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Manipulation Strategy to Increase Expression Level of Soluble Recombinant Protein Penicillin G Acylase in *Escherichia coli* Bacteria: A Review Article

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ABSTRACT

The large-scale production of Penicillin G Acylase (PGA) at high levels of soluble protein content is achievable through recombinant genetic techniques and expression within a specific host organism. *Escherichia coli (E. coli)* remains a popular choice as a bacterial host due to its rapid growth, cost-effectiveness, and high expression rate. Despite its advantages, using *E. coli* as a production host presents disadvantages, notably in the proper folding of recombinant proteins, often resulting in biological inactivity. Various strategies have been developed to overcome these issues. These include selecting specific host strains (such as *E. coli* HB101 & JM109), utilizing fusion proteins to enhance the recovery of soluble proteins (e.g., MBP & NusA), optimizing fermentation conditions (e.g., low-temperature incubation), optimizing the protein isolation process for the recovery of active PGA (e.g., Freeze-thawing technique), and optimizing pH, temperature, and substrate specificity during the synthesis of β-lactam class antibiotics. This study proposes a solution to increase the expression of soluble PGA protein within biological hosts. By replacing the expression host and employing genetic engineering techniques, the study aims to achieve a high expression level of the PGA enzyme in an active form while simplifying the purification process.

Keywords: Expression, *E. coli,* PGA, Soluble Protein,

INTRODUCTION

Penicillin G acylase (PGA), derived from microorganisms like bacteria, fungi, yeast, and actinomycetes, is a unique and important enzyme in industrial settings. Its primary function involves catalyzing the enzymatic hydrolysis of various penicillins by breaking the amide side chain, thereby producing 6-aminopenicillanic acid (6-APA) (Tian *et al*., 2020). Penicillin G is a substrate that is enzymatically converted for the synthesis of new β-lactam antibiotics such as amoxicillin (Pan *et al*., 2020). The enzymatic hydrolysis method is preferred over chemical synthesis due to its specificity, cost-effectiveness, and feasibility under milder conditions (Buchholz, 2016). The conversion process of penicillin G into 6-APA and phenylacetic acid (PAA) primarily occurs through PGA's action, especially under alkaline pH conditions. 6-APA, a crucial intermediate molecule resulting from this process, plays a pivotal role in the synthesis of semi-synthetic antibiotics, exhibiting diverse antibacterial and pharmacological characteristics (McDonald *et al*., 2019).

Penicillin G acylase (PGA) is one of the most extensively employed commercial enzymes globally worldwide (Sambyal & Singh, 2021). Initially, penicillin G, a β-lactam antibiotic, was discovered for its efficacy in combating gram-positive bacteria. However, the widespread and

prolonged use of antibiotics has led to the development of resistance among numerous pathogens. Addressing this challenge involves various strategies, including structural modifications in the synthesis of new semisynthetic antibiotics (Ashraf *et al*., 2015; Buchholz, 2016). Present efforts in creating new semisynthetic antibiotics primarily rely on utilizing the function of the PGA enzyme. Currently, the production of PGA enzymes involves employing recombinant genetic methodologies, wherein the pac gene responsible for encoding the PGA enzyme is cloned into specific vectors and subsequently expressed in bacterial hosts like *E. coli* (Mayer *et al*., 2019). The choice of the expression host strain and plasmid vector assumes significance in PGA enzyme production. Moreover, careful analysis and optimization of the environmental conditions during fermentation are essential to achieve the optimum conditions for PGA enzyme production.

E. coli, commonly utilized as an expression host, presents several drawbacks, notably the tendency for recombinant protein expression to yield incorrect conformations. Proteins folding incorrectly may interact with each other, rendering the protein biologically inactive. This issue arises due to the reducing conditions within the cytosol of *E. coli* and the limited availability of chaperone proteins, which fail to cope with the high levels of recombinant protein expression (Maksum *et al*., 2022). The challenge of ensuring proper protein conformation during expression in the host is critical in achieving substantial bioactive PGA recombinant protein mass. To overcome this hurdle in acquiring high quantities of bioactive PGA recombinant proteins, a strategy has been devised. This strategy involves the utilization of protein fusion and co-expression of chaperones to enhance the production of active soluble proteins (Costa *et al*., 2014). The objective of this approach is to promote the precise folding conformation of soluble recombinant PGA proteins at high levels, thereby ensuring their biological activity.

In this discussion, we aim to describe the advantages and disadvantages associated with various strains of *E. coli* hosts, alongside genetic manipulation methods employed for generating substantial quantities of biologically active soluble PGA enzymes. The great hope is for this review to serve as a guide for advancing research methodologies concerning the expression and isolation of biologically active soluble PGA enzymes on a large scale. This endeavor seeks to offer valuable insights applicable to laboratory-scale investigations.

Early Discovery of PGA

Enzymes, as naturally occurring biocatalysts provided by the environment, offer a light and easy process in various industrial applications, rendering them of utmost importance (Gonzalo & Lavandera, 2021; Marešová *et al*., 2014). The enzyme PGA was first discovered by Sakaguchi & Murao (1950) within the mycelium of *Penicillium chrysogenum* Q176. This enzyme exhibits the potential to catalyze the hydrolysis of penicillin G into phenylacetic acid (PAA) and other organic compounds such as 6-aminopenicillanic acid (6-APA) (Fierro *et al*., 2022). Numerous microbial strains, including *E. coli*, *Pseudomonas melanogenum*, *Bacillus megaterium*, *Kluyvera* sp., and *Proteus rettgeri*, possess the natural capability to produce PGA. Notably, according to to Vélez *et al*., (2014) & Sambyal & Singh, (2021), the PGA enzymes that is in great demand in both research and industry settings originate from *E. coli* and *B. megaterium* due to their exceptional efficiency.

In 1960, the hydrolysis of penicillin G utilizing PGA isolated from *E. coli* was first documented, revealing the enzyme's capability to hydrolyze penicillin G into 6-aminopenicillanic acid (6-APA) (Buchholz, 2016). Numerous reports underscore the potency of PGA enzyme from *E. coli* (PGAEc) in hydrolyzing various types of phenyl acetyl substituted compounds (Avinash *et al*., 2016a). Earlier studies have highlighted the significance of 6-APA as an important intermediate, which, under acidic pH conditions, can be converted by PGA into a new semisynthetic antibiotic derived from penicillin G (Sawant *et al*., 2020a). Consequently, PGA enzymes sourced from *E. coli* are in great demand for large-scale industrial production, catering to commercial purposes.

Increased Expression Levels of Soluble Recombinant Proteins PGA in *E. coli*

The microbial production of PGA, particularly from *E. coli* isolates, has been a longstanding practice (Torres-Bacete *et al*., 2015). PGA is categorized as an intracellular enzyme, necessitating its production for the growth of bacterial strains on standard media, which typically includes a carbon source and inducer control at specific concentrations. Furthermore, research into nutrient sources, chemical additives, and cultivation methodologies are pivotal in

optimizing conditions for protein expression during fermentation. This optimization process also aims to mitigate the production of potentially harmful or toxic substances from bacterial strains (Illanes & Valencia, 2016; Velasco-Bucheli *et al.*, 2020).

The design for industrial-scale production of PGA should improve engineering techniques with a strong approach to manipulation of host strain expression, recombinant vectors that are safe from toxic proteins, fermentation methods, and optimization of downstream processes(Ajamani *et al.*, 2019; Rajendran *et al.*, 2015). The overexpression of soluble PGA protein in *E. coli* can be successfully carried out if the optimization process in several lines as previously described can be improved (Figure 1). The strategies that can be done, are as follows.

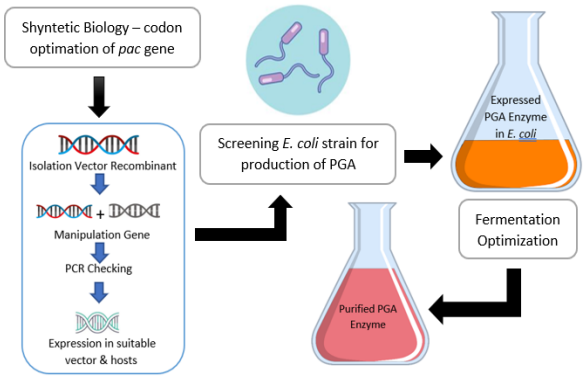

Figure 1. Development of optimization expression of PGA enzyme in a bacterial host.

Co-expression Chaperon Protein: High Activity and Expression of PGA

Chaperones are proteins that play crucial roles in monitoring the conformation and stabilizing non-native protein structures while assisting the folding process of recombinant proteins. It is important to note that chaperones do not become integral parts of the final native structure of the monitored protein. Instead, they primarily serve to enhance the efficiency and optimize the folding of non-native proteins without adding structural information during the folding process. These chaperones are distributed across various cellular compartments, where they function as sites dedicated to facilitating proper protein conformation (Kaur *et al.*, 2018; Thirumalai *et al.*, 2020).

Chaperones play a pivotal role in stabilizing folding intermediates effectively by binding to non-native proteins, thereby preventing their intramolecular or intermolecular interactions that could lead to folding errors. This interaction serves to mitigate protein folding errors and the formation of protein aggregates (Balchin *et al.*, 2020). Chaperons themselves have three main functions including as holdases that act to stabilize the conformation of non-native proteins, foldases that help the folding process to the native state, and unfoldases that act to help the process of opening proteins that fail to fold properly (Chatterjee *et al.*, 2018; Wu *et al.*, 2022).

The Trigger Factor (TF) is currently recognized as the sole chaperone in bacteria, known for its association with ribosomes (Deuerling *et al.*, 2019). This cytosolic protein is expressed abundantly and constitutively, typically found in quantities two to three times greater than the relative molar amount of proteins undergoing the folding process on the ribosome (de Geyter *et al.*, 2020; Kumar *et al.*, 2020). TF binds to the ribosome at a 1:1 stoichiometry, utilizing the ribosomal protein L23 as its primary docking site. This positioning places TF on the exit side of the ribosome, where the elongating polypeptide chain emerges from the ribosome into the crowded cytosolic environment cytosol (Hoffmann *et al.*, 2010). Optimizing the expression of recombinant proteins through co-expression with chaperones such as TF is expected to enhance the production of soluble target proteins like PGA. However, the effectiveness of each approach may vary from one protein recombinant to another soluble protein recombinant and it requires an empirical optimization.

Fusion Maltose-binding Protein (MBP) on PGA Expression

The Maltose-Binding Protein (MBP) is a periplasmic protein in E. coli with a molecular weight of 40.6 kDa and notably lacks cysteine residues (Greenfield *et al.*, 2020; Subroto *et al.*, 2022). MBP is often utilized as a fusion tag linked to the N-terminal region of recombinant proteins, primarily to enhance protein solubility (Fang *et al.*, 2018; Greenfield *et al.*, 2020). Purification of MBP-fused proteins can be achieved through affinity chromatography owing to MBP's inherent affinity for maltose (around 10 mM) (Lebendiker & Danieli, 2017; Nguyen *et al.*, 2020). MBP functions as a holdase, engaging in temporary interactions with exposed protein sites, effectively preventing the premature aggregation of the protein into insoluble aggregates. This interaction persists until

spontaneous folding, mediated by endogenous chaperones, facilitates proper folding of the protein, increasing solubility (O'Neil *et al*., 2018; Wruck *et al*., 2018).

Maltose-binding protein with its main characteristics fused with the *pac* gene produces PGA recombinant protein in large quantities and with the right conformation to increase the enzyme activity obtained. This process involves several stages crucial for obtaining mature PGA enzymes, including post-translational modification, folding, and periplasmic processing. MBP fusion aids in the folding process, ensuring the acquisition of the correct conformation for PGA. The fusion with MBP serves a dual purpose: firstly, it reduces the formation of inclusion bodies, thereby increasing the yield of recombinant proteins, as noted by Fang *et al*., (2018). Secondly, MBP's role is transient, temporarily preventing improper folding of the recombinant protein until spontaneous folding occurs with the assistance of endogenous chaperones (Bhatwa *et al*., 2021; Paraskevopoulou & Falcone, 2018). Studies by Maksum *et al*., (2022) and Fang *et al*., (2018) have reported that overexpressing heterologous recombinant proteins in the *E. coli* BL21(DE3) host, in conjunction with MBP sequences in the recombinant plasmid construct, significantly enhances solubility and stability of protein expression compared to traditional overexpression methods without MBP insertion.

Studies conducted by Ko *et al*., (2021) & Nemergut *et al*., (2021) align with previous findings indicating that the addition of maltose-binding protein (MBP) to the recombinant plasmid construct significantly enhances the solubility of heterologous recombinant protein expression. This enhancement allows for the isolation of the target protein in a soluble form and facilitates the extraction of larger quantities of the protein of interest. Following the expression of the MBP-fused recombinant protein, the protein isolation stage typically involves purification using an affinity column column (Greenfield *et al*., 2020). This purification process aids in obtaining relatively pure protein preparations. Referring to the research that has been reported, it is possible to overexpress the *pac* gene, encoding the PGA enzyme in a soluble form with high levels. Moreover, there is one thing that needs to be considered, MBP will be fused in the target protein so that the molecular weight of the recombinant protein will increase (Fang *et al*., 2018) so the target protein requires certain engineering or further purification processes to obtain pure target protein results.

Fusion of N-utilizing substance A (NusA) for Alternative Production of Soluble

NusA, a multi-domain protein with a molecular weight of 55 kDa, is involved in transcriptional processes, specifically in transcription termination and anti-termination. It acts upon the RNA polymerase during both elongation and transcription termination phases phases (Costa *et al*., 2014). NusA can increase soluble protein expression by slowing down the translation process through the transcriptional pause's mechanism by RNA polymerase so that it can provide more time for the protein folding step (Kang *et al*., 2019; Kaur *et al*., 2018). Unlike some other fusion tags, NusA does not possess intrinsic affinity for purification. Therefore, it often requires the addition of a His-tag for efficient purification processes. Additionally, NusA operates in a passive capacity in protein folding, meaning it enhances the solubility of recombinant proteins without directly influencing the structure of proteins prone to aggregation (Lebendiker & Danieli, 2017; Zhu *et al*., 2022).

Several previous studies have demonstrated that fusing recombinant proteins with NusA enhances protein solubility. For instance, Andler *et al*., (2019) employed NusA fusion with the recombinant protein $Lcp1_{vh2}$, resulting in significantly high expression levels of the targeted recombinant protein in soluble properties. Similarly, Hemmati & Ranjbari, (2019) reported a substantial increase in soluble expression of IGF-1 by using NusA fusion in E. coli. NusA recombinant protein fusion proves beneficial for expressing heterologous proteins, even those intended for the periplasmic compartment. Ahmad *et al*. (2018) and Hemmati & Ranjbari (2019) observed increased solubility of recombinant proteins Scorpine and IGF-1, designed for expression in the periplasm, upon fusion with NusA. These findings serve as a valuable reference for considering PGA recombinant protein fusion with NusA, expecting an increase in protein solubility. The assumption is that the soluble protein has a targeted biological activity in the form of a PGA enzyme. However, it is important to note that NusA requires a His tag linker to provide affinity during the purification process. Thus, careful consideration of the recombinant interplay between NusA, the target protein, and the His tag linker is crucial. Failure in this process might lead to difficulties in purifying

the highly expressed target protein, despite achieving high expression levels.

Recombinant Host Strain Development

E. coli boasts rapid growth, high cell density, and simple metabolism and physiology, making it an appealing host for recombinant protein expression. However, the endogenous promoter of the *pac* gene responsible for encoding the PGA enzyme in *E. coli* exhibits weak properties, thus proving inadequate for large-scale production. Consequently, it becomes imperative to manipulate this system for the enhanced production of PGA recombinant protein. To achieve this, manipulation involves facilitating native overexpression of the *pac* gene via high copy episomal plasmids. This approach aims to increase the number of gene copies, thereby enhancing all expression stages regulated by robust promoter systems. Such systems govern key steps like transcription, translation, translocation, periplasmic processing, and folding. An emphasis is placed on modifying the ribosome binding site to augment mRNA stability for the *pac* gene. This modification ultimately facilitates the production of mature PGA recombinant proteins exhibiting heightened enzymatic activity (Illanes & Valencia, 2016; Rajendran *et al*., 2015).

The heterologous expression of *pac* genes from bacterial sources other than *E. coli*, such as *B. megaterium*, *Acinetobacter faecalis*, *Kluyvera crysophila*, and *Thermus thermophilus*, has been explored. PGA enzymes originating from these diverse bacterial strains exhibit distinct characteristics compared to PGA from *E. coli*, including a broader operational range, enhanced molecular stability, and increased tolerance to environmental conditions (Srirangan *et al*., 2013). Efforts to increase PGA production in various recombinant *E. coli* hosts and understand the factors contributing to the reduction in post-translational PGA yield are crucial. An initial observation indicates that the enzyme production process is constrained by the intracellular proteolytic degradation of newly synthesized PGA precursors. This phenomenon is associated with decreased cell growth rates and impedes the protective fusion and efficient translocation of PGA through the plasma membrane (Rajendran *et al*., 2015).

To increase PGA expression at the molecular level within recombinant *E. coli* strains, it is crucial to address intracellular proteolysis and optimize translocation efficiency, along with considering the host system and suitable culture media. Cultivating *E. coli* BL21(DE3), known for lacking the ATP-dependent proteinase Lon and the outer membrane proteinase OmpT, on protein-free media can enhance PGA expression results (Falak *et al*., 2022; Hausjell *et al*., 2018). Moreover, studies have indicated that *E. coli* strains like HB101 and JM109 exhibit promising performance in PGA production, yielding PGA with high biological activity (Narayanan *et al*., 2006).

E. coli HB101 has been chosen as a preferred host for recombinant protein expression due to specific advantageous properties. Notably, it harbors a recA13 gene mutation that aids in stabilizing gene insertions, lacks the T7 RNA Polymerase gene, resulting in lower copy number expression, and is widely used for gene expression predominantly centered in the periplasm (Srirangan *et al*., 2013). Another alternative strain, *E. coli* JM109, is also used for recombinant protein expression. According to (Kim *et al*., 2004), *E. coli* JM109 demonstrates proficient expression of the PGA enzyme, particularly when cultivated at 26°C. By utilizing a lac promoter and various inducers, this strain exhibits differences in protein yield and varied enzyme activities. Furthermore, E. coli strains, like JM109, have shown capabilities to express the poli-γ-glutamic acid protein in substantial quantities while retaining functional enzymatic properties for synthetic purposes (Cao *et al*., 2013).

Optimization of the Fermentation Process

The fermentation process is very important in achieving optimal PGA production (Pan *et al*., 2018). Analysis of environmental conditions during fermentation is crucial. Elevated fermentation temperatures may lead to the formation of inclusion bodies due to excessive protein expression, resulting in the aggregation of recombinant proteins and hindering efficient periplasmic translocation processes (Huleani *et al*., 2022; Zhou *et al*., 2018). In addition, optimal pH conditions during the fermentation process must also be maintained because if there are changes, the metabolic and physiological processes of the bacteria will be disrupted which disrupts the stability of bacterial growth (Pontrelli *et al*., 2018). This will have an impact on bacterial conditions that are not optimal in the expression of recombinant proteins. Optimal aeration will also result in high levels of recombinant protein expression. The carbon source for growth energy and recombinant protein expression from bacteria

must also be considered because if the carbon source used is not suitable, it will reduce the average growth of bacteria (Manan & Webb, 2017).

The fermentation incubation process conducted at lower temperatures, specifically below 30°C, has demonstrated the ability to mitigate the formation of inclusion bodies (Gaciarz *et al*., 2017; Slouka *et al*., 2019). At lower temperatures, the periplasmic environment benefits from the assistance of endogenous chaperones, notably the DegP chaperones, which play a pivotal role in aiding proper protein folding with the proper conformation chaperons(Harkness *et al*., 2021; Šulskis *et al*., 2021). Moreover, lower temperatures during fermentation contribute to higher levels of soluble proteins (Petrus *et al*., 2019), directly impacting the enzymatic activity of the PGA recombinant protein. This increase in soluble protein levels correlates with enhanced enzyme activity. The efficiency of the maturation process and periplasmic processing is significantly improved at lower temperatures. This efficiency boost reduces inclusion body formation and enables the production of recombinant proteins with the desired conformation at elevated levels (A. Singh *et al*., 2020; Tripathi & Shrivastava, 2019).

Recovery of Biologically Active Recombinant Protein PGA

Inclusion bodies refer to insoluble protein aggregates commonly observed in recombinant DNA technology, particularly in the expression host *E. coli*. These aggregates typically form due to protein overexpression, leading to misfolded or partially folded proteins that experience errors during the folding process. The lack or absence of chaperones exacerbates this issue, preventing proteins from achieving their native conformation within the reduced cytoplasmic environment. As a result, these proteins are prone to degradation by proteases (Chatterjee *et al*., 2018; Xu *et al*., 2005). PGA, being a periplasmic protein, accumulates in the periplasm during its production as a recombinant protein. However, the accumulation process during overproduction in *E. coli* often results in the formation of inclusion bodies (Ayakar & Yadav, 2019).

Inclusion bodies are presumed to contain a relatively pure and high quantity of recombinant protein. Due to their homogeneous nature, the purification process becomes simpler (Singh & Panda, 2005). Biologically active inclusion bodies are sometimes referred to as non-classical inclusion bodies. In general, inclusion bodies are characterized by a loose arrangement of protein molecules, making them easy to dissolve using denaturation at various concentrations. The composition of active molecules within inclusion bodies is contingent upon the environmental conditions prevalent during protein expression. The quality of these inclusion bodies can be influenced by changes in the expression temperature. Lower expression temperatures, for instance, tend to favor the formation of non-classical inclusion bodies (Singh *et al*., 2015).

The dissolution of inclusion bodies via the freeze-thawing method often leads to protein denaturation and loss of activity. When subjected to freezing, salt crystals form within the buffer, resulting in protein concentration. Increasing the protein concentration during clotting may induce protein aggregation; however, higher protein quantities can enhance protein stability due to the existence of a self-stabilizing mechanism inherent to the protein itself. Interestingly, the freeze-thawing process typically induces minimal or negligible alterations in the protein's secondary structure (Pikal-Cleland *et al*., 2000).

The report by Qi *et al*., (2015) describes a dissolution method for inclusion bodies involving the freeze-thawing process. This method involves freezing the inclusion bodies and subsequently thawing them, combined with the addition of urea and utilizing a buffer with a certain pH range. According to their findings, the inclusion bodies were dissolved using potassium phosphate buffer within a pH range of 5 to 10. The dissolution process included varying urea concentrations from 1 to 8 M. The inclusion bodies were frozen overnight at -20 °C and subsequently thawed at room temperature. The study suggests that this Freeze-Thawing method can effectively dissolve recombinant proteins commonly expressed in the form of inclusion bodies within *E. coli* hosts.

Substrate Specificity, pH, and Temperature of PGA Enzyme Activity

The specificity of the PGA enzyme towards its substrate, particularly penicillin G, exhibits a notably high value, up to 100-fold greater compared to other substrates such as cephalothin, cephalexin, amoxicillin, and (Rajendhran & Gunasekaran, 2007). However, it is important to note that PGA does not demonstrate the specific activity with substrates like penicillin V, methicillin, and cephalosporin C (Balci *et al*., 2014; Rajendhran & Gunasekaran, 2007). The active site of the PGA enzyme primarily comprises

hydrophobic residual bonds. This characteristic enables PGA to effectively hydrolyze substrates possessing large phenylacetyl side chains, such as penicillin G. The preference and specificity of PGA towards its substrate can be determined by evaluating the Kcat/Km value.

The optimal functioning of the PGA enzyme is notably dependent on favorable pH conditions. Being an amphoteric molecule, PGA's performance is significantly influenced by the pH level in its environment. The pH value can alter the conformation of the hydrophobic bonds in PGA through electrostatic interactions, hydrogen bonding, and other molecular patterns. When PGA is at its optimum pH, the enzyme's conformation is not affected by the amount of H+ ions in the system. Therefore, the enzyme conformation is suitable for substrate binding to produce a higher potency. The α-amino groups in PGA possess a pKa range of 6.8 to 7.9. Thus, for the hydrolysis of penicillin G, a pH range typically between 7.5 and 8.0 or even higher is utilized. However, during the synthesis of antibiotics, an ideal pH range of 6.0 to 6.5 is preferred (Giordano *et al*., 2006).

The enzymatic activity of PGA is notably influenced by the temperature range within its environment. Previous studies have revealed varied optimal temperature conditions for different PGA enzymes. For instance, *Bacillus badius* PGA demonstrated maximum activity at 50°C and pH 7.0, exhibiting approximately 80% overall activity. This thermostability was sustained across buffer conditions within the pH range of 6.0 to 8.5. However, *E. coli* PGA exhibited reduced total activity after a 30-minute incubation at the same temperature and pH. Similarly, PGA from *Bacillus megaterium* and *Achromobacter viscosus* was inactivated at 50°C (Cheng *et al*., 2006; A. Li *et al*., 2021; S. Li & Cao, 2014; Terreni *et al*., 2007). According to reports by Rajendhran & Gunasekaran (2007), the PGA enzymes from *Alcaligenes faecalis* and *Achromobacter xylosoxidans* displayed superior thermostability compared to *E. coli* PGA. The enhanced stability of Alcaligenes faecalis PGA is attributed to the presence of disulfide bridges in its β subunit. Conversely, *Achromobacter xylosoxidans* PGA's robustness is credited to its numerous salt bridges, contributing significantly to its thermostability (Cheng *et al*., 2006).

Kafshnochi (2010) suggests that while disulfide bridges and salt bridges contribute significantly to PGA's thermostability, other factors also play pivotal roles. These additional parameters include a high arginine/lysine ratio, a lower presence of thermolabile amino acids, the presence of proline - which rigidifies the conformation - in the β curvature, and a higher count of ion pairs. These factors, along with various independent interactions, collectively contribute to PGA's thermostability. Moreover, Kafshnochi highlights the success of a modified consensus approach in identifying stable amino acid residue positions. This approach involves site-specific comparisons between meso-stable and heat-stable PGAs.

Effect of Mutagenesis on the PGA Enzyme Activity

Modifications to enzymes become crucial when specific properties are not naturally inherent. In nature, such modifications predominantly occur among microorganisms that adapt to environmental changes changes (Choi & Geletu, 2018; Sklyarenko *et al*., 2017). Nevertheless, advancements in molecular biology and genome sequencing have introduced new ways for engineering or modifying enzymatic properties, allowing for the deliberate customization of enzymes to attain desired characteristics (Avinash *et al*., 2016b; Sawant *et al*., 2020a).

Modification of PGA enzyme characteristics can be achieved through a method known as site-directed mutagenesis (Pan *et al*., 2018). To identify the amino acid residue for mutation, high-resolution crystal structure instruments are crucial in determining its position within the protein structure (Avinash *et al*., 2016b). These instruments play a pivotal role in determining the structure-function relationship within a protein, elucidating the enzyme's catalytic and stability mechanisms (Kubiak *et al*., 2021; Mayer *et al*., 2019). PGA mutagenesis aims to optimize its activity by (1) increasing temperature and pH stability (Pan *et al*., 2020) and (2) increasing substrate affinity for the enzyme's active site enzyme (Yang *et al*., 2014). In the process of antibiotic synthesis, mutagenesis targets elevating the affinity of activated acyl donors and the S/H ratio (indicating the product/mole of donor side chains formed from hydrolysis), while reducing the hydrolytic activity of antibiotic products already formed. PGA with minimal secondary hydrolytic activity provides greater product accumulation (Pan *et al*., 2018).

The intrinsic properties of the PGA enzyme significantly impact the efficiency of antibiotic synthesis reactions (Pan *et al*., 2018; Ye *et al*.,

2019). Through site-directed mutagenesis, alterations in Pheβ24A led to increased PGA activity, a higher synthesis-to-hydrolysis ratio, and enhanced resistance to Phenyl acetic acid (PAA) inhibition (Deng *et al*., 2016). The βF24A+αF146Y mutant of *E. coli* PGA exhibited higher conversion rates in the synthesis of cephalexin, cephalexin, and sefprozil (Cecchini *et al*., 2007, 2012). This indicates that mutagenesis can effectively enhance PGA activity by increasing the ratio between synthesis and hydrolysis reactions. Several positions, specifically αR145, αF146, and βF24 in *E. coli* PGA, as well as αR144, αF145, and βF24 in *B. megaterium* PGA, have been identified as crucial sites for improving synthetic PGA enzyme activity These positions serve as promising targets for enhancing the performance of synthetic PGA enzymes. (Deng *et al*., 2015; Jager *et al*., 2007; Pan *et al*., 2018).

Role of PGA Enzyme in Antibiotic Production

Over the past three decades, the chemical technology involved in producing antibiotics of the β-lactam class has progressively been replaced by biocatalyst technology which is cheaper and tends to be safer because of minimal side effects (A. Li *et al*., 2021). This shift is attributed to several factors contributing to the significant growth and development of biocatalysts are related to (1) the high quality of the final product; (2) the undeniable ecological benefits of biocatalysts such as mild reaction conditions (pH, temperature), excluding toxic reagents and providing increased product/waste ratios (decreased organic solvents and reagents); (3) increase in economic efficiency and competitiveness of enzymatic technology due to the improvement of each aspect (strain, biocatalyst, enzymatic transformation), as well as due to optimal process integration in the application of the one pot process (Alemzadeh *et al*., 2010; Sklyarenko *et al*., 2015).The current focus in the production of β-lactam antibiotics includes the synthesis of ampicillin, amoxicillin, cephalexin, cefachlor, and cefazolin. Several approaches have been explored to enhance the efficiency of β-lactam acyl transfer synthesis are (1) optimization of pH, ionic strength, and temperature (Deng *et al*., 2016; Pan *et al*., 2020); (2) the use of excess molar acylating agents over antibiotic cores (Deng *et al*., 2016); (3) use of solvents (Cerqueira Pereira *et al*., 2012; Illanes *et al*., 2005); (4) synthesis in a biphasic/ two-phase system (Zhu & Cao, 2014); (5) increasing the concentration of reagents (Illanes & Valencia, 2016); (6) changes in the catalytic properties of enzymes by site-directed mutagenesis (Alkema *et al*., 2002; Jager *et al*., 2007; Pan *et al*., 2020); (7) changes in biocatalyst properties by chemical modification and immobilization of enzymes (Deng *et al*., 2016; Pan *et al*., 2018).

Several microorganisms are commonly employed as producers of penicillin G acylase (PGA) for the synthesis of antibiotics. Among them, *E. coli* stands out as the most dominant, along with *B. megaterium*, *Achromobacter xylosoxidans* (high thermal stability), and *Providencia rettgeri*, each contributing distinct characteristics to PGA (Pan *et al*., 2020); (Ye *et al*., 2019). The choice of microorganisms significantly impacts the activity and efficacy of the resulting PGA. PGA-driven synthesis has been particularly successful in producing amoxicillin and ampicillin antibiotics. In these processes, 6-aminopenicillanic acid (6-APA) serves as the β-lactam core, while phenylglycine methyl ester (PGME), hydroxyphenylglycine methyl ester (HPGME), or D-PGA function as the acyl donors (Pan *et al*., 2020).

The synthesis of β-lactam antibiotics using PGA can be approached through two main systems: thermodynamic systems (Estruch *et al*., 2008) and kinetic control systems (KCS) (Giordano *et al*., 2006). These strategies have been extensively discussed and reviewed in previous studies (Giordano *et al*., 2006; Valencia *et al*., 2010). However, it is worth noting that the kinetic control system generally offers higher synthesis yields in comparison to thermodynamically controlled synthesis (Cerqueira Pereira *et al*., 2012; Cuthbertson *et al*., 2019; Terreni *et al*., 2007). Therefore, approaches based on kinetic control are usually preferred for the synthesis of β-lactam antibiotics (Giordano *et al*., 2006; Pan *et al*., 2022; Ye *et al*., 2019).

Synthesis under kinetic control (KCS) involves a complex strategy. Conditions in KCS for β-lactam antibiotic synthesis have been optimized across various parameters such as pH (Sawant *et al*., 2020b), ionic strength (D. Yang *et al*., 2016), temperature (Sklyarenko *et al*., 2015), molar ratio of acyl donors and nucleophiles (Dai *et al*., 2001; Deng *et al*., 2015), and the composition of the medium with organic cosolvent (Deng *et al*., 2016), all aimed at enhancing the synthesis/hydrolysis ratio (S/H). This optimization has shown success in the synthesis of cephalologlysin in methanol, as well as ampicillin and cephalexin in glycol (Zhu & Cao, 2014).

Structural changes in enzymes exert a stronger influence on amidase than synthetase activity due to the complex mechanism involved in amide hydrolysis (Buchholz, 2016; Sambyal & Singh, 2021). Organic solvents contribute to increasing substrate solubility, influencing reaction pK values for the formation of non-reactive ions, and aiding synthesis by reducing water activity (Deng *et al*., 2015).

Synthesizing β-lactam antibiotics like ampicillin, amoxicillin, and cephalexin at high substrate concentrations has shown an increase in product yield (Pan *et al*., 2020; Valencia *et al*., 2010). However, excessively high substrate concentrations pose a challenge due to limited solubility at suboptimal pH and temperature conditions (Pan *et al*., 2020). Under the kinetic control system, the hydrolysis rate appears more temperature-sensitive compared to the synthesis rate (Dai *et al*., 2001; Giordano *et al*., 2006). Higher temperatures can increase the hydrolysis activity of PGA, while synthesis activity can increase at lower temperatures (Chandel *et al*., 2008; da Rocha *et al*., 2022). The temperature usually used for synthesis is 4 °C, which is for the synthesis of cephaloglysin (Terreni *et al*., 2007), cephalexin (Estruch *et al*., 2008), cefadrochlor, cephalothin (Estruch *et al*., 2008); 14-20 °C for cephalexin synthesis (Illanes *et al*., 2005; Illanes & Valencia, 2016); and 25-28 °C for the synthesis of cefadroxil and sefprozil ((Liu *et al*., 2020; Pan *et al*., 2018).

Increasing substrate concentration often leads to an extended time to achieve maximum yield, accompanied by a subsequent decrease in this maximum yield. The effect of substrate concentration on product yield is closely tied to the solubility of the β-lactam core, such as 6-APA. Increasing the concentration of PGME is a common approach to enhance the solubility of nucleophiles (Valencia *et al*., 2010). In addition, increasing substrate concentration ensures a sustained decrease in substrate availability due to its hydrolysis by PGA. Penicillin acylase breaks down PGME, generating a by-product, phenylglycine (Illanes & Valencia, 2016; K. Li *et al*., 2020; Schimek *et al*., 2020; Valencia *et al*., 2010). Lower substrate-to-product ratios are generally preferred due to their enhanced economic value and their role in streamlining downstream processes.

CONCLUSION

The enhancement of soluble recombinant protein PGA expression in the bacterial host E. coli can be achieved through strategic manipulation using genetic engineering techniques, specifically co-expressing chaperones such as MBP and NusA fusion protein. Optimal conditions for the highest PGA production were observed in *E. coli* HB101 and JM109 strains when incubated at lower temperatures (below 30 °C). These particular hosts were selected due to their widespread utilization in expressing recombinant periplasmic proteins. To optimize protein isolation results, the Freeze-Thawing method can be employed to recover biologically active PGA. The ideal characteristics of PGA enzymes suitable for synthesizing β-lactam class antibiotics include pH stability ranging from 7.0 to 8.0 for hydrolysis reactions and 6.0 to 7.0 for synthesis processes. In addition, the optimum temperatures for hydrolysis reaction lie between 30 and 37°C, while for the synthesis reaction, it ranges from 4 to 30°C. PGA exhibits high specificity towards Penicillin G substrate, generating 6-APA, along with possessing high S/H kinetic parameters. The pivotal role of PGA in antibiotic synthesis underscores the importance of optimizing enzymatic antibiotic synthesis parameters. This involves determining factors such as pH, ionic strength, and temperature, as well as employing a molar excess of acylated substances over the antibiotic core, using solvents, conducting synthesis in a biphasic system, increasing reagent concentrations, changing enzyme catalytic properties through site-directed mutagenesis, and modifying biocatalyst properties through chemical modifications and enzyme immobilization.

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CONFLICT OF INTEREST

All authors declare that they do not have any conflicts of interest.

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