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INCREASING RECOMBINANT PENICILLIN G ACYLASE PRODUCTION: GENETIC, PROTEIN ENGINEERING, AND PRODUCTIVITY IMPROVEMENT

Peningkatan Produksi Penisilin G Asilase Rekombinan: Rekayasa Genetik, Protein, dan Optimasi Produksi

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ABSTRACT

β-lactam derived antibiotics are the most used globally for treatment against different infections caused by pathogenic bacteria and comprises 65% of the world antibiotics. Recently, penicillin G acylase (PGA) is used as biocatalyst for those β-lactam antibiotics production by which 6-aminopenicillanic acid (6-APA) or 7-aminodeacetoxycephalosporanic acid (7-ADCA) as the building blocks is produced. Commercialized PGA from native microbial resources are still limited to E. coli. *Therefore, genetic engineering approach such as cloning and expression in other microbial hosts were assessed to enhance bacterial strains that produce PGA. However, such improvement could increase immature precursors accumulation and lowering the enzyme yield, activity, or stability. This review focus on the review of PGA recombinant produced by several microbial host, their expression levels, and improvement achieved by some modification such as replacement of signal peptide and promoter continued to protein engineering to utilize the enzymes in synthetizing amoxicillin rather than to hydrolyses Penicillin G.*

Keywords: *β-lactam antibiotics, catalytic activity, hydrolysis activity, Penicillin G Acylase, recombinant protein.*

ABSTRAK

Antibiotik turunan β-laktam adalah kelompok antibiotik yang paling banyak digunakan dalam pengobatan infeksi bakteri patogenik dan mencakup 65% dari penggunaan antibiotik di seluruh dunia. Enzim Penisilin G Asilase (PGA) digunakan sebagai biokatalis dalam produksi senyawa bahan baku antibiotik β-laktam berupa asam-6-aminopenisilinat (6-APA) dan asam-7-aminodeasetoksisefalosporanat (7-ADCA). Enzim PGA komersial masih sangat terbatas dan lebih banyak bersumber dari *E. coli*. Peningkatan produksi PGA dapat dilakukan melaui rekayasa genetika seperti kloning dan ekspresi PGA dengan menggunakan mikroorganisme lain. Namun demikian, rekayasa tersebut dapat menyebabkan akumulasi prekursor dalam sel yang mengakibatkan rendahnya perolehan enzim yang didapat, maupun rendahnya aktivitas dan stabilitas enzim. Tinjauan ini berfokus pada potensi peningkatan produksi PGA rekombinan dari berbagai sel mikroorganisme yang dapat dicapai melalui modifikasi sekuens sinyal peptida dan promoter hingga rekayasa protein dengan tujuan mendapatkan enzim PGA untuk sintesis antibiotik amoksisilin daripada hidrolisis PGA.

Kata Kunci: Aktivitas hidrolisis, antibiotik β-laktam, katalisis, Penisilin G Asilase, protein rekombinan.

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INTRODUCTION

Antibiotics group from β-lactam derivatives are widely used to treat several bacterial infections across the world. It represents the most important group of antibiotics which comprises 65% of the world antibiotics with amounts of annual sales of around US$ 15 billion (Rodriguez-Herrera et al. 2019). As the high occurrence of antibiotic resistance, the need of semi-synthetic antibiotics increased (Sawant et al. 2020). Nowadays, penicillin G acylase (PGA) is used as a biocatalyst in the production manner of β-lactam antibiotics, which results in the formation of intermediate molecules such as 7-amino deacetoxy cephalosporanic acid (7-ADCA) or 6-aminopenicillanic acid (6-APA). Penicillin G acylase (PGA, EC 3.5.1.11; penicillin acylase; penicillin amidohydrolase) is a hydrolytic enzyme that works on the amide bond of penicillin G, cephalosporin G, and other β-lactam antibiotic compounds, generating 6-APA or 7-ADCA and its side chain (Bečka et al. 2014). Those molecules served as the building blocks to produce several semi-synthetic antibiotics (Figure 1). The PGA consumption for industries has recently reached 10 to 30 million tons annually with up to 65% of them toward 6-APA production (Sawant et al. 2020).

Enzymatic reaction occurs more specific and is preferable in milder conditions, generating less waste and toxic chemicals compared to chemical reactions (Zhang et al. 2016, Marešová et al. 2017). Utilizing penicillin G or benzylpenicillin as the main substrate, PGA belongs to the type II penicillin acylases which acts specifically to an aromatic phenylacetyl moiety (Type IIa) or to an aliphatic moiety (Type IIb). The other type is owned by Type I, that uses penicillin V or phenoxymethyl penicillin as the main substrate, and thus referred to as penicillin V acylase (PVA) (Avinash et al. 2014, Li et al. 2020). Due to the fact that roughly 65% of 6-APA generated enzymatically is derived from penicillin G, type IIa PGAs are the most extensively used in industry.

