

## Optimization and Scaling up of Penicillin Acylase Production Process by *Escherichia coli*

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**Abstract:** In the present study, the standard strain of *Escherichia coli* ATCC 11105 was used to develop an industrial process for penicillin G acylase (PAC) production. Among different media used, media composed of tryptone, yeast extract, sodium chloride and glucose yielded the highest volumetric PAC production of 27.5 U mL<sup>-1</sup> after 24 h. Medium optimization studies showed that glucose in concentration not exceeding 4 g L<sup>-1</sup> is necessary to support cell growth and volumetric PAC production. Whereas, study of the effect of yeast extract concentration in the production medium exhibits that addition of yeast extract in concentration of 5 g L<sup>-1</sup> was necessary also for cell growth and enzyme production. Higher concentrations of glucose or yeast extract decreased both cell growth and PAC production. On the other hand, medium osmolarity played also significant role as important as medium composition. The optimal medium osmolarity for PAC production was 360 mOsmol kg<sup>-1</sup>. The optimized medium developed in this work not only increased the volumetric enzyme production but also decreased PAC production time from 24 h to only 19 h in shake flask cultures. Further development in the production process was achieved upon transferring the production process to 15-L stirred tank bioreactor. The maximal volumetric PAC obtained in the bioreactor culture was 53.6 U mL<sup>-1</sup> after only 10 h cultivations. Moreover, not only the volumetric production increased but also the specific enzyme production [ $Y_{PXX}$ ] was higher by about 50% in bioreactor than those obtained in shake flask under the same cultivation conditions.

**Key words:** *Escherichia coli* · Medium optimization · Penicillin acylase · 6-APA

### INTRODUCTION

Penicillin G acylase (EC.3.5.1.11) is an enzyme commercially used for the hydrolysis of penicillin G and cephalosporin G to 6-amino penicillanic acid (6-APA) and 7-amino-3-deacetoxy-cephalosporanic acid (7-ADCA), which are precursors for the production of semisynthetic penicillins and cephalosporins. This enzyme is also able to catalyze other reactions including the hydrolysis of phenylacetyl derivatives of peptides, acylation of 6-APA, 7-ADCA, 7-ACA and mono lactamic acid [1]. Since the

first report on penicillin acylase (PAC) in 1950 by Sakaguchi and Murao [2], this enzyme is considered as one of the main catalysts in antibiotic industries. PAC is produced by wide range of microorganisms belong to mold, yeast, bacteria and actinomycetes [3-5]. However, bacterial strains belonging to *Escherichia coli*, *Bacillus sp.* and *Alcaligenes sp.* are the most potent enzyme producers [6-8]. Nowadays, the industrial production of PAC is carried out either by wild type or recombinant *E. coli* [9-12]. The catalytically active enzyme derived from *E. coli* is in form of  $\alpha$ ,  $\beta$  heterodimer localized in the

periplasmic space of cell. The native enzyme is formed by proteolytic processing of a pro-enzyme yielding two polypeptide chains with molecular weight 69 and 20.5 kDa. [13]. For PAC industrial production, it is usually important to enhance both of volumetric and specific production of the strain by manipulating the environmental conditions. As previously reported by many authors, optimization of medium composition and cultivation conditions are very essential for the successful industrial production of bacterial PAC [6,14-16]. In general, two key parameters are usually governing the expression of PAC in *E. coli* in the cellular level. First, control of cultivation temperature at 30°C is necessary for enzyme production. If cultivation is carried out at elevated temperature, the *pac* gene is transcribed and translated but the precursor polypeptide is incorrectly folded resulting in an enzyme inactivation [17]. Second, the production of PAC is inhibited by glucose when added to the cultivation medium in high concentration [18]. The glucose repression effect on *pac* gene in *E. coli* was confirmed at the transcriptional level [14]. The aim of the present work was to optimize the medium composition for PAC production for the production of this industrially important enzyme in small scale. Furthermore, the production of PAC in 15 L pilot scale bioreactor was carried out to evaluate the scalability of the production process under proper mixing and aeration conditions as those applied in large scale production.

## MATERIALS AND METHODS

**Micoorganism:** *Escherichia coli* (ATCC 11105), obtained from The American Type Culture Collection (ATCC, Manassas, VA, USA) was used throughout this study. This strain was delivered as lyophilized culture and was firstly activated in nutrient agar medium. Cells were cultivated in an incubator for 48 h at 30°C to activate the cells. The grown colonies were harvested in 50% glycerol solution ( $w v^{-1}$ ) for cell banking at -80°C. The viability of the deep-frozen culture was periodically controlled by plating the cells on nutrient agar medium to ensure cell viability. For experimental work, one deep frozen vial was activated on nutrient broth for 24 h and used as inoculum for each experiment to minimize the variability of result based on the inoculum quality.

**Inoculum Preparation:** Inoculum was prepared in LB broth composed of ( $g L^{-1}$ ): Tryptone, 10; Yeast extract, 5 and NaCl, 10. After sterilization for 15 min. at 121°C, 50 ml LB broth was inoculated with 200  $\mu$ L of glycerol culture.

The inoculated flasks were cultivated for 24 h at 30°C on incubator shaker at 200 rpm. The obtained vegetative cells were used to inoculate either shake flask or bioreactor with concentration of 5% ( $v v^{-1}$ ).

**PAC Production Media:** For the first experiment of media screening, five different media were used. The compositions of these media were as follows ( $g L^{-1}$ ): Medium (1), Peptone 5, meat extract 3. Medium (2), Casein 4; yeast extract 8;  $K_2HPO_4$  4.2;  $KH_2PO_4$  3. Medium (3), Tryptone 10; yeast extract 5; NaCl 10. Medium (4), Tryptone 10; yeast extract 5; NaCl 10; glucose 0.3. Medium (5), Tryptone 10; yeast extract 7.5; NaCl 10; glucose 0.3. The pH values of all media were adjusted to 7.5 before sterilization.

**Shake flask and Bioreactor Cultivations:** The flask cultivations were performed in 250-ml Erlenmeyer flask with working volume of 50 ml. The inoculated flasks were incubated at 200 rpm and 30°C on rotary shaker (Innova 4230, New Brunswick Scientific, NJ, USA) with shaking eccentricity of 20 mm. The bioreactor cultivations were carried out in 15-L stirred tank bioreactor (Biostat-C, Sartorius BBI Systems, Melsungen, Germany) with a working volume of 9 L. The stirrer was equipped with two 6-bladed rushton turbine impellers ( $d_{(impeller\ diameter)} = 85\text{ mm}$ ;  $d_{(tank\ diameter)} = 214\text{ mm}$ ,  $d_i d_t^{-1} = 0.397$ ). Unless other wise mentioned, the agitation speed was 200 rpm. During cell cultivation, aeration was performed using filtered sterile air and supplied continuously to the bioreactor with rate of  $1\ v v^{-1}\ min^{-1}$ . Air flow was adjusted and controlled using mass flow controller (F102D, Bronkhorst High-Tech B.V., Nijverheidstraat, The Netherlands) coupled with the control console CDU of the bioreactor. Foam was suppressed by the addition of the antifoam agent Struktol (Schill+Seilacher Gruppe, Hamburg, Germany). During the cultivation process, pH and dissolved oxygen in culture were determined using pH and DO polarographic electrodes, respectively (Ingold, Mittler-Toledo, Switzerland).

**Sample Preparation and Determination of Cell Dry Weight:** Samples, in form of 2 flasks containing 50 ml each, or 30 ml of broth in case of bioreactor, were taken at different times during the cultivation in centrifugation tubes (Falcon, USA). Immediately after sampling, the optical density was determined at  $OD_{600}$ . The cell dry weight (CDW) was measured by centrifuging 1 ml of broth at 12000 rpm for 5 min and then the precipitate was washed twice with distilled water. The cells were dried in

oven at 110°C for constant weight. The obtained OD<sub>600</sub> value of the cells was correlated with the cell dry weight via the calibration curve. The sample was then centrifuged at 5,000 rpm for 20 min. and the supernatant was frozen at -20°C for enzyme determination.

**Determination of Pencillin Acylase (PAC):** The penicillin G acylase in culture was determined using colorimetric method proposed by Kornfeld [19]. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmole of 6 APA per min.

### RESULTS AND DISCUSSION

**Production of PAC in Different Cultivation Media:** Based on literature survey, cell growth and PAC production was studied using five different cultivation media formulations as in the materials and methods part. As shown in Fig. 1, all media supported cell growth and PAC production in

different extents. After 24 h cultivation, cell growth in all media was more than 2 g L<sup>-1</sup>. The maximal cell mass of about 3.4 g L<sup>-1</sup> was obtained in medium II which composed of casein, yeast extract and potassium monhydrogen and potassium dihydrogen phosphate. The superiority of growth in this medium was due to the presence of rich phosphate source compared to other medium. On the other hand, the maximal PAC production of 27.5 U mL<sup>-1</sup> was obtained in medium number IV which composed of tryptone, yeast extract, sodium chloride and glucose. The less enzyme production, in spite of high cell growth in medium II, may be due to the presence of casein which is less suitable substrate for PAC production due to its protein nature [15,20]. For better understanding of the cell efficiency for PAC production, the yