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Medium Optimization for Penicillin Acylase (PAc) Production by Recombinant *Bacillus megaterium MS941* Containing *pac* Gene from *Bacillus thuringiensis* BGSC BD1 Using Response Surface Methodology

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Penicillin G acylase (PAc) hydrolyses of the amide bond of benzylpenicillin (Pen-G) releasing PAA and 6-APA, key intermediate in the production of various semisynthetic penicillins. In this study, we optimised the PAc production medium by RSM using two variables (xylose as inducer and $CaCl_2$ as divalent cations) to obtain the optimum PAc specific activity from *Bacillus megaterium* bt*pac*BD1. For this purpose, combinations of five different xylose concentrations (0.13 – 0.87 %) and five different $CaCl_2$ concentrations (0.64 – 4.36 mM) were analysed, in a total of 22 experiments. CCD used for the analysis showed that in shake flask cultivations, xylose and $CaCl_2$ gave significant effects on PAc volumetric activity and the quadratic model was in good agreement with the experimental results (R^2= 0.86 (p-value < 0.0001)). The maximum specific activity (130.669 ± 50.241 units mg protein^{-1}) was reached when xylose and $CaCl_2$ concentrations were 0.49% and 2.4 mM, respectively, and medium pH was around 7. Under such conditions, the activity of PAc and protein concentration achieved were 1.318 ± 0.406 units mL^{-1} and 0.01 ± 0.01 mg mL^{-1}. The shake flask validation experiments demonstrated that with such medium composition the volumetric activity, protein concentration and specific activity achieved were 1.294 ± 0.171 units mL^{-1}, 0.0102 ± 0.0003 mg mL^{-1} and 125.91 ± 13.31 units mg^{-1}, respectively . When the optimum medium composition was applied in 10 L bioreactor, the optimum volumetric activity (2.0687 ± 0.0820 units mL^{-1}) and protein concentration (0.0078 ± 0.0008 mg mL^{-1}) were achieved 48 h after the start of the cultivation. However, the optimum PAc specific acivity (1260.52 ± 27.57 units mg protein^{-1}) was achieved 18 h after the start of the cultivation.

Key words: *Bacillus megaterium* bt*pac*BD1, CCD, $CaCl_2$, Penicillin acylase, xylose

Penisilin asilase (PAc) menghidrolisis ikatan amida dari benzilpenisilin (Pen-G) dan menghasilkan PAA dan 6-APA, bahan dasar produksi bermacam antibiotik penisilin semisintetis. Pada penelitian ini, kami mengoptimasi media untuk produksi PAc menggunakan RSM menggunakan dua variabel (xilosa sebagai induser dan $CaCl_2$ sebagai kation divalent) untuk mendapatkan aktivitas spesifik PAc maksimal dari *B. megaterium* bt*pac*BD1. Untuk mencapai tujuan ini, kombinasi dari lima konsentrasi xilosa (0,13 – 0,87%) dan lima konsentrasi $CaCl_2$ (0,64 – 4,36 mM) yang berbeda telah dianalisa sebanyak 22 percobaan. CCD digunakan untuk menganalisa pada kultivasi skala labu erlenmeyer yang menunjukkan bahwa xilosa dan $CaCl_2$ memberikan pengaruh yang nyata terhadap aktivitas PAc dan model kuadratik dipilih berdasarkan hasil percobaan dengan (R^2=0,86 (nilai p < 0,0001)). Aktivitas spesifik PAc tertinggi (130,669 ± 50,241 unit mg^{-1}) didapatkan pada konsentrasi xilosa dan $CaCl_2$ masing-masing sebesar 0,49% dan 2,40 mM pada pH medium sekitar 7. Pada kondisi ini, aktivitas PAc dan kadar protein yang dihasilkan adalah 1,318 ± 0,406 unit mL^{-1} dan 0,01 ± 0,01 mg mL^{-1}. Verifikasi hasil komposisi media optimal pada labu erlenmeyer dengan komposisi media hasil optimasi dapat menghasilkan aktivitas, kadar protein dan aktivitas spesifik masing-masing sebesar 1,294 ± 0,171 unit mL^{-1}, 0,0102 ± 0,0003 mg mL^{-1} dan 125,91 ± 13,31 unit mg^{-1}. Komposisi media optimal diaplikasikan pada skala bioreaktor 10 L, aktivitas tertinggi (2,0687 ± 0,0820 unit mL^{-1}) dan kadar protein tertinggi (0,0078 ± 0,0008 mg mL^{-1}) dihasilkan pada waktu kultivasi 48 jam. Sedangkan aktivitas spesifik tertinggi (1260,52± 27,57 unit mg protein^{-1}). dihasilkan pada waktu kultivasi 18 jam.

Kata kunci: *Bacillus megaterium* bt*pac*BD1, $CaCl_2$, CCD, Penisilin asilase, xilosa

Penicillin G (pen-G) was the most widely used antibiotics in the world market (approximately 19 %) (Parmar *et al.* 2000). Pen-G inhibits the enzyme transpeptidase involved in peptidoglycan synthesis, so that bacterial cell wall formation does not occur

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(Madigan *et al*. 2003). Overuse of pen-G led to a bacterial pathogen resistance due to the production of β-lactamase which hydrolyzes β-lactam ring of penicillin rendering it inactive (Navarro *et al.* 2004). Pen-G's sensitivity to β–lactamase, its limited activity to gram positive bacteria and possibility to cause allergy raised problems of using this antibiotic (Madigan *et al.* 2005).

In industry, penicillin G acylase (penicillin amidohydrolase EC 3.5.1.11/ PAc) catalyzes the conversion of benzyl penicillin (Pen-G) via hydrolysis of the amide bond in the benzyl penicillin side chain, releasing phenylacetic acid (PAA) and 6-aminopenicillanic acid (6-APA). 6-APA is the key intermediate in the production of various semisynthetic penicillins (methicillin, ampicillin and amoxicillin) and the β-lactamase inhibitor (clavulanic acid) (Wilson 1982).

Several bacterial isolates are known to produce PAc, for examples *Escherichia coli* (Cole 1969), *Alcaligenes faecalis* (Verhaert *et al*. 1997), *Arthrobacter viscosus* (Ohashi *et al.* 1989), *Bacillus megaterium* (Chiang and Bennett 1967), *B. thuringiensis* BGSC Huazhongensis serovar strain 4CC1, 4BD1, 4AJ1 and 4AW1 (Nurhayati 2009). However, those that have been used commercially to produce PAc enzymes are only *E.coli* and *B. megaterium* (Vandamme and Voets 1974). The production of PAc can be improved by genetic engineering and/or bioprocess engineering. Our study focused on the production of PAc from recombinant *B. megaterium. B. megaterium* was chosen as the production host due to its relatively low extracellular protease activity (Wittchen and Meinhart 1995) and its capability to secrete PAc directly into the medium, facilitating its purification and consequently decreasing the downstream processing and final production costs since the PAc could be harvested simply by cold centrifugation (Yang *et al.* 2006; Hammond 1978). This is of course a great improvement in comparison to *E. coli* expression system, which retains the expressed protein intracellularly. The other disadvantage of *E. coli* was the presence of lipopolysaccharide (LPS) as a component of the outer membrane. LPS is endotoxin and harmful to humans.

The strain used in this study contained a recombinant plasmid pMMbt*pac*BD1. The plasmid contained *pac* gene isolated from the genomic DNA of *B. thuringiensis* BGSC BD1. The gene, *btpac-bd1*, was inserted into pMM1525 plasmid, by removing the lipase signal peptide (SPlipA) from the plasmid. The *btpac-bd1* gene was predicted to have its own signal peptide that would lead the secretion of recombinant proteins out of the cell (Nurhayati 2009).

According to Yang *et al.* (2006), *B. megaterium* YYBm1 produced the highest PAc with the addition of 0.5 % xylose and 2.5 mM $CaCl_2$ in medium. In contrast to Yang *et al*. (2006) who used the conventional methodology, this study used response surface methodology (RSM) via the Design-Expert software for the medium optimization, thus improving the accuracy and minimizing errors (Box and Draper 2007). In this project, in order to increase PAc production, we tried to optimize the medium by addition of two variables, inducer and divalent cation, based on the previous research (Yang *et al.* 2006).

This study aimed to determine the optimum medium composition by xylose and $CaCl_2$ addition, try PAc production using bioreactors at its optimum conditions, and determine the best harvesting time.

MATERIALS AND METHODS

Microorganism. The strain used in this study (*B. megaterium* bt*pac*BD1) was from the culture-collection of Centre for Bioindustrial Technology, BPPT, Indonesia. Stock cultures were maintained in 75% (v v^{-1}) glycerol.

Seed Medium Composition. Luria Broth (LB) agar contained 0.5% NaCl, 1% tryptone, 0.5% yeast extract and 0.75% agar. LB liquid contained 0.5% NaCl, 1% tryptone and 0.5% *yeast* extract. Initial pH was adjusted to 7. Media was autoclaved at 121°C for 15 min. LB tetracycline (LB-tet) medium was made by adding tetracyclin (12.5 μg mL^{-1}) to the medium as a selection pressure.

Production Medium Composition. The minimal medium contained 50 mM MOPS (pH 7.0), 5 mM Tricine (pH 7.0), 520 μM $MgCl_2.6H_2O$, 276 μM K_2SO_4, 50 μM $FeSO_4.7H_2O$, 2.5 mM $CaCl_2$, 100 μM $MnCl_2$.4H2O, 50 mM NaCl, 10 mM KCl, 37.4 mM NH_4Cl, 1.32 mM K_2HPO_4, 0.4 % (w v^{-1}) glucose, 1 mL L^{-1} trace element solution, and 1 mL L^{-1} vitamin solution. The initial pH was adjusted to 7 and the medium was autoclaved at 121°C for 15 min. Antibiotic tetracyclin (12.5 μg mL^{-1}) was added to the medium as a selection pressure.

The trace element solution contained 3.708 mg (NH4) $6Mo7O_{24}.4H_2O$, 24.73 mg H_3BO_3, 7.137 mg $CoCl_2$, 2.497 mg $CuSO_4$, 15.832 mg $MnCl_2$, and 2.875 mg $ZnSO_4$ L^{-1}. The vitamin solution consisted of 6 mg

biotin, 20 mg niacin amid, 20 mg p-amino benzoate, 10 mg Ca-panthotenate, 100 mg pyridoxal/HCl, 20 mg folacid, 50 mg riboflavin, 50 mg DL-6,8-thioctic acid and 10 mg thiamine dichloride L^{-1}. The minimal medium was supplemented with concentration of amino acid solution (1x). The stock solution (10x) was prepared separately according to the optimum solubility of amino acids in water as: 10 mg alanine, 10 mg arginine, 1 mg aspartic acid, 1 mg cysteine, 40 mg glycine, 4 mg isoleucine, 2 mg leucine, 10 mg lysine, 5 mg methionine, 5 mg proline, 5 mg serine, 5 mg threonine, 1.6 mg glutamic acid, 8 mg valine, 4 mg histidine, 3 mg asparagines and 3 mg glutamine L^{-1} *(Yang et al. 2006)*. All chemicals used for enzyme activity and protein assays were analytical grade.

Inoculum Preparation and Shake Flask Experiments. Seed culture was prepared by inoculating a single colony from a 24 h LB-tet plate into 20 mL of LB-tet liquid medium (seed medium), followed by incubation at 37°C, 180 rpm for 6 h until OD_{578nm} was 0.6 –0.8. After that, inoculum was prepared by inoculating 10 mL of seed culture to 90 mL of seed medium at 37°C, 180 rpm for 3 h until OD_{578nm} was 0.6 – 0.8.

Batch cultivation was carried out as reported by Rajendhran *et al.* (2003) with 180 rpm agitation, 28°C for 48 h in 500 mL erlenmeyer flasks containing 90 mL of cultivation medium. Each flask was then inoculated with 10 mL of inoculum.

Experimental Design. Respon surface methodology (RSM) with two variables, *i.e.* xylose as inducer and $CaCl_2$ as divalent cations, were used to obtain the maximum and specific activity of PAc produced by *Bacillus megaterium* bt*pac*BD1. For this purpose, five different xylose concentrations (ranging from 0.13% to 0.87%) and five different $CaCl_2$ concentrations (ranging from 0.64 mM to 4.36 mM) (Table 1) were analysed in combinations in a total of 22 experiments (Table 2). Design expert Software Version 9.0 was used for the experimental design and statistical analysis of the experimental data.

Batch Cultivation in 10 L Bioreactor. The batch cultivation was carried out in a 10 L Biostat bioreactor equipped with three six-bladed disc impeller and oxygen under the following conditions: medium volume 5 L, inoculation volume 10% (v v^{-1}), 28°C, 1.0 vvm aeration, 150 agitation and pH medium was 7. The pH of the medium was controlled by addition of sterile NaOH and HCl. Vegetable oil was also added as an antifoaming agent.

To determine the best harvesting time, *B. megaterium* bt*pac*BD1 was cultivated for 60 h and the volumetric activities were measured every 6 h. Samples of the culture were withdrawn at 0, 6, 12, 18, 24, 30, 36, 42, 48, 54 and 60 h after the inoculation.

Samples of the culture were harvested every 6 h and the cultivations stopped at the best time from the result of the best harvesting time's experiment.

Assay of PAc Volumetric Activity. Culture were centrifuged at 4°C and 6000 rpm for 15 min (Hitachi CR216, Rotor R10A2) as reported by Rajendhran *et al.* (2003). The supernatant was used as the source for the enzyme analysis. The PAc activity in the clear supernatant was assayed at 50°C using Penicillin G as substrate. One unit of PAc activity was defined as the amount of enzyme required to release 1 µmol of 6-APA min^{-1} under the assay condition as reported by Balasingham *et al*. (1972).

Protein Concentration. Protein assays were performed using bovine serum albumin as protein standard as reported by Bradford (1976).

Specific Activity. Specific activity of PAc (units mg^{-1}) was expressed as units mg^{-1} of protein concentration or ratio from PAc volumetric activity (units mL^{-1}) and protein concentration (mg mL^{-1}).

RESULTS

Response Surface Methodology. The strategy used to attain the goal of this work was to explore the experimental space around the previously selected medium compositions. An experimental design was implemented in order to better evaluate the interactions between the variables. The CCD was chosen as the one that allows the fitting of several mathematical models from the data obtained.

Response 1: PAc Volumetric Activity. The analysis of variance (ANOVA) of the response surface model showed that when *B. megaterium* strain bt*pac*BD1 was cultivated in shake flasks, xylose and $CaCl_2$ combination had significant effects on PAc volumetric activity. The quadratic model and two factors factorial design could explain the data achieved significantly (p-value < 0.0001) at 95% confidence level. The lack of fit test showed that only the quadratic model gave the incompatibility (p-value > 0.1142 and R^2= 0.86). The ANOVA of the quadratic response surface model demonstrated that the response surface quadratic model could explain the data (p-value < 0.0001). The variables that affected the model at 99% confidence level was indicated by p-value < 0.01. The application of the quadratic response model showed an

Table 1 Independent variable in the experimental plan

Variable	Coded level					
	Coded	-1.861	-1	0	1	1.861
Xylose	X_1	0.13	0.3	0.5	0.7	0.87
$CaCl_2$	X_2	0.64	1.5	2.5	3.5	4.36

Table 2 The CCD matrix employed for two independent variables

Experiment Number	X_1	X_2
1	1	-1
2	1	1
3	-1	1
4	-1	-1
5	-1	-1
6	0	0
7	0	0
8	-1	1
9	1	-1
10	1	1
11	1	-1
12	-1	1
13	-1	-1
14	1	1
15	0	0
16	-α	0
17	0	0
18	0	-α
19	α	0
20	0	α
21	0	0
22	0	0

Note: -1: lower limit; 0: the midpoint; 1: the upper limit; α: Starting point

empirical relationship between PAc activity and the test variables. The relationship could be expressed in the following equation:

$$Y = 1.132 - 0.324\,X_1^2 - 0.129\,X_2^2 - 0.257\,X_1 * X_2$$

where, Y is PAc volumetric activity (units mL^{-1}), X_1 is xylose concentration (%) and X_2 is $CaCl_2$ concentration (mM). The response surface and contour plot of xylose and calcium ions combinations on PAc volumetric activity was presented in Fig 1A.

Response 2: Protein Concentration. The ANOVA of the response surface model by treatment combinations showed that when *B. megaterium* strain bt*pac*BD1 was cultivated in shake flasks, xylose and $CaCl_2$ did not have significant effects on protein concentration (p-value > 0.0191).

Response 3: PAc Specific Activity. The ANOVA of the response surface model showed that when *B. megaterium* strain bt*pac*BD1 was cultivated in shake flasks, xylose and $CaCl_2$ had significant effects on PAc specific activity. The quadratic model and two factors factorial design could explain the data from this results significantly (p-value < 0.0001) at 95% confidence level. The lack of fit test showed that only the quadratic model gave the incompatibility (p-value > 0.2688 and R^2= 0.79). The ANOVA of the quadratic response surface model demonsrated that the quadratic model could explain the data (p-value < 0.0001). The variables that gave the effects on the model at 99% confidence level was indicated by p-value <0.01. The application of the quadratic response model showed an empirical relationship between PAc activity and the test variables. The relationship could be expressed in

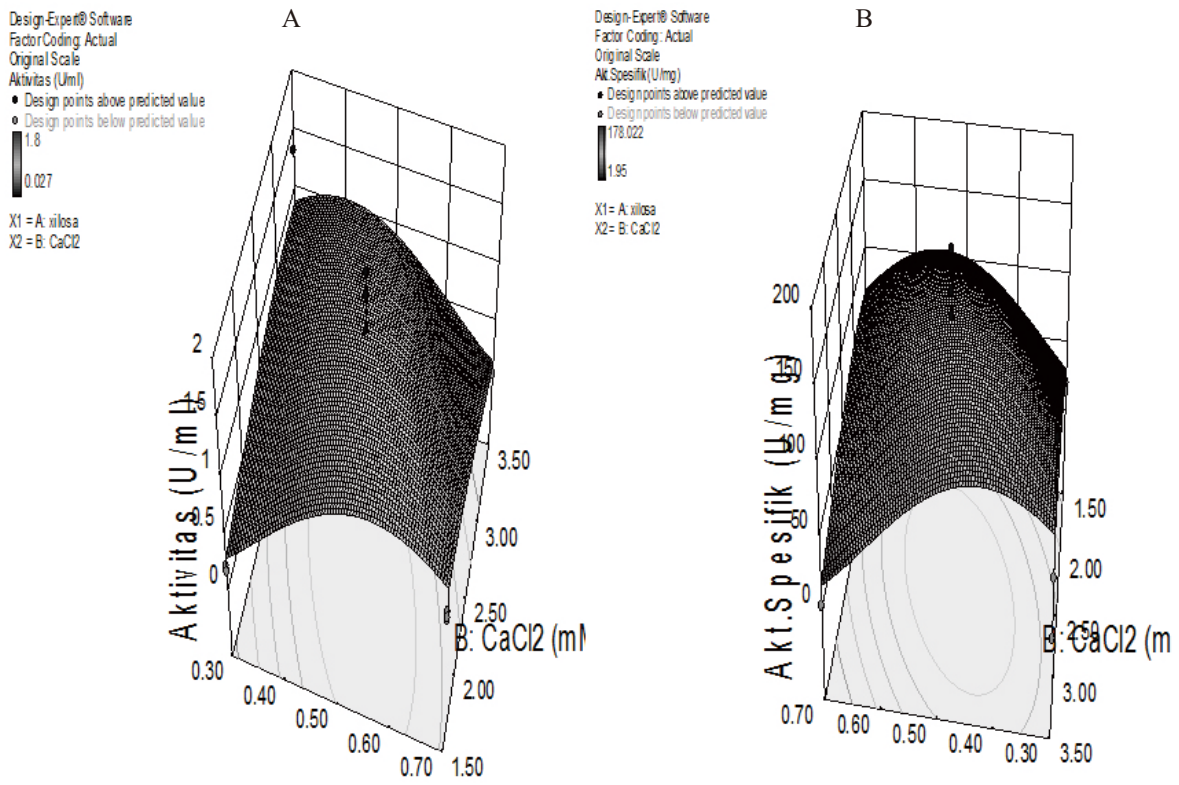

Fig 1 Response surface and contour plot of xylose and calcium ions combinations on PAc volumetric activity (A) Response surface and contour plot of xylose and calcium ions combinations on PAc specific activity (B).

