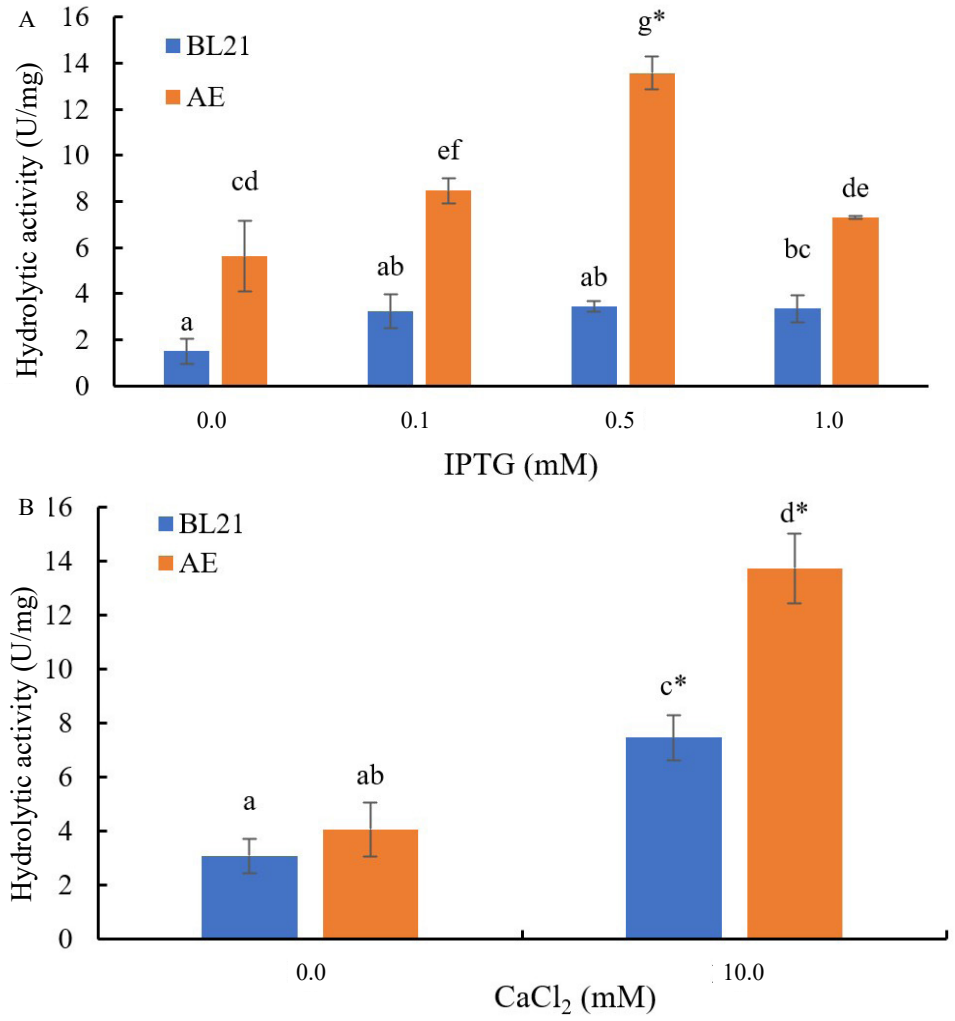

Figure 2. Effect of IPTG induction (A) and $CaCl_2$ supplementation (B) on active PGA expressed in *E. coli* BL21 (■) and AE (■). *p<0.05 was considered significantly different. Same letters indicate an insignificant difference

letters (a-g) indicates that the difference within IPTG concentration was significant in AE but less prominent in BL21. At 0 mM concentration of IPTG, AE expressed detectable hydrolytic activity of 5.6 U/mg, significantly higher than that of BL21 (1.5 U/mg), suggesting basal or leaky expression from T7 promoter in AE. The PGA activity further increased with the induction of IPTG at 0.1 and 0.5 mM but decreased at 1 mM IPTG, indicating that excess IPTG does not enhance and may even suppress active enzyme accumulation.

Further optimization of active PGA expression was performed by supplementing the culture media containing 0.5 mM IPTG without or with 10 mM $CaCl_2$. As presented in Figure 2B, the addition of 10 mM $CaCl_2$ to the culture media resulted in a significant increase of active PGA in both *E. coli* strains. Without addition of $CaCl_2$ (0 mM) AE exhibits a higher basal PGA activity (4.04 U/mg) in comparison to BL21 (3.07 U/mg), as indicated by the letter grouping (a-ab). Upon addition of 10 mM $CaCl_2$ PGA activity significantly rises to 7.44 U/mg (d*) and 13.74 U/mg (e*) for BL21 and AE, respectively.

3.2. Effect of Co-Expression of Chaperones

Expression analyses of axPGA in recombinant *E. coli* strains grown in LB medium without and with $CaCl_2$ or Terrific Broth were analyzed qualitatively by SDS-PAGE. SDS-PAGE profiles in Figure 3 reveal distinct differences in the accumulation of PGA in *E. coli* BL21 and AE, which are indicated by the presence of the predicted β-subunit band (~62 kDa) of axPGA under those various culture conditions. In the case of *E. coli* BL21 (Figure 3A), induction with 0.5 mM IPTG

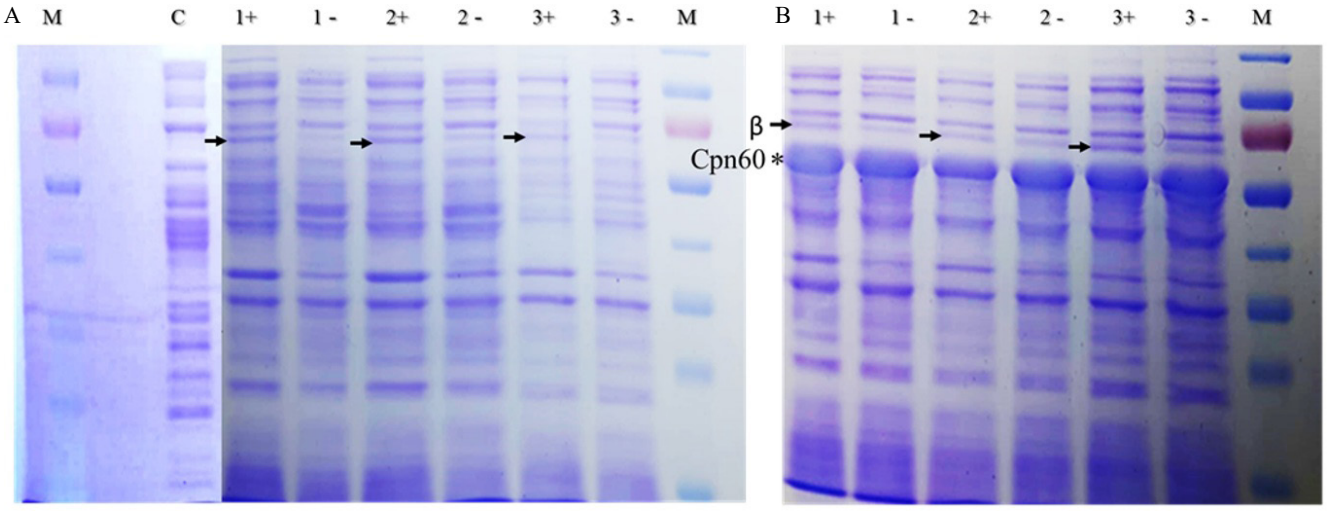

Figure 3. SDS-PAGE analysis of axPGA expression of (A) *E. coli* BL21 and (B) *E. coli* AE grown in LB media without $CaCl_2$ (1), with $CaCl_2$ (2), and TB media without $CaCl_2$ (3) Cultures induced with 0.5 mM IPTG and uninduced control are indicated by plus (+) and minus (-) symbols, respectively. C indicates a control culture of *E. coli* without a plasmid

into culture (Figure 3A, lanes 1+, 2+, 3+) generated clear bands at the predicted ~62 kDa molecular weights of β-subunits of axPGA, which were not or weakly present in the uninduced controls (Figure 3A, lanes 1-, 2-, 3-). Expression of AxPGA in TB medium (lane 3+) demonstrates a more intense β-subunit band than in LB medium (lanes 1+ and 2+). Meanwhile, the effect of $CaCl_2$ addition (lane 2+) can be seen in LB medium, where the β-subunit band appears stronger than in LB without $CaCl_2$ (lane 1+). In comparison to Figure 3A, SDS-PAGE profile of *E. coli* AE lysates (Figure 3B) showed in addition to the target bands of β-subunits of AxPGA, a prominent protein band at about 60 kDa corresponding to the constitutively co-expressed cold-adapted chaperonin Cpn60. The targeted β-subunit band of AxPGA appears in all culture conditions including the control uninduced ones (lanes "-"). This pattern of detected ~62 kDa band aligns with the quantitative enzymatic activity data, in which AE consistently exhibits higher PGA activity than BL21, including measurable activity even in the absence of IPTG induction. The appearance of the β-subunit in uninduced AE fractions supports AE's enhanced capacity for basal expression and its improved folding efficiency facilitated by co-expressed chaperonins Cpn60/Cpn10, which collectively result in higher levels of active PGA. Notably, expressions in TB medium (Figure 3B, lane 3+) showed a more intense band of β-subunit compared to LB medium (Figure 3B, lanes 1+ and 2+), suggesting that TB medium supports higher biomass accumulation and protein yield due to its richer nutrient composition.

3.3. Subcellular Accumulation of Active AxPGA

The PGA-hydrolytic activities of cytoplasmic and periplasmic protein extracts were analyzed to predict the subcellular accumulation of the active PGA. Periplasmic protein fraction was extracted from the cell pellet using a cold-osmotic procedure, which allowed the soluble axPGA to be harvested from the clear supernatant. The remaining cell pellet was then extracted by ultrasonication to collect the cytoplasmic protein fraction. The hydrolytic activity data presented in Table 1 highlight that active PGA predominantly accumulated in the periplasmic fraction, which reached almost 90% in both strains of *E. coli*. The periplasmic fraction of *E. coli* BL21 and AE exhibited significantly higher specific productivity of 16.59±0.01 U/OD and 27.33±0.93 U/OD, respectively compared to their cytoplasmic counterparts of 1.42±0.13 U/OD and 3.71±0.03 U/OD, respectively. This pattern was aligned with the volumetric activity data, confirming that the majority of catalytically active enzyme was localized within the periplasm.

4. Discussion

The *Achromobacter xylosoxidans* Penicillin G Acylase (axPGA) was chosen owing to its ability to synthesize ampicillin and amoxicillin efficiently (Bečka *et al.* 2014). The gene encoding axPGA encodes 862 amino acid residues of axPGA consisting of signal

Table 1. Yield of active PGA from periplasmic and cytoplasmic fraction

	E. coli BL21(DE3)		*E. coli* arctic express	
	Periplasmic	Cytoplasmic	Periplasmic	Cytoplasmic
Volume (mL)	4.53	0.50	2.43	0.50
OD_{600} Cell (OD)	2.38±0.02	2.38±0.02	0.80±0.09	0.80±0.09
Protein conc. (mg/mL)	0.17±0.00	0.28±0.03	0.13±0.04	0.41 0.08
Vol. Activity (U/mL)	8.70±0.00	6.42±0.07	9.19±0.07	5.90±0,00
Spec. Activity (U/mg)	51.50±0.00	22.68±0.24	70.73±0.52	4.22±0.00
Spec. Productivity (U/OD)	16.59±0.01*b	1.42±0.13*a	27.33±0.93*d	3.71±0.03*c

All assay reactions were conducted in triplicates. *p<0.05, were considered significantly different between PGA-hydrolytic activity obtained from periplasmic (b, d) and cytoplasmic fraction (a, c) within strains

peptide (21 aa), α- subunit (230 aa), linker peptide (54 aa), and β-subunit (557 aa). This recombinant AxPGA was successfully cloned into the pET28b plasmid system and transformed into *E. coli* BL21 (DE3) and *E. coli* Arctic Express. Recombinant expressions using IPTG induction often involve a trade-off between expression level and the folding/solubility and thus activity of the target enzyme. In this study, it can be observed that *E. coli* AE BL21 exhibits higher PGA hydrolytic activity across all IPTG concentrations (0-1 mM) in comparison to its counterpart, BL21 strain. This observation reflects optimized folding machinery in *E. coli* AE, which is particularly facilitated by the cold-adapted Cpn10/Cpn60 chaperonins system, which enhances soluble protein expression at low temperature (Ferrer *et al.* 2003). These chaperonins likely minimize misfolding and aggregation of the pre-pro-PGA precursor, thereby enhancing the proportion of the enzyme that is correctly folded and can be processed into the active α/β subunits.

The observed decrease in activity at high IPTG concentrations (1.0 mM) in AE suggests metabolic or folding stress related to strong induction. This aligns with previous research, which has shown that excessive IPTG can overwhelm translational and folding machinery, eventually reducing the yield of functional enzyme (Baneyx & Mujacic 2004; Rosano & Ceccarelli 2014). Conversely, moderate induction (0.5 mM IPTG) optimized expression kinetics, resulting in the highest levels of soluble and active PGA.

In the case of BL21, its relatively flat activity response across various IPTG concentrations suggests a limited folding capacity at a low temperature of 20°C, resulting in the accumulation of misfolded or inactive precursors. This strain lacks the specialized chaperones found in AE, which accounts for its significantly lower enzymatic activity, regardless of IPTG concentration. Previous studies have demonstrated that PGA maturation is highly sensitive to folding efficiency and necessitates periplasmic translocation and autocatalytic processing to produce an active enzyme (Scherrer *et al.* 1994). Inadequate precursor folding in the cytoplasm likely limits the amount of protein that can be exported and processed, leading to poor activity in BL21.

These findings are consistent with earlier research indicating that moderate levels of IPTG support a balance between transcription and translation rates, which helps reduce the formation of inclusion bodies and metabolic stress while optimizing the production of soluble protein (Rosano & Ceccarelli 2014; Dvorak *et al.* 2015). When IPTG concentrations are too high, the resulting substrate toxicity and increased translation burden can lead to protein misfolding and aggregation, ultimately affecting the recovery of active enzymes (Gatti-Lafranconi *et al.* 2011). These findings align with broader literature emphasizing the importance of finely tuned induction intensity to balance expression load and protein folding efficiency in recombinant enzyme production (de Marco *et al.* 2007; Estrabagh *et al.* 2022). Keeping the IPTG in a lower concentration also meets the industrial purpose as IPTG is expensive and toxic compound (Lopes *et al.* 2019).

Furthermore, Calcium was found to be an essential component in the production of functional AxPGA. By adding 10 mM of $CaCl_2$ to the culture medium, the yield of active AxPGA in both *E. coli* strains significantly increased up to 2.4 and 3.4-fold for BL21 and AE, respectively. Similar observations were reported from *Kluyvera citrophila* and *Thermus thermophilus* PGAs (Jiang *et al.* 2007; Torres *et al.* 2012). These observations are consistent with the established role of Ca^{2+} ions in membrane transportation and PGA maturation, assisting proper folding of the precursor enzyme structure (Hewitt *et al.* 2000; Kasche *et al.* 2005; Tishkov *et al.* 2010). Ca^{2+} is known to bind specific sites in the penicillin G acylase precursor, stabilizing intermediate conformations required for correct autocatalytic cleavage into the mature α- and

β-subunits (Brannigan *et al.* 1995; Duggleby *et al.* 1995). Supplementation with exogenous Ca^{2+} likely enhances this processing step, resulting in a larger population of correctly folded, active PGA. Calcium ions are well-known for stabilizing the tertiary structure and folding intermediates of many secreted or periplasmic enzymes, reducing susceptibility to proteolysis, and enhancing pro-peptide maturation in the penicillin-acylase family (Kasche *et al.* 2003; Baneyx and Mujacic 2004; Cai *et al.* 2004; Kasche *et al.* 2005; Ignatova *et al.* 2005).

The activity enhancement observed in *E. coli* AE was higher than that of its counterpart, suggesting a synergistic relationship between Ca^{2+} and the cold-adapted Cpn10/Cpn60 chaperonin system. At low expression temperatures (10°C), these chaperonins reduce misfolding and aggregation of the PGA precursor, improving the likelihood that Ca^{2+} can stabilize the intermediate forms that proceed to successful maturation (Ferrer *et al.* 2003). The combination of enhanced folding capacity and Ca^{2+}-mediated stabilization therefore explains the pronounced improvement in AE compared to BL21.

Subcellular fractionation revealed that Artic Express produced slightly higher periplasmic PGA activity compared to BL21, aligning with the function of cold-adapted chaperonins (Cpn60/10) in increasing protein folding efficiency at low temperature. Despite this, achieving a higher protein concentration (~1.5x) of active PGA underlines the necessity of targeting proteins to the periplasm to obtain active PGA, as periplasmic fractions demonstrated significantly higher volumetric and specific activities than cytoplasmic fractions in both strains. This observation is consistent with earlier research suggesting that the oxidizing environment of the periplasm and certain foldases facilitate the proper formation of disulfide bonds and the maturation of PGA (Scherrer *et al.* 1994; Hewit *et al.* 2000; Ignatova *et al.* 2003).

Overall, these findings suggest that a synergistic combination of optimized IPTG induction, calcium addition, and host strain selection significantly impacts the expression of active PGA. From an industrial biotechnology perspective, choosing a strain for PGA production must consider yield, stability, production cost, and scalability. The results indicate that *E. coli* Arctic Express produced significantly higher levels of active PGA than BL21 under similar induction conditions, primarily due to the co-expression of cold-adapted chaperonins (Cpn60/10) that improve folding efficiency at low temperatures (10°C). This implies that AE has a superior intrinsic ability to produce soluble and active PGA, which is crucial for commercial production where refolding inclusion bodies is costly and inefficient (Rosano & Ceccarelli 2014). However, cultivating Arctic Express at 10°C increases operational costs due to the significant cooling requirements in large-scale fermenters. Low-temperature cultivation leads to slower growth and extended fermentation times, which in turn reduces volumetric productivity. On the other hand, BL21(DE3) can be grown at moderate temperatures (20–30°C), with strong growth and high cell density in standard fed-batch processes, minimizing energy costs and maximizing biomass productivity. Although BL21(DE3) yields lower native soluble PGA, supplementation strategies (e.g., $CaCl_2$ addition, optimized induction, periplasmic targeting) can significantly enhance enzyme activity while keeping production costs manageable.

In conclusion, this research demonstrates that inducing at 0.5 mM IPTG results in the highest levels of soluble and active axPGA in both *E. coli* BL21 and Arctic Express strains. Notably, Arctic Express exhibits significantly greater activity due to improved folding facilitated by cold-adapted chaperonins. Adding 10 mM $CaCl_2$ notably enhanced PGA activity in both strains, highlighting that Ca^{2+} supplementation is an effective strategy to enhance the yield of functional PGA. Subcellular localization studies identified the periplasm as the optimal site for active enzyme accumulation. From an economic perspective, BL21 is attractive for large-scale PGA production because of its strong growth and lower cultivation expenses, while Arctic Express is better suited for laboratory-scale or specialized applications that require maximum solubility. Future research should focus on developing scale-up fermentation strategies, exploring autoinduction media, alternative inducers, and optimizing supplementation to further enhance yields for industrial purposes.

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