High demand for β-lactam semisynthetic antibiotics will push the research on PGAs, including isolation, identification, and characterization of novel microbial sources producing PGA, genetic and protein engineering in heterologous hosts, large-scale production, or biochemical and structural characterization of the recombinant enzymes (Buchholz 2016, Sambyal and Singh 2021). Further recombinant DNA technology study showed that *Escherichia coli* became the first choice of bacterial host system for heterologous protein expression of PGA and large-scale fermentation (Vélez et al. 2014). Although microbial production of PGA is relatively an established industrial biotechnology, however, there are still technological issues and constraints to be solved. For instance, PGA producing bacterial strains can be improved through genetic manipulations to increase the enzyme productivity. On the contrary,

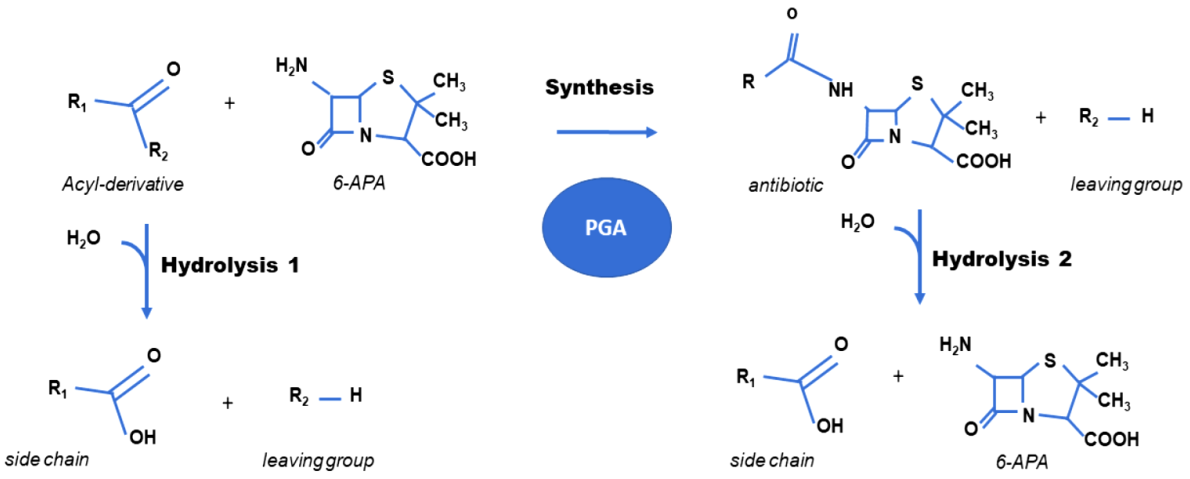

Figure 1. Schematic of the enzymatic reaction in the hydrolysis and synthesis of (H/S) β-lactam antibiotic by penicillin G acylase

these manipulations could potentially build up immature precursors or protein misfolding and inclusion bodies formation that occurred in the expression of PGA, resulting in low enzyme yield, activity, and/or stability (Lee 2017). In recent years, alternative hosts such as *Bacillus* sp. or *Pichia pastoris* emerged as potential hosts, however, the review about this microbial host is still limited. Therefore, in this mini review, we focus on recombinant PGA including the cloning and the expression of the PGA gene not only in dominant host such as E. coli, but also in other potentially emerged hostsm such as *Bacillus* sp. and *Pichia pastoris*, the improvement achieved by some modification of signal peptide, promoter, in the expression process, as well as the protein engineering to achieve better character of PGAs produced.

MICROBIAL SOURCES OF PGA

Penicillin G acylase cleaves the substrates (penicillin G or cephalosporin G) amide bond, and results in an intermediate as products to further generate semi-synthetic β-lactam antibiotics. They are found either as extracellular or intracellular protein. However, mature enzymes all need heterodimer conformation that contains a small α subunit (23-27 kDa) and a large β subunit (59-62 kDa). The mature enzyme originally came from a single polypeptide (94 kDa) precursor harboring a signal peptide and a spacer peptide that autocatalytically processed (Lakowitz et al. 2018, Sambyal and Singh 2021).

Diverse microorganisms are identified as the producer of PGA either extracellular, intracellular, or periplasmic (Table 1). In Gram-negative bacteria such as *E. coli, Kluyvera cryocrescens, Alcaligenes faecalis*, and *Achromobacter xylosoxidans* most of PGA are expressed intracellular or periplasmic. Recovery of PGA from different fraction needs different method according to corresponding protein, as cell permeability is required for extraction PGA from periplasmic (Illanes and Valencia 2017). On the other hand, Gram-positive bacteria tend to produce PGA either intracellular or extracellular such as *Bacillus megaterium* (Pan et al. 2022). In fungi such as *Mucor*

Table 1. PGA microbial sources and enzyme characteristics

Sources	Characteristics	References
E. coli	Periplasmic heterodimer, 23 & 62 kDa at temperature 40ºC and pH 8.0	Scaramozzino et al. 2005
Bacillus badius	Heterodimer 25 & 62 kDa optimum at 55ºC	Rajendran et al. 2014, Torres et al. 2012
Bacillus thermotolerans	Heterodimer 24754.51 & 61602.24 Da	Mayer et al. 2019
Bacillus sp. FJAT-27231	Heterodimer 24822.02 Da & 62 kDa	Mayer et al. 2019
Bacillus megaterium	Extracellular PGA with optimum temperature at 60ºC and pH 7.0	Priya and Suganya 2013
Alcaligenes faecalis	Periplasmic penicillin G acylase (signal peptide α subunit 23 kDa)-spacer (37 aa)-(β subunit 62.7 kDa) at temperature 55ºC and pH 8.0.	Varshney et al. 2012
Kluyvera cryocrescens/ Kluyvera citrophila	Intracellular penicillin G acylase with 87% sequence similarity to *E. coli* PGA and tolerate higher temperature	Varshney et al. 2013
Providencia rettgeri	α subunit 23.7 kDa & β subunit 62 kDa	
Achromobacter xylosoxidans	Intracellular thermostable at 55-60ºC. Activity decreased when the pH was lowered to 5.3. (α subunit 27 kDa - β subunit 62.4kDa)	Li et al. 2021, Torres et al. 2012
Achromobacter sp. CCM	Maximum acitivity at pH 8.0 and 60ºC while at pH of 6.0 the maximum temperature was shifted to 65ºC	Bečka et al. 2014
Thermus thermophilus	The optimum activity was recorded at 75ºC and pH 4, with the half-life in 9.2 h at this temperature.	Torres et al. 2012
Mucor griseocyanus	Extracellular PGA at 570 $IU.L^{-1}$ after 72 horus of fermentation. Showed high identitity percentage with PGA from *Alcaligenes faecalis*	Cano-cabrera et al. 2021

griseocyanus, PGA is produced extracellular (Pan et al. 2022). Cell disruption is the primary step in protein recovery as the protein is located inside the cell. Thus, many contaminants such as lipid, host cell proteins, and DNA are released along with the product. In term of cost effective, the extracellular recombinant protein is more favorable as it decreases the complexity in downstream processing (Kastenhofer et al. 2021, Burdette et al. 2018).

PGA characteristics also different between microbial source. PGA from *A. xylosoxidans* and *A. faecalis* more stable at higher temperature than *E. coli* (Panigrahi et al. 2015). The most thermophilic and thermostable PGA comes from *Thermus thermophilus*, with the optimum temperature 75ºC and had half life 9.5 hours at this temperature that has been functionally expressed in (Torres et al. 2012).

The maturation of PGA is the most important factor influencing the enzyme's catalytic activity. Improper translocation, incorrect folding, and non-specific proteolysis in cytosol or periplasmic by peptidase are the major causes of the low catalytic activity of the enzyme (Pan et al. 2018). βSer1 of the N-terminal α-amino group plays a major role in catalytic activity of PGA as well as stabilization of transition states and catalytic residues (Grigorenko et al. 2014). To overcome this, wild type PGA from various sources were engineered to overexpress this enzyme using different host cell. Although there are many PGA reports, however, large-scale production of PGA is still limited to *E. coli* as the major platform (Vélez et al. 2014, Sambyal and Singh 2021). The fast growth growth kinetics, easily achieved high cell density, and relatively conventional metabolism are the main reasons in choosing *E.coli* as host organism to produce recombinant protein (Rosano and Ceccarelli 2014).

HOSTS FOR CLONING AND EXPRESSION

PGA enzymes are naturally produced by various microorganisms (bacteria, yeast, and fungi), however, most of industrial production using wild type microorganism is limited. Industrial production of PGA should be cost effective particularly in related to the enzyme activity and yield (Buchholz 2016) Therefore, effort to increase productivity through genetic engineering have been tried by many researchers. The selection of recombinant host cells is important for the improvement of PGA expression since it is closely related to intracellular or extracellular expression abilities, or the costs of large-scale inducible plasmid system (Hassan 2016). Several PGA encoding gene constructs have been developed in various vector systems and overexpressed in various hosts. PGA-producing bacterial strains can be improved through genetic manipulations, which usually need a host for expressing the target PGA gene. However, such improvement could increase immature precursors accumulation involved in the expression of PGA, and as the result lowering the enzyme yield, activity, or stability (Tishkov et al. 2010). PGA accumulation as inclusion bodies seen in *E. coli* using rapid expression T7 promoter system with IPTG induction due to imbalance in PGA formation pathway (Srirangan et al. 2013). On the hand, PGA expression in *Bacillus* gains attention where natural PGA also found and posses necessary post-translational modification in PGA maturation (Rajendran et al. 2014, Srirangan et al. 2013).

In the development process, genetic engineering and cloning techniques are generally aimed to increase the enzyme productivity by over-expression, or to increase cost efficiency of enzyme production by considering upstream processes such as fermentation and downstream processes such as protein purification and concentration. The following aspects must be considered to carry out a large-scale fermentation process: the availability of tools, materials, costs, and energy efficiency as well as efficiency of downstream processes such as protein purification and recovery processes. For instance, protein extraction from host cells by intracellular protein or periplasmic expression mechanism requires a process of cell breakdown and protein purification. Meanwhile, proteins from extracellular expression generally have to go through a concentration process.

Table 2. Microbial host for recombinant PGA

Host	Original sources	Maximum activity	References
E. coli (intracellular)	*Kluyvera citrophila*	9.6 U.mL^{-1}	Wen et al. 2005
	Providencia rettgeri	18 U.mL^{-1}	Pan et al. 2018
	Alcaligenes faecalis	75 U.mL^{-1}	Pan et al. 2018
	Achromobacter xylosoxidans	10 U.mL^{-1}	Pan et al. 2018
Bacillus megaterium	*Bacillus thermotolerans*	0.22 U.mL^{-1}	Mayer et al. 2019
(extracellular)	*Bacillus* sp. FJAT-27231	0.55 U.mL^{-1}	Mayer et al. 2019
	Bacillus megaterium	0.9 U.mL^{-1}	Panbangred et al. 2000
	Mutant *B. Megaterium*	20.4 U.mL^{-1}	Panbangred et al. 2000
	Bacillus thuringiensis	2.0687 U.mL^{-1}	Putri et al. 2015
Pichia pastoris	*Achromobacter* sp. CCM 4824	5.880 U.mL^{-1}	Marešová et al. 2017
(extracellular)	*Providencia rettgeri*	26.5 U.mL^{-1}	Šenerović et al. 2009

Further, in the aspect of PGA recombinant expression, there are several microorganisms that familiar as host for over expression. They are including *E. coli, Bacillus,* and yeasts. However, production using *E. coli* hosts via periplasmic area is the state-of-art technology predominantly used on industrial scale. In addition, recombinant strain harboring foreign protein might turns out toxic to the cell especially in *E. coli* (Jia and Jeon 2016).

Aside from *E. coli*, various potential PGA genes from different microorganisms have been cloned into *A. faecalis, A. xylosoxidans, B. megaterium*, and *K. citrophila*. On top of that, *E. coli* still remain being the most routine host for cloning and expression, since its system is well known, and the growth is relatively fast. However, in order to optimize the expression of heterologous proteins (including PGA) in *E. coli*, numerous aspects must be taken into account, including the size of the heterologous protein, codon biasing, post-translational modification of the protein, and the protein's origin site. *E. coli* is a Gram-negative bacterium that cannot secrete protein out of cells, besides the recombinant protein retain in periplasmic area through Twin-arginine translocation (Akkaya et al. 2012). They also cannot support the post translational modification especially for signal peptide cleavage in the case extracellular PGA that is needed to construct mature PGA. Downstream process of recombinant PGA in *E. coli* needs purification step to extract the protein using various methods such as detergent or lysozyme addition, osmotic shock, electric pulse or heat treatment (Lee 2017). These become important consideration, since large scale production of PGA must be established cost economically. Srirangan et al. (2013) reported a recent step in enhancing heterologous PGA synthesis, specifically in the *E. coli* system.

An alternative to periplasmic expression in *E. coli* is PGA production extracellularly from the genus of *Bacillus* i.e *Bacillus subtilis* (Lakowitz et al. 2018) or *B. megaterium* (Yang et al. 2006, Priya and Suganya 2013, Putri et al. 2015, Mayer et al. 2019). *B. megaterium* was used as host for expression and production PGAs from other *Bacillus* such as *B. megaterium, B. hermotolerans,* and *Bacillus* sp. FJAT-27231. PGA from these strains was characterized and their structure was compared. With the temperature-sensitive expression system of *B. subtilis* Phage phi105, recombinant B. subtilis harboring PGA gene from *B. megaterium* (BmPGA) produced extracellular recombinant enzyme with increased activity from 2.55 U.mL-1 in shake flask to 2.7 U.mL-1 and 4 U.mL-1 in bioreactor cultivation and in a three-phase fluidized bed bubble column, respectively (Chen and Cen 2005). In bioreactor batch-cultivations, recombinant six-protease deficient *B. subtilis* WB600 secreted PGA from *A. faecalis* (AfPGA) and from site-directed mutations BmPGA (BmPGAb24F) as much as 378 U.L-1 and 0.92 U.mL-1, respectively (Zhang et al. 2006). For comparison, the same strain expressed PGA up to 1960 U.L-1 when fed-batch cultivation applied. Other expression hosts for recombinant PGA with the related activity are shown in Table 2.

However, plasmid instability occurs in the majority of bacterial expression systems, making continuous production strategies quite challenging. Additionally, the production of

Table 3. Promoter modification in recombinant PGA

Original sources	Host	Promoter	Increase of activity	References
Providencia rettgeri	*E. coli*	Substitution to MalE promoter	0.06→18 U.mL-1	Pan et al. 2018
Alcaligenes faecalis	*E. coli*	Substitution to pelB promoter	10→75 U.mL-1	Pan et al. 2018
Achromobacter xylosoxidans	*E. coli*	Substitution to pelB promoter	0.28 →10 U.mL-1	Pan et al. 2018
E. coli ATCC 11105	*E. coli χ6212/pRT4*	Substitution to *trc* promoter	43 $U.g^{-1}$ → 1000 $U.g^{-1}$	Vohra et al. 2001
Alcaligenes faecalis	*E. coli*	Rhamnose-inducible promoter	4500 $U.L^{-1}$	Deak et al. 2003

enzymes as inclusion bodies might be a concern when employing a high-level expression system (Bhatwa 2021). PGA amplified from the genome of *Achromobacter* sp. CCM 4824 (Gram-negative bacteria) and cloned in *P. pastoris* X-33 expressed both intracellular and extracellular (Bečka et al. 2014, Marešová et al. 2017). Codon optimization is required for improved expression, which uses the codon typically employed by *P. pastoris*. Extracellular expression, on average, generates higher activity than intracellular expression. Protein secretion to the extracellular media decreases the impurity of the protein and reduces the complexity of dowstream processing (Burdette et al. 2018).

FURTHER ENGINEERING

As previously described, *E. coli* is used very often as a model in order to increase PGA overexpression, either using *E. coli* as an expression host or using PGA gene derived from *E. coli* (EcPGA). EcPGA ATCC11105 was cloned into recombinant *E. coli* X6212/pRT4 and produced 1000 units.g-1 cell dry weight, with a 23-fold increase in activity higher than that of wild-type *E. coli*. The pac promoter was replaced to a strong ptrc promoter, resulting in higher yield of PGA (Vohra et al. 2001). *E. coli* produce PGA and retained it in the periplasmic area in which proteolytic degradation highly occurs and decreases the yield of enzyme product. *E. coli* strain BL21 (DE3) might be an alternative to become the expression host as it is a natural deficient of ATP-dependent proteinase (Sambyal and Singh 2021). PGA from chromosomal DNA of *Achromobacter xylososxidans* (AcPGA) consists of α and β-subunit with the size of 27.0 and 62.4 kDa respectively were inserted into the pBluescriptIISK(+) then introduced into *E. coli* DH5α. The gene was then retransformed into *E. coli* BL21(DE3) with T7 lac promoter to manage PGA expression (Cai et al. 2004). Various promoters used in order to obtain high yield and productivity of PGA production (Table 3). Generally, strong promoter led to higher level of expression, but it also can led to accumulation of insoluble protein or problem in protein folding for *E. coli* expression system. Other alternative is choosing less strong promoter coupled by selecting the optimal vectors that can concentrate the PGA expression (Tishkov et al. 2010). The other choice is optimization of cultivation or product recovery to maximize the yield. PGA from Alcaligenes faecalis was expressed in *E. coli* under the rhamnose inducible control promoter that acted also as carbon source. The recombinant *E. coli* grown in minimal medium expressed PGA at 4500 U.L-1 to the culture medium (Deak et al. 2003). Rhamnose inducible promoter will directly reducing production cost in which minimal medium and inducer compounds are less expensive compared to lac operon system that use IPTG as inducer.

Site directed mutagenesis can simplify the work to obtain specific mutant that can produce PGA efficiently. αR141, αF142, and βF24 positions of PGA from A. xylosoxidans PX02 were mutated and in result, βF24 strain mutant have better enzymatic activity while mutations at αR145L give 2-fold higher PGA activity in *E. coli* (Pan et al. 2020).

Bacillus is another favorable host for protein production because of the secretion system of protein to the medium. PGA gene from *B. megaterium* ATCC14945 cloned into

pMM1522 under xylose inducible promoter control in combined with signal peptide LipA replacing original PGA signal peptide. Xylose-deficient strain *B. megaterium* MS941 used as expression host. LipA was acted as signal peptide that can export PGA to the media. Changing the signal peptide and expression system led to increasing PGA yield from 230 U.g-1 to 380 U.g-1 (Yang et al. 2006). Another strategy related to changing promoter system and signal peptide was also done by using dual promoter system from PamyE and Pcdd used to export PGA originated from *B. megaterium* and *B. subtilis* WB800 expression system. A dual promoter was generated by inserting a second promoter fragment into a single promoter upstream or downstream. This dual promoter system combined with PGA signal peptide yielded 135.58 U.mL-1 of PGA (Kang et al. 2020).

Producing protein in different host cell might cause incompatibility due to proteolytic activity of the cells as it is recognized as foreign object. In other case, the original strain might harbour repressor gene resulting small yield of product. PGA from the *B. megaterium* UN-1 was amplified and inserted into the pTF6 then transformed to the *B. megaterium* UN-1 that already been mutated to knock-out the original sequence of pac gene in the genome. This strategy resulting in 20-fold better activity of PGA at 20.9 U.mL-1 to the medium compared to parent strain UN-1 at 0.9 U.mL-1. Thus, using the original host can increase PGA yield in which PGA expression not disturbed by repressor gene or proteolytic activity of the host cell (Priya and Suganya 2013).

One of the well-known expression host that capable of creating a large amount of protein is yeast. Apart from their capacity for high biomass production, recombinant proteins can also undergo post-translational modification in yeast. As a result, in the process of large-scale protein synthesis the employment of yeast is more convenient rather than *E. coli* (Baghban et al. 2019). Several kinds of yeast, including *Pichia pastoris*, *Hansenula polymorpha*, and *Saccharomyces*, are frequently used as hosts for extracellular protein expression. Recombinant PGA gene from *Providencia rettgeri* was constructed by subcloning pac ORF from pGOB-2T expression plasmid into pPIC9 plasmid and expressed in *Pichia pastoris*. Recombinant PGA expressed in *P. pastoris* has lower specific activity compared to *S. cerevisiae* as expression host, but in turn, due to glycosylation, it has thermostable characteristics. Other work done by Šenerović et al (2009), established four copy of PGA gene from *P. rettgeri* integrated into *P. pastoris* GS115 genome that proteinase PEP4 has been knock-out using integrative vector. Following 120 hours of methanol induction, its PGA activity reached 26.5 U.mL-1. Marešová et al. (2017) optimized a codon in *P. pastoris* for extracellular expression of PGA. The wild-type pac gene of *Achromobacter* sp CCM4824 was synthesized with α-mating factor sequence 30 bp before the N-terminus subunit for *P. pastoris* codon usage. This sequence was then cloned to pPICZαA and transformed into *P. pastoris* X-33 under AOX1 promoter (Marešová et al. 2017).

FERMENTATION TECHNIQUE

Biomass formation as consequently high-level gene expression containing soluble enzyme in high activity level with correctly folded protein is the main parameter that need to achieve (Vélez et al. 2014). Other than genetic and protein engineering, fermentation technique also plays important role. Since a decrease in temperature promotes the formation of properly folded proteins, it is known that growth temperature affects the rate of protein synthesis. Recombinant *E. coli* BL21 DE3 harboring PGA in pT101/D-TOPO reached specific enzyme activity of 3000 U that increased 6-fold when it cultivated at 20ºC (Vélez et al. 2014). In comparison, recombinant PGA expressed in *E. coli* BL21 DE3/pET28b with IPTG induction in batch system fermenter exhibit 12 gDCW.L-1 and 0.42 IU.mL-1h-1 product formation at 28ºC (Wen et al. 2005). In addition, to increase the solubilization of expressed protein, the use of *E. coli* host that has been modified to grow in lower temperature than 20ºC such as *E. coli* BL21 (DE3) Arctic-express strain can be considered.

Continuous fed-batch fermentation with complex media and lactose induction exhibit high biomass at 100 gDCW.L-1 with higher rate of product formation at 7.8 IU.mL-1h-1 (Vélez et al. 2014). Another work done by Lin et al. (2002), produced recombinant PGA in fed-batch system and with IPTG induction.

Obtained high cell density was 75 at OD600 with high specific PGA activity at 500 U.L-1OD600-1 by *E. coli* MDPP7/pTrcKnPAC2902.

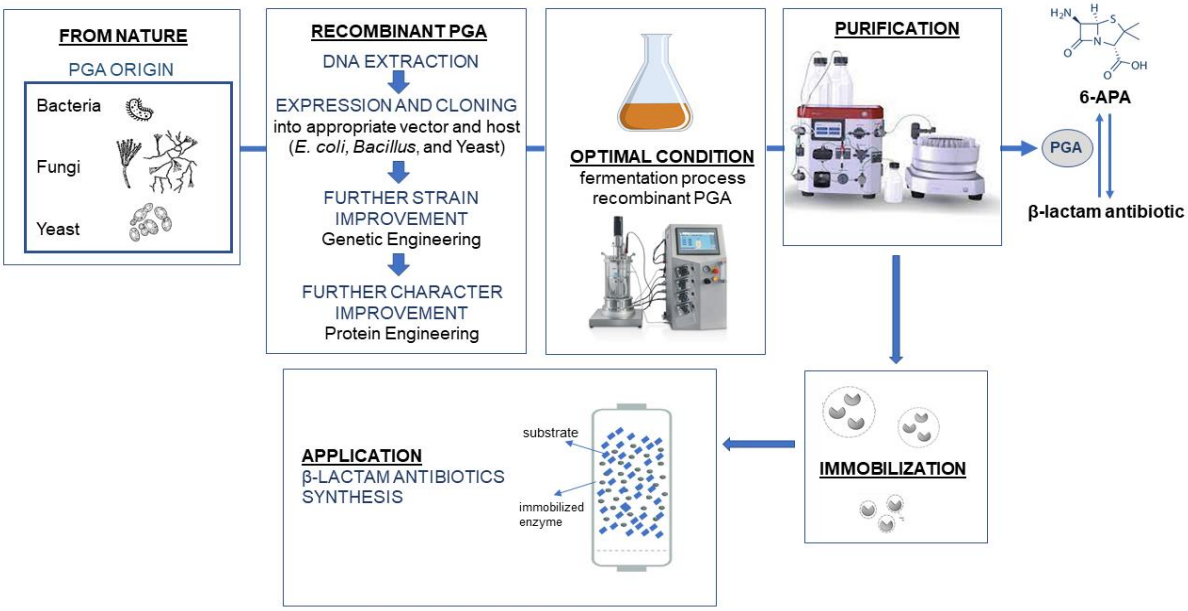

Figure 2. Schematic strategy of increasing the production of recombinant penicillin G acylase for the synthesis of β-lactam antibiotics