the following equation:

$$Y = 11.191 - 3.368\,X_1^2 - 1.519\,X_2^2 - 2.025\,X_1 * X_2$$

where, Y is PAc volumetric activity (units mL^{-1}), X_1 is xylose concentration (%) and X_2 is $CaCl_2$ concentration (mM). The response surface and contour plot of xylose and calcium ions combinations on PAc specific activity was presented in Fig 1B. The maximum specific activity was reached when xylose and $CaCl_2$ concentrations were 0.49% and 2.4 mM, respectively, and medium pH was around 7. Under such conditions, the volumetric activity of PAc achieved was 1.318 ± 0.406 units mL^{-1} with protein concentration 0.0101 ± 0.01 mg mL^{-1} and specific activity 130.669 ± 50.241 units mg^{-1}. The validation experiment in shake flask demonstrated that with such medium composition the volumetric activity achieved was 1.294 ± 0.171 units mL^{-1}, protein concentration 0.0102 ± 0.0003 mg mL^{-1} and specific activity 125.91 ± 13.309 units mg protein^{-1}.

Batch Cultivation in 10 L Bioreactor. The optimum medium composition was applied in 10 L bioreactor.

The growth of *B. megaterium* bt*pac*BD1 and the volumetric activities were measured every 6 h for 60 h (Fig 2). The exponential (log) growth phase occured between 0 – 30 h of cultivation. According to Yang *et al.* (2006), the lag phase of the *B. megaterium* was 1 – 5 h after the start of cultivation. The adaptation (lag) phase might not have been detected in our data because the observation period was longer than the adaptation phase. *B. megaterium* bt*pac*BD1 underwent the stationary phase between 30 – 42 h before subsequently entered the death phase. Fig 2 also showed that PAc secretion started in the exponential phase and reached maximum (2163 units mL^{-1}) in the stationary phase (48 h), before eventually declining. Since we aimed to obtain the highest possible enzyme activity on harvesting, then 48 h was selected as the best time point to harvest the enzyme, hence used in subsequent experiments as the final time point.

The growth of *B. megaterium* bt*pac*BD1, PAc volumetric activity, protein concentration and PAc specific activity during cultivation are presented in Fig 3. As seen in Fig 3 and Fig 3A, the bacterial growth and PAc volumetric activity showed the same pattern as previously observed (Fig 2). At 48 h, the maximum PAc volumetric activity was 2.069 ± 0.0820 units mL^{-1} which reached in the s meaning that 1 mL of PAc enzyme harvested could degrade 2.0687 ± 0.0820 µmol pen-G to 6-APA per minute at 50°C. The PAc volumetric activity was relatively constant between 18 – 42 h.

Fig 3B showed that the protein concentration started to increase in the exponential phase and continued to increase until the cultivation was terminated at 48 h

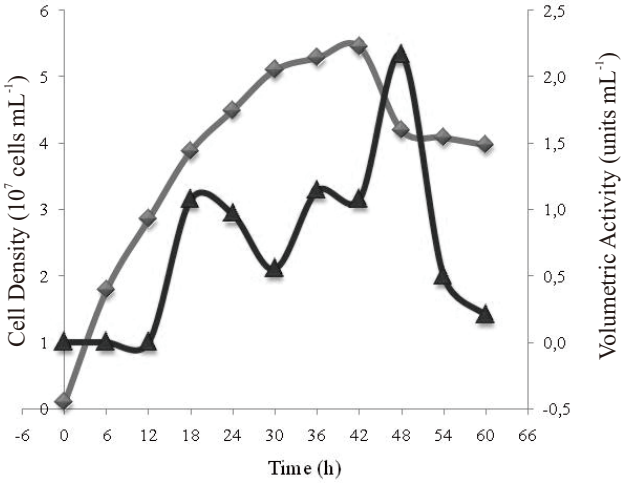

Fig 2 *Bacillus megaterium* cell density (◆) and PAc volumetric activity (▲) in optimum medium at 28°C, 1.0 vvm aeration and 150 rpm agitation for 60 hours cultivations.

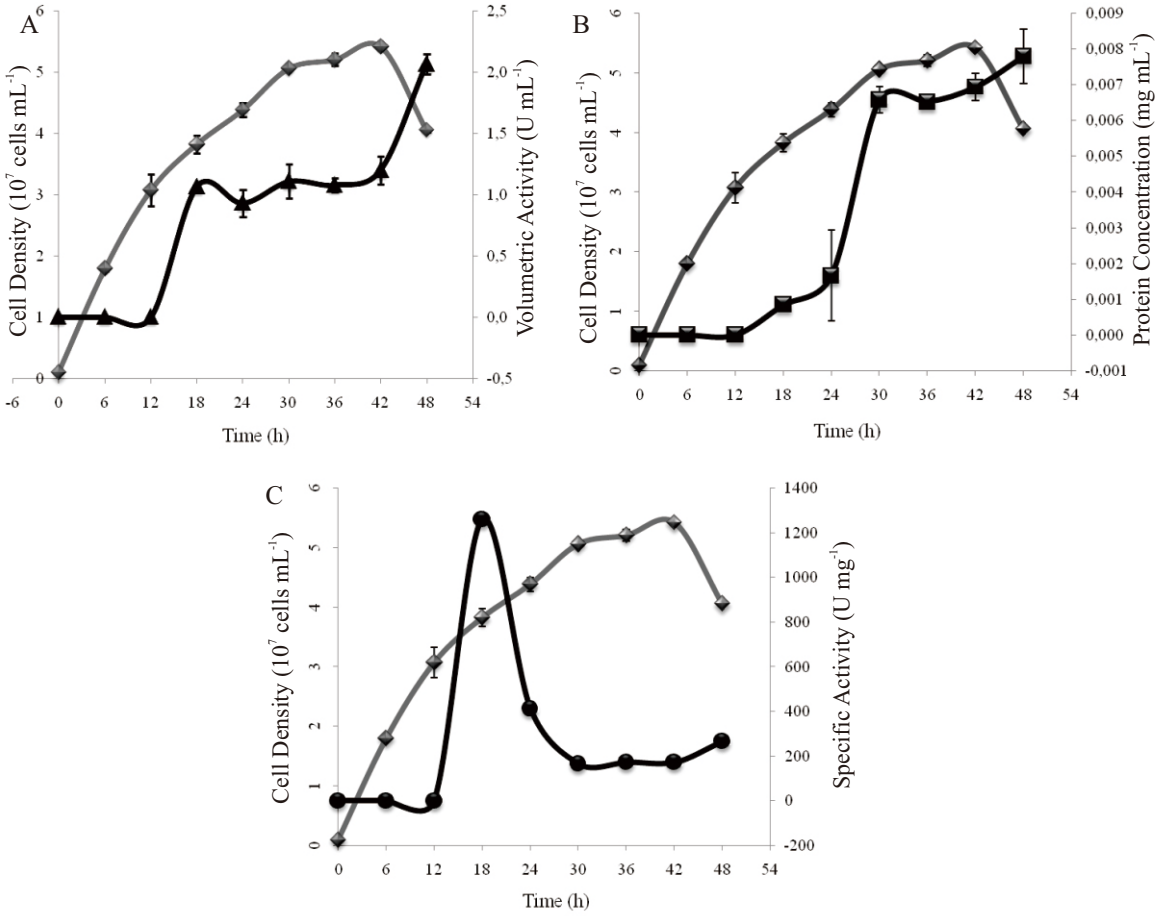

Fig 3 *Bacillus megaterium* cell density (◆) and PAc volumetric activity (▲) (A) protein concentration (■) (B) and PAc specific activity (●) (C) in optimum medium at 28°C, 1.0 vvm aeration and 150 rpm agitation. Each data point represents the average of three experiments, each experiment was performed in at duplicate/ triplicate.

when the protein concentration was 0.0078 ± 0.0008 mg mL^{-1}.

Fig 3C showed that the PAc specific activity was maximum during the exponential phase (18 h). The highest PAc specific activity achieved was 1260.521 ± 27.5711 units (mgprotein)$^{-1}$.

DISCUSSION

Design Experts Software version 9.0 (Stat Ease

inc. USA) was used to determine the optimum xylose and $CaCl_2$ concentrations. As clearly and thoroughly presented above, the results indicated that xylose and $CaCl_2$ concentrations affected the protein concentration and the activity of PAc enzyme synthesized by *B. megaterium* containing pMMbt*pac*BD1 plasmid that had the strong *xylA* promoter. Gene *btpac-bd1* was cloned so that its expression was directed by *xylA* promoter. So, when xylose was added to the medium, the promoter started trancription (MoBitec Gmbh 2012). The influence of calcium ions on the activity of *B. megaterium* PAc was also tested. Previous research showed that the calcium ions was a cofactor for membrane transport and important factor influencing the protein folding and maturation (Ignatova *et al.* 2005; Kasche *et al.* 2005). Ca^{2+} is a divalent cationic positive modulator, influencing the conformation of the catalytic enzymes, thus facilitating the enzyme-substrate interaction and enhancing the enzyme catalytic activity (Susanti 2003).

Although the PAc activity started to increase during the exponential phase (12 – 18h), the activity remained relatively constant between 18 – 42h (Fig 2, 3a). However, when bacteria entered the death phase, the PAc activity seemed to have increased rather significantly. Presumably, the enzyme that was previously accumulated in the cell was released via cell lysis into the cultivation medium directly, without secretory process.

This result might indicate that the protein was not secreted as expected. As the vector signal peptide gene had been removed, protein secretion had relied entirely on the host's secretion machinery. However, there is possibility that the host's secretion machinery does not suit that of the expressed protein, rendering the retention of the protein inside the cell, hence the stagnant activity during the exponential phase followed by the sudden increase of activity during the death phase Optimization of the medium composition by Yang *et al.* (2006) who used conventional methodology achieved volumetric activity of 1600 units L^{-1}. Our experiment using RSM achieved higher volumetric activity (2069 ± 82 units L^{-1}). However, this result was still lower than that of Acevedo and Cooney (1973), who achieved 3000 units L^{-1} and Panbangred *et al.* (2000), who achieved 9000 units L^{-1}. Differences in the results might be caused by the differences in the origin of the *pac* gene, the plasmid construct, the medium composition, the cultivation conditions and PAc assays.

In our study, the protein concentration was determined using crude extract enzyme without any purification step, so the measured protein concentration might not only contain PAc but also other impurities accumulating during cultivation. This might have been the main cause of the significant reduction of the PAc specific activity. When the cells started to lyse during death phase, releasing the intracellular proteins, the amount of impurities might increase significantly.

This study was performed using *B. megaterium* MS941, which had been engineered by eliminating the extracellular proteases. So, when the cells were intact, extracellularly expressed protein can be protected from protease degradation. However, when the bacteria entered the death phase, the bacterial cells lysed releasing numerous proteins that might include various intracellular proteases into cultivation medium, causing hydrolysis of PAc, further reducing the enzyme's specific activity.

Previous study by Leonita (2013), who used the same strain as the one used in our study but did the optimization using conventional methodology, reported that the PAc specific activity achieved was 1018.83 units mg^{-1}, 36 h after the start of the cultivation. However, Leonita (2013) used different medium with different composition so the result might not be comparable.

In conclusion, it is clear that the methodology used for optimization of medium compositions, particularly the concentration of xylose and $CaCl_2$, affect the PAc-like activity. As clearly and thoroughly presented above, optimum medium composition as defined by the RSM aided optimization increased the PAc-like activity produced. This is a better methodology for the optimization of PAc production from *B. megaterium* bt*pac*BD1 than the conventional methodology. However, further research must be performed to optimize the cultivation conditions, such as pH, agitation, aeration and temperature on a pilot scale in order to achieve a better PAc volumetric activity and specific activity.

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