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Characterization of Extracellular Penicilin G Acylase Produced by A New Local Strain of *Bacillus subtilis* BAC4

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Penicillin G acylase (PGA) which catalyses penicillin G hydrolysis reaction is a key enzyme for the industrial production of penicilin G derivatives used in therapeutics. A new local strain of *Bacillus subtilis* BAC4 was found capable of producing extracellular PGA. However, characteristics of this extracellular PGA are not known. The goal of this research was to characterize the extracellular PGA produced by *B. subtilis* BAC4. Enzyme production was carried out by batch fermentation, followed by enzyme purification and characterization of the PGA. The PGA activity was determined by the Kornfeld method, with optimal activity for hydrolysing penicillin G observed at 43 °C and pH 8.5. The activation energy of penicillin G hydrolysis by the PGA of *B. subtilis* BAC4 was determined as 4.9 kcal.mol^{-1} and V_{max} and K_m values were found to be 0.7 µmole.min^{-1}.mg^{-1} and 3.5 mM respectively. PGA catalytic activity was competitively inhibited by phenylacetic acid with an inhibition constant, $K_{i(PAA)}$, of 347.2 mM. It was concluded that the extracellular PGA of *B. subtilis* BAC4 can hydrolyse penicillin G efficiently.

Key words: PGA, extracellular, *Bacillus*, local

INTRODUCTION

At the end of the 19th century the first scientific studies appeared in which the antagonistic effect of chemical compounds on the growth of microorganisms was described. These compounds were later referred to as antibiotics. Nowadays, antibiotics are used as chemotherapeutic agents in the treatment of a variety of infectious diseases. Many compounds with antibiotic activity are produced in nature and represent a wide variety of chemical classes including peptides, nucleosides, and phenols. These antibiotics inhibit bacterial growth by interfering with cellular processes such as cell wall synthesis (penicillins and cephalosporins), protein synthesis (tetracycline, streptomycin, and erythromycin) and RNA synthesis (rifamycin) (Chandel *et al*. 2008).

One of the first antibiotics that was discovered was penicillin G, which belongs to the well-known class of the β-lactam antibiotics. Penicillin G consists of a phenylacetic acid moiety connected via an amide bond to 6-aminopenicillanic acid (6-APA), a heterocyclic ring system formed by a β-lactam ring fused to a thiazolidine ring. The phenylacetic acid moiety is commonly referred to as the side chain of the antibiotic and heterocyclic ring system is called the nucleus. The reactive part of the antibiotic is the four membered β-lactam ring as illustrated in Figure 1. The carbonyl carbon C7 of the amide bond in the ring is highly susceptible to nucleophilic attack due to the unfavourable cyclic configuration of the ring and the unusual geometry of the cyclic amide bond; this decreases the resonance that normally stabilises amide bonds (Alkema *et al*. 2002).

The β-lactam antibiotics are desirable therapeutic agents because they interfere with a part of the bacterial metabolism that does not have an equivalent in human biochemistry, *i.e*. the synthesis of the bacterial cell wall (Jager *et al*. 2007). The bacterial cell wall is a membrane made up of a lipid bilayer surrounded by a peptidoglycan layer. The presence of peptidoglycan in the cell wall is characteristic of almost all prokaryotes. It is composed of alternating units of two aminosugars, N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM). The NAM unit consists of a sugar with a variable peptide chain attached to its 3-hydroxyl group via which the different peptidoglycan chains are crosslinked.

The development of resistance by microorganisms to penicillin G and other β-lactam antibiotics has prompted the search for new β-lactam antibiotics. Other important factors were the need for antibiotics against a broader spectrum of organisms and the need for antibiotics with improved pharmacochemical properties such as increased resistance against acid degradation. Numerous penicillin G variants with diverse structure have now been isolated from nature and can also be synthesised chemically. In general, modification of the side chain as well as the nucleus has been proven successful. However, only a limited amount of these new β-lactam antibiotics are produced by nature with the majority synthesised chemically (Nicholas *et al*. 1995).

At this moment, the most important enzyme in the production of semi-synthetic β-lactam antibiotics is penicillin G acylase. The enzyme catalyses hydrolysis of the amide bond in penicillin G to produce 6-APA and phenylacetic acid (PAA;

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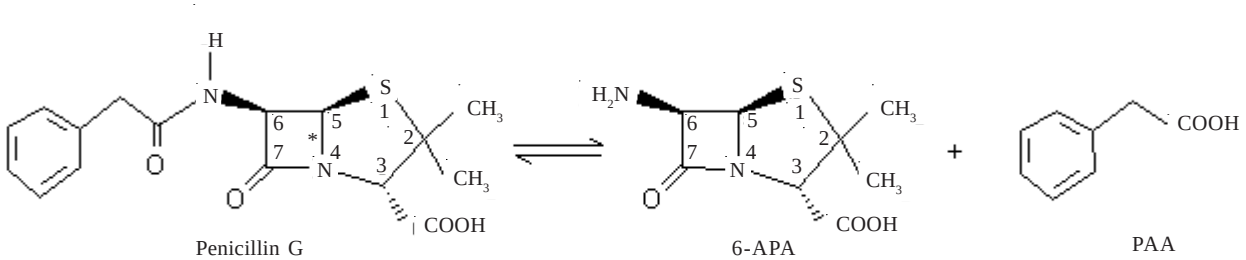

Figure 1. Hydrolyse of penicillin G catalysed by penicillin G acylase (*: β-lactam ring as a heterocyclic system) (Alkema *et al*. 2002).

Figure 1). This enzymatic conversion is used for the industrial production of 6-APA that is used for producing semi-synthetic penicillins. More than 60% of commercial 6-APA is produced enzymatically using PGA and world production is estimated at 30,000 tons.year^{-1} (Souza *et al*. 2005; Chandel *et al*. 2008). More importantly, the enzyme can also be used to synthesise new semi-synthetic antibiotics, a process that uses 6-APA as a substrate (Holownia & Noworyta 2008). In this process, penicillin G acylase catalyses the condensation reaction between an activated synthetic side chain and a 6-APA or 7-aminocephalosporanic acid (7-ACA) derived β-lactam nucleus, yielding semi-synthetic penicillins and cephalosporins, respectively. Therefore, penicillin G acylase (also referred to as PA, penicillin amidohydrolase, E.C.3.5.1.11) is the key enzyme in the industrial production of semi-synthetic β-lactam antibiotics. More than fifteen semi-synthetic penicillins derived from 6-APA are marketed today, from which ampicillin (D-phenylglycil-6-APA) and amoxicillin (*p*-hydroxy-D-phenylglycil-6-APA) are of particular commercial significance (Zhang *et al*. 2006). The application and potentials of PA has been the subject of recent review (Jager *et al*. 2008).

PGA enzyme from the *E. coli* strain ATCC 11105, the *Bacillus megaterium* strain ATCC 14945, and from mutants derived from these two strains are the most commonly used in industry. The enzyme is produced by wide variety of microorganisms even after eliminating those which also exhibite â-lactamase activity. The microorganisms most frequently reported in the literature for PGA production are strains of *E. coli*, *Proteus rettgeri*, and *B. megaterium* (Jager *et al*. 2008). Protein of the PGA is a heterodimer from the α- and β-subunits, which is unusual for proteins of prokaryotic origin. Successful processing and the formation of the αβ heterodimer are required to produced active periplasmic enzyme (Klei *et al*. 1995). The PGA from *E. Coli* has been extensively studied and most industrial biocatalysts use this enzyme. The enzyme is a periplasmic protein produced by growth of the organism at pH 7 and 24-30 °C in a medium which includes phenylacetic acid (PAA) as an inducer. Two important conditions for the process are the requirement for low oxygen transfer and the absence of fermentable sugars. After biomass production, penicillin G acylase biocatalysts may take various forms either as free or immobilized microorganisms or as immobilized enzyme, after partial purification (Silva *et al*. 2006). Penicillin G acylase is one of the few immobilized enzymes in use at the industrial scale, with annual sales of $6 million, below those of glucose isomerase with $20 million dollars (Anming *et al*. 2007).

A new local strain of *Bacillus* sp. BAC4 that can produce PGA extracellularly has been identified. This strain had also been characterised as being most closely related to *B. subtilis* (Kusumaningrum 1999) although the characteristics of the extracellular PGA were not investigated. An investigation into the nature of extracellular PGA produced by *B. subtilis* BAC4 is strongly needed as an effort to making efficient use of the local strain. In this study the extracellular PGA produced was recovered, purified and characterised.

MATERIALS AND METHODS

Materials. Casamino acid, casein hydrolysate, glucose, glycerol, phosphates, sodium sulphate, acetylacetone, p-dimethylaminobenzaldehide, and other mineral salts were purchased from Merck (Darmstadt, Germany). PAA, 6-APA, penicillin G (potassium salt), sepharose 4B, and 1,6-hexanediamine were obtained from Sigma (St. Louis, Missouri, USA). Bactopepton, and bactoagar were obtained from Difco (Detroit, Michigan, USA). All reagents were analytical or microbial grade.

Organism. *Bacillus subtilis* BAC4 selected for PGA production was obtained from the microbiology laboratory at ITB Bandung. The strain was maintained on slants of casein hydrolysate-glucose agar at pH 7 and 4 °C.

Fermentation Conditions and Media Composition. Fermentations were carried out batchwise at pH 7.0 and 37 °C in 1 litre flasks with 0.25 litres of medium and 10% v/v of inoculum in an orbital shaker at 150 rpm for 10 hours. All media were designed for carbon limitation, and contained in 1 litre: 7.5 g glycerol, 2 g casamino acid, 9 mg Na_2SO_4, 4.5 g KH_2PO_4, 5.3 g $Na_2HPO_4 \cdot 2H_2O$, and 10 ml solution of sulfur-free mineral salts (75 mg $MgCl_2 \cdot 6H_2O$, 55 mg $CaCl_2 \cdot 6H_2O$, 14 mg $FeCl_3 \cdot 6H_2O$, 12 mg $CoCl_2 \cdot 6H_2O$, 10 mg $MnCl_2 \cdot 4H_2O$, 1 mg $CuCl_2 \cdot 2H_2O$, 1 mg $Na_2MoO_4 \cdot 2H_2O$, and 1 mg $ZnCl_2$).

Enzyme Recovery and Characterization. PGA was recovered from fermentation broth by centrifuging at 15,000 g 4 °C for 20 min in a Sorvall RC-5B high speed centrifuge. The cell-free supernatant was freeze dried then dialized overnight against water at 5 °C. The clean, concentrated supernatant was purified by affinity chromatography column using sepharose 4B as a solid support and 1,6-hexanediamine as a spacer arm with PAA as a ligand. The column was eluted with 20 mM sodium phosphate buffer pH 6.8 and 100 mM sodium chloride. Temperature and pH activity of PGA were determined and kinetic parameters evaluated by initial rate studies under selected conditions. Buffer types tested were phosphate

(pH 7-7.9), tris-Cl (pH 8.2-8.8), and carbonate-bicarbonate (pH 9.1-9.7). All buffers were used at concentration of 0.1 M.

Analitycal Methods. The PGA activity was assayed by measuring the initial rate of penicillin G hydrolysis, as determined by the formation of 6-APA. 6-APA concentration or amount was determined using Ehrlich's reagent according to Kornfeld (1978). Ehrlich's reagent consisted of *p*-dimethylaminobenzaldehyde, 4.0 g; 95% ethanol, 380 ml; and HCl, 80 ml. 100 µl of enzyme was added to a screwcapped tube containing 400 µl of 15 mg.ml^{-1} penicillin G potassium salt solution in phosphate buffer pH 8.0 before being agitated and incubated at 40 ^{o}C for 20 min. Next, 100 µl of 2,4-pentanedione and 1.4 ml of 0.5 M sodium phosphate buffer pH 6.8 were added to the reaction mixture and agitated briefly with a vortex. After 20 min of heating, the tubes were quickly cooled under running tap water and placed in ice bath. Color was produced by adding 1.5 ml of Ehrlich's reagent. The same enzyme was used as a control after incubating in a boiling water bath for 5 min. The absorbance of the reaction mixture was measured at λ 542 nm aginst a blank. PGA activity was determined using standard curves of 6-aminopenicillanic acid (6-APA). One international unit of PGA activity was defined as the amount of enzyme that needed to produce 1 µmol 6-APA per minute from 15 mg.ml^{-1} penicillin G at 40 ^{o}C and pH 8.0.

The protein content was determined according to Lowry *et al.* (1951). Prepare a series of tubes including: (i) a blank containing 500 µl H_2O; (ii) a set of standard tubes each containg appropriate aliquote of a protein standard solution (100 µg albumine per ml) that will yield separate tubes containing 10, 20, 30, 40, and 50 µg of protein, all in final volumes of 500 µl; and (iii) a set of assay tubes containing appropriate dilution of the protein solution of unknown concentration in final volumes of 500 µl. Separately prepare 100 ml of fresh alkaline copper reagent by mixing, in order, 1 ml of 1% $CuSO_4 \cdot 5\,H_2O$, 1 ml of 2% sodium citrate and 98 ml of 2% Na_2CO_3 in 0.1 N NaOH. Add, and immediately mix in, 4.5 ml alkaline copper reagent to each tube. After 10 min at room temperature add, and immediatly mix in, 250 µl of Folin-Ciocalteu reagent to each tube. After 30 more min, read the absorbance of the standard and assay tubes at λ 745 nm against the blank.

The purity of enzyme was analyzed by using SDS-PAGE electrophoresis carried out in a vertical mini-unit protean (Pharmacia Biotech) using 12% acylamidegels, 10 µl of concentrated sample was loaded, with 5 µl protein standard containing phosphorylase b (94 kDa), albumine (67 kDa), ovalbumine (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumine (14.4 kDa). Electrophoresis was carried out at voltage of 125 volt for 55 min before gels were stained using coomassie brilliant blue R250.

RESULTS

Previous experiments to determine the optimum conditions for maximum enzyme production indicated that activated *B. subtilis* BAC4 cells were needed to stabilize the extracellular PGA production. Active cells of *B. subtilis* BAC4 can be obtained by subculturing cells at least 10 times. In contrast to other bacterial strains, the presence of PAA in the medium did not induce PGA production in *B. subtilis* BAC4 and inorganic phosphate concentration, [Pi], in the fermentation medium of more than 18 g.l^{-1} appeared to disturb the PGA production. Optimal temperature and pH values for the PGA production were observed at 37 ^{o}C and 7.0, respectively.

Production of extracellular PGA by *B. subtilis* BAC4 in glycerol-casamino acid medium and subsequent purification from other proteins are described in Table 1. The highest production of extracellular PGA achieved was 2.05 U per litre of medium with a specific activity of 0.4 U.mg^{-1}. The isolation of PGA was carried out from the clean-concentrated crude extract by affinity chromatography column with PAA as a ligand. A profile of elution and PGA activity are illustrated in Figure 2. Purification of PGA by affinity chromatography column gave a total yield of PGA of 0.46 U (22.47%) with specific activity of 0.85 U.mg^{-1}.

Kinetic parameters values were determined using soluble purified PGA eluted from the affinity chromatography column. The total protein content of the purified PGA fraction is 0.04 mg.ml^{-1}. Effect of PAA on the PGA activity for penicillin G hydrolisis was studied. Reactions were carried out using 50 mM PAA in 50 mM phosphate buffer pH 8.0 with penicillin G at concentration of 5, 12.5, 15, 20, 25, and 40 mM at temperature of 40 ^{o}C. The kinetic parameters were calculated from a Eadie-Hofstee plot and are shown in Table 2.

The relationship between temperature and activity of purified PGA was determined by measuring the initial reaction

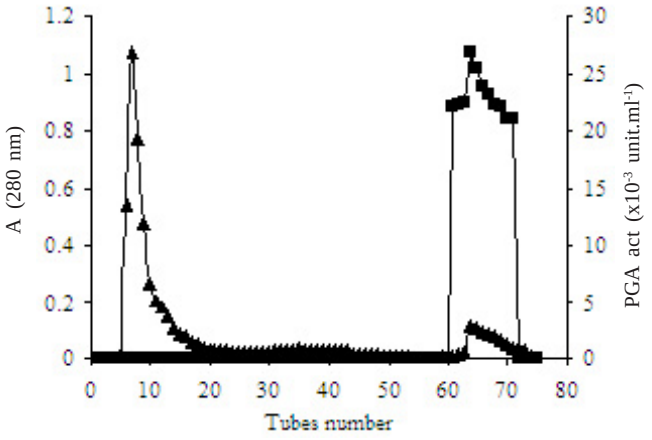

Figure 2. Elution profile of PGA activity using affinity chromatography. The column was eluted with 20 mM sodium phosphate buffer pH 6.8 and 100 mM sodium chloride. ▲: proteins and ■: PGA activity.

Table 1. The production and isolation results of extracellular PGA by affinity chromatography column with phenylacetic acid ligand

Steps	Volume (ml)	Total of PGA activity (U)	Total of protein (mg)	Specific PGA activity (U.mg^{-1})	Yield (%)	Purification (x)
Crude	60	2.05	5.15	0.4	100	1
PGA fraction	18	0.46	0.54	0.85	22.47	2.13

rate at pH 8.0 at temperatures ranging from 27 to 55 °C. The purified PGA and penicillin G substrate used were at concentration of 0.05 and 15 mg.ml^{-1} in 50 mM phosphate buffer pH 8.0 respectively. As illustrated in Figure 3, the relative initial reaction rate increased with increasing temperatures as far as 43 °C with the highest activity was obtained at 43 °C. Relative initial reaction rates were calculated using the initial reaction rate at 43 °C as 100%. The activation energy of penicillin G hydrolysis by the purified PGA was determined as 4.9 kcal.mol^{-1} using as Arrhenius plot (ln *v* versus 1/T) as shown in Figure 4.

The effect of pH on the activity of purified PGA was determined by measuring the initial reaction rates at 40 °C and at pH values ranging from 7.0 to 9.7. The purified PGA and penicillin G substrate used were at concentration of 0.03 and 15 mg.ml^{-1} respectively. The activity profile is shown in Figure 5. The highest initial reaction rate was obtained at pH 8.5 and the initial reaction rate at other pH values were expressed as relative reaction rate taking the value at pH 8.5 as 100%.

Analysis of the purified PGA using SDS-polyacrylamide gel electrophoresis revealed two protein bands at 57.8 and 62.1 kDa (Figure 6). This result shows that the relative molecular mass (Mr) of the extracellular PGA of *B. subtilis* BAC4 was estimated to be 120 kDa.

Table 2. Kinetic parameters values (K_m, $V_{max.}$, and $K_{i(PAA)}$) obtained by the Michaelis-Menten adjusment made to experiment points of the penicillin G hydrolyse catalyzed by soluble *B. subtilis* BAC4 PGA

Kinetic parameters	Estimate values
K_m	3.5 mM
V_{max}	0.7 (µmol 6-APA.min^{-1}.mg^{-1})
$K_{i(PAA)}$	347.2 mM

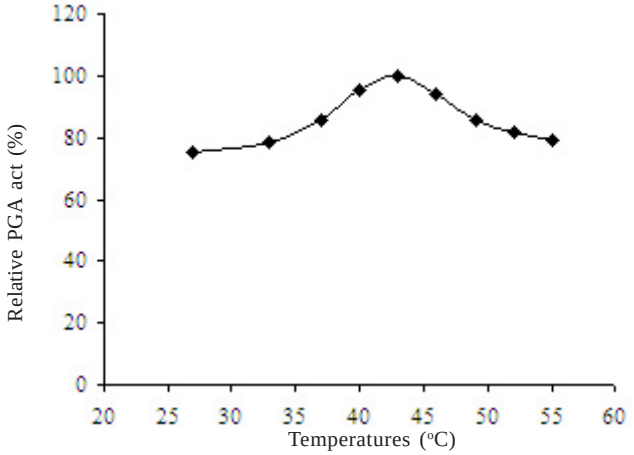

Figure 3. Effects of temperature on the PGA activity for penicillin G hydrolysis. ◆: relative PGA activity (%).

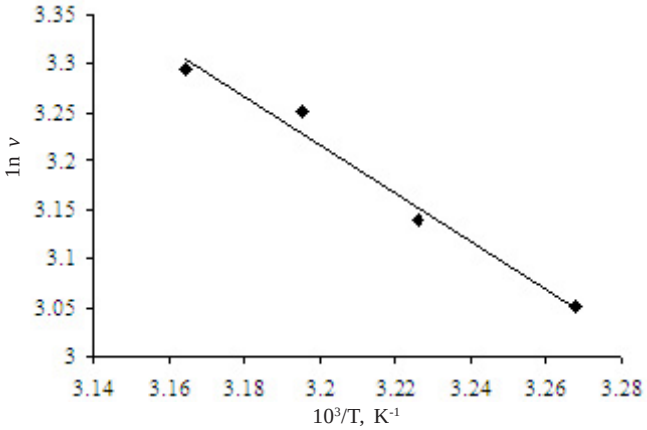

Figure 4. Arrhenius plot for determination of activation energy of extracellular *B. subtilis* BAC4 PGA [♦: ln *v*].

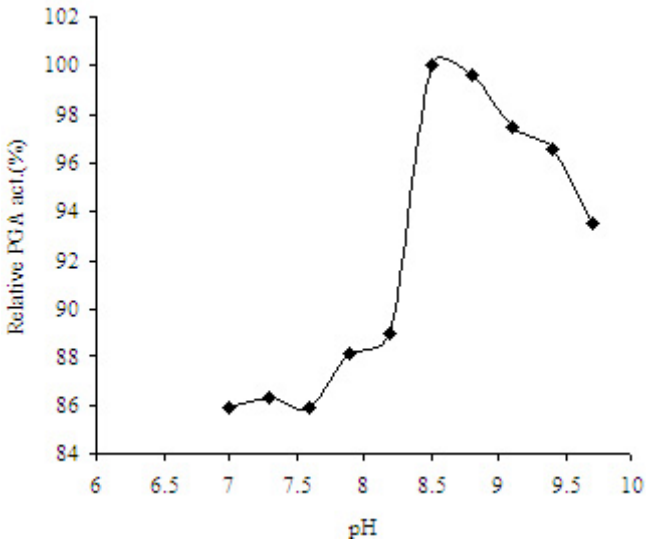

Figure 5. Effect of pH on the PGA activity for penicillin G hydrolysis [♦: Relative PGA activity (%)].

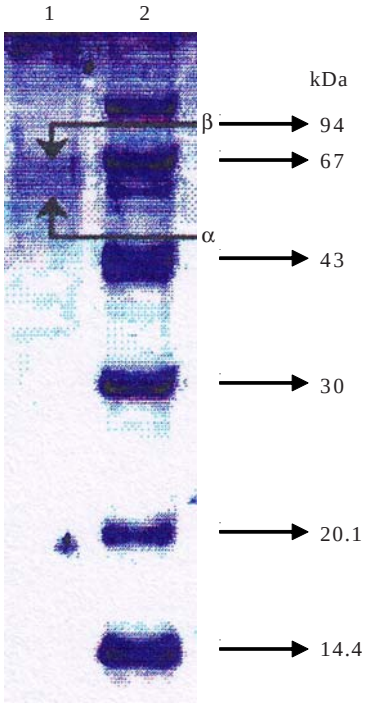

Figure 6. SDS-PAGE analysis of *B. subtilis* BAC4 PGA [Lane 1. PGA from *B.subtilis* BAC4; Lane 2. Markers: phosphorylase b (94 kDa), albumine (67 kDa), ovalbumine (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumine (14.4 kDa)].

DISCUSSION

The isolation of *B. subtilis* BAC4 PGA from the medium was performed by affinity chromatography column using the PAA as a ligand. The elution profile of the proteins and PGA eluted from affinity chromatography column is shown in Figure 2. It can be seen that the PGA protein was separated well from other proteins. Yield of the PGA was as 22.46% with purification of 2.13 times.

The maximal velocity (V_{max}) and Michaelis-Menten constant (K_m) values of the extracellular PGA of *B. subtilis* BAC4 were calculated from a Eadie-Hofstee plot as 0.7 (µmol 6-APA.min^{-1}.mg^{-1}) and 3.5 mM pencillin G, respectively. The competitive inhibition by PAA and the inhibition constant were also determined from a Eadie-Hofstee plot in the presence of 50 mM PAA. The inhibition constant ($K_{i(PAA)}$) for PAA (produced by the hydrolysis of penicillin G) was calculated as 347.2 mM. Furthermore, at same pH and temperature the V_{max}, K_m, and $K_{i(PAA)}$ values of the PGA of *B. megaterium* ATCC 145945 were estimated to be 1.1 (µmol 6-APA.min^{-1}.mg^{-1}), 4.5 mM, and 450 mM, respectively (Chiang & Bennett 1967). Therefore, the V_{max}, K_m, and $K_{i(PAA)}$ values of the *B. subtilis* BAC4 PGA are comparable with those of the extracellular PGA of *B. megaterium* ATCC 145945. However, the K_m value for *B. subtilis* BAC4 was smaller than that for *B. megaterium* ATCC 145945. In a separate study, Souza *et al*. (2005) obtained V_{max} and K_m values for *B. megaterium* ATCC 145945 PGA of 0.165 (µmol 6-APA.min^{-1}.mg^{-1}) and 1.83 mM respectively at 37 ^{o}C and pH 8,0 also using penisillin G as a substrate. Moreover, the V_{max}, K_m, and $K_{i(PAA)}$ values of the PGA of *E. coli* ATCC 11105 were reported as 9.63 (µmol 6-APA.min^{-1}.mg^{-1}), 2.33 mM, and 4.46 mM respectively (Erarslan *et al*. 1991). Since the Michaelis-Menten constant measures the affinity between enzyme and substrate, the values suggest that *B. subtilis* BAC4 PGA had the higher affinity to penicillin G.

The optimal temperature and pH of the PGA activity were determined for penicillin G hydrolysis. As shown in Figure 3 and 5, the optimal temperature and pH values were obtained at 43 ^{o}C and 8.5 respectively. These values are comparable with those determined other groups for the extracellular PGA of *B. megaterium* ATCC 14945 e.g 45 ^{o}C and pH 8.5 (Chiang & Bennett 1967), and 37 ^{o}C and pH 8 (Souza *et al*. 2005). In contrast, the PGA of *E. coli* ATCC 11105 shows optimal temperature of 55 ^{o}C and pH of 8.0 (Erarslan *et al*. 1991) and the optimal temperature and pH of the PGA from *Kluivera citrophila* were reported as 55 ^{o}C and 7.3 respectively (Martin *et al*. 1990).

The activation energy of penicillin G hydrolyse by the PGA of *B. subtilis* BAC4 was determined as 4.9 kcal.mol^{-1} from the Arrhenius plot as swhon in Figure 4. It is much lower than the value for the PGA of *E. coli* ATCC 11105 that was found to be 10.55 kcal.mol^{-1} (Erarslan *et al*. 1991). This indicates that the extracellular PGA of *B. subtilis* BAC4 can hydrolyse penicillin G more efficiently than the PGA of *E. coli* ATCC 11105.

Analysis of the PGA proteins on SDS-PAGE was carried out by using polyacrylamide gel at concentration of 12%. After staining the gel using coomassie brilliant blue R250 given two protein bands at 57.8 and 62.1 kDa as shown in Figure 6. This result shows that the extracellular PGA of *B. subtilis* BAC4 consisted of two subunit, α and β. The smaller subunit (α) has a molecular weight of 57.8 kDa, and the bigger subunit (β) has a molecular weight of 62.1 kDa. Therefore, the relative molecular mass (Mr) of the extracellular PGA of *B. subtilis* BAC4 was estimated to be 120 kDa. Furthermore, the PGA of *B. megaterium* ATCC 14945 shown Mr about 85.6 kDa and consisted of two subunit, subunit α at Mr of 24.2 kDa and subunit β at Mr of 61.4 kDa (Verhaert *et al*. 1997). PGA of *E. coli* ATCC 11105 consisted of two subunit, subunit α at Mr of 20.5 kDa and subunit β at Mr of 65 kDa. So, the Mr of PGA of *E. coli* ATCC 11105 was estimated as 85.5 kDa (Erarslan *et al*. 1991). Moreover, PGA of *Alcaligenes faecalis* shown a Mr of 85,6 kDa and consisted of two subunit, subunit α at Mr of 23 kDa and subunit β at Mr of 62.7 kDa (Verhaert *et al*. 1997). PGA of *Providencia rettgeri* shown a Mr of 85.89 kDa and consisted of two subunit, subunit α at Mr of 23.73 kDa and subunit β at Mr of 62.16 kDa (Klei *et al*. 1995). This is encouraging for the studies that will follow, which consider mutation using acridine orange for increasing the PGA production (Bernal *et al*. 2002).

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