As a Gram-negative bacterium, *E. coli* surrounded by outer membrane which comprise lipopolysaccharide and served as permeability barrier. The barrier control molecule transfer in or out of the cell, thus become one of the limiting factors of producing extracellular protein. Taking advantage of the limited extracellular protein, by targeting recombinant proteins into the extracellular compartment, the level of contaminant proteins from the host cells can be significantly reduced by which simplifying the following downstream process. For that reason, Orr et al. (2012) developed an integrated production and subsequently one step purification of PGA overexpressed extracellular in *E. coli* grown in cultivation medium containing lowered salt and protein content. Glucose, Casamino acid, and MOPS buffer were added to the cultivation medium providing sufficient nutrients for sustaining cell growth and recombinant protein overexpression as well as a sufficiently low conductivity to allow direct application of the extracellular fraction to the anion-exchange chromatography column. This production strategy yielded a highly PGA specific activity of 871 U.g-1 DCW of which more than 90% was localized in the extracellular medium.

Protein secretions remain the bottleneck of producing high yield protein even in eukaryotic cell, in which the protein retained inside the cell. Yeast can be alternative host cell as it can secrete recombinant protein to extracellular and perform post-translational modifications such as glycosylation that give thermostable characteristic (Šenerović et al. 2009). Borčinová et al. (2020) divided the changes of specific rates in PGA production over time in *Pichia pastoris* culture into 3 stages (initial, transition, and saturation phase) with 3 important indicators there are specific rate of: product secretion, total production, and product retention in cells. Specific rates of product secretion reach the higher stable point in saturation phase or after 100 hours of methanol feeding (Borčinová et al. 2020). Above all, experiments of PGA production using yeast as production host remain limited.

B-LACTAM ANTIBIOTICS CATALYSIS

Penicillin G acylase can undergo synthetic and hydrolytic activity. As described above, PGA hydrolyzes penicillin G, cephalosporin G, or other β-lactam substances to become 6-APA or 7-ADCA and PAA. In conversion, PGA also synthesizes β-lactam antibiotics in a reaction mixture consisting of activated acyl-donor and nucleophile (6-APA or 7-ADCA) (Pan et al. 2018). Since PGA can

catalyze both synthetic and hydrolysis of antibiotics, using the same enzyme to produce 6-APA or 7-ADCA and antibiotic compounds is the primary idea to be applied in industry (Figure 2). However, due to this mechanism, producing semi-synthetic antibiotics often results in a low yield of the product. To control this, the synthetic rate and hydrolysis rate (S/H) ratio is crucial in producing semi-synthetic antibiotics (Deng et al. 2016).

The loss of the activated side chain of the precursor resulted in reduced efficiency of the acyl transfer reaction in the hydrolysis reaction by water. Higher S/H ratio is preferable economically and possesses sufficiently high enzyme activity (Van Der Laan et al. 2010). The condition and mixture where the reaction takes place are the main determining factor of the S/H ratio. The S/H ratio represents the ratio of the initial rate of the acyl-transfer products generation to the activated acyl donor hydrolysis. In addition, 6-APA nucleophilic reactivity, a ratio of enzyme to target product and acyl donor, and initial concentration of reagents play the main role to determine synthetic activity (Wang et al. 2021). To date, two options can be used to determine the hydrolysis activity of PGA, there are spectrophotometric analysis using NIPAB reaction (Vélez et al. 2014) and H+ titration as performed originally by Balasingham et al. (1972). On the other hand, synthetic analysis is primarily done by HPLC (Sari et al. 2020)

Enzymatic synthesis of β-lactam antibiotics takes place in either a regulated thermodynamic or kinetic synthesis. Thermodynamically controlled synthesis aimed to push the reaction equilibrium towards the product with non-activated acyl donor (free caboxylic acid) is being used (Pan et al. 2022, Cobos-Puc et al. 2020). Alternatively, the kinetically controlled synthesis is commonly focused on activated acyl donor (ester or amide of carboxylic acid), which should be in molar excess to drive the reaction towards synthesis that requires further reagents (Dorr and Fuerst 2018). Among those two synthesis method, kinetically controlled synthesis will generate higher amounts of antibiotics as the high molar ratio of activated acyl donors and β-lactam cores not restricted to equilibrium conversion. Hydrolysis of acyl donor is the critical point in approaching product synthesis, as it is spontaneously hydrolyzed at pH above 6.0 or by the enzyme (Bečka et al. 2014). However, from the economical point of view, molar ratio of acyl donor and β-lactam nucleus should be at the lowest point but at the same time, this low ratio should not affect the product synthesis.

Currently, PGA derived from *E. coli* is the prominent source to generate semi-synthetic β-lactam antibiotics. Ampicillin and amoxicillin synthesis can be achieved in kinetically controlled synthesis with 3:1 ratio of acyl donor/β-lactam nucleus with pH 7.0 at 25ºC under constant stirring and 40% glycerol (Pan et al. 2020). By reacting PGME with 7-amino-3-deacetoxycephalosporanic (7-ADCA) with a 4:1 molar excess of the ester, Travascio et al. (2008) obtained the highest yield of cephalexin. The unwanted hydrolytic reactions of β-lactam enzyme synthesis can be repressed by reducing the water activity in the medium. 1-ethyl-3-methylimidazolium triflimide and 1-butyl-3-methyl-imidazolium hexafluorophosphate or other ionic liquids can replace aqueous media used in the reaction and increases the S/H ratio (Pereira et al. 2012). Several PGA variants have beenable to synthesize cephalexin under a broad range of substrate concentrations (Harris et al. 2022).

Large scale PGA utilization in antibiotics synthesis using the enzyme in two-step cascade system including hydrolysis of penicillin and synthesis of penicillin is economically important (Deng et al. 2016). It is possible to lower the cost of PGA by immobilizing the enzyme, which allows it to be used frequently and easy to recover the product (Shi et al. 2014). The review related to the source, classification, structure, and catalytic mechanism of PGA, the studies the development of immobilization methods, immobilized carriers, reaction media, enzyme activity regeneration, and reactors of immobilized PGA in recent years have been described by Li et al. (2020). The immobilization of PGA can be accomplished using a range of methods such as entrapment, adsorption, cross-linking, encapsulation, covalent and non-covalent bonding. These methods carry different benefits and characteristics to the enzyme, which must be optimized to find the best technique for enzyme immobilization

Table 4. Matrix and characteristics of immobilized PGA

Original sources	Host	Method of immobilization	Characteristic	References
E. coli ATCC 11105 (wildtype)	-	Whole-cell entrapment in chitosan-glutaraldehyde matrix	Recover 90% of initial activity after 20 cycles	Bagherinejad et al. 2012
Providencia rettgeri	*P. pastoris*	Covalent immobilization using poxidic polymers and aminic polymers	Higher and more stable enzyme in aminic polymers matrix	Šenerović et al. 2009
Alcaligenes faecalis (*β*F24G mutant)	*E. coli*	Covalent bonding to epoxy carrier		Deng et al. 2016
E. coli	-	Physical adsorption of PGA into mesocellular siliceous foams (MCFs) surface with 3-aminopropyltriethoxysilane (APTS) and covalent binding with chitosan and glutaraldehyde	85% of activity retained after five operation cycles and no activity loss in the pH range 7.9-10.0 at 37 to 57ºC	Shi et al. 2014
		Phisical adsorption of PGA into immobilized functional ionic liquids Cl^-, BF_4^-, PF_6^-, and Tf_2N^- on silica nanoparticles and composite materials	70% of PGA activity observed after 9 operation cycles	Zhou et al. 2012a
E. coli (mono-cysteine mutants)	*E. coli*	PGA immobilized on tailor-made disulfide epoxy supports (Eupergit)	PGA retained its half-life for 250 h under 60% of dioxane at pH 7.0 and 4ºC	Grazu et al. 2012

(Nawaz et al. 2021). For example, in covalent bonding, this procedure involves free amino groups of the enzyme with activated aldehyde groups via interaction of protein surface that usually rich in lysine residue which is abundant near the active site of the PGA enzyme (Orrego et al. 2020). This procedure might affect enzyme catalytic activity as it is limiting the substrate-active site contact. Addition of tag consisting lysine residue far from active site might increase PGA stability on immobilization matrix (Grazu et al. 2012)

Various method of PGA immobilization results in different characteristics as described by Bagherinejad et al. (2012), Deng et al. (2016), Shi et al. (2014), Zhou et al. (2012a), Grazu et al. (2012), and etc (Table 4). Penicillin G acylase immobilized on surface-modified magnetic nanoparticles exhibit high effective activity and less affected by pH and temperature. An external magnetic field used in order to separate solutions and magnetic nanoparticles (Liu et al. 2016). Other methods of PGA immobilization used macro-mesoporous silica or silica nanotube because of large surface area and non-reactive material of silica (Zhou et al. 2012b).

CONCLUSION

Penicillin G Acylase is an important enzyme in β-lactam antibiotics synthesis as an alternative and promising approach replacing chemical synthesis. PGA is naturally found in many species but not many of them are suitable to use in industry. Various techniques to fulfill industrial demand are not only limited to finding a potential strain that exhibits high PGA activity, but also genetic engineering including site-directed mutagenesis, expression system optimization, fermentation strategies, and establishing the best catalytic condition of the enzyme. Selecting an expression system such as low-temperature to grow modified *E. coli*, *Bacillus*, or yeast over traditional *E. coli* may offer a number of advantages; however, more study and validation must be undertaken. Due to the fact that PGA exhibit both synthesis and hydrolysis activity, the synthesis/hydrolysis ratio of PGA is the primary parameter used to govern semi-synthetic antibiotic production. Combining a two-step cascade system to hydrolyze substrates and synthesis of β-lactam antibiotics using recombinant or wild-type

microbial-derived PGA, is a cutting-edge technique that can fulfill industrial demands.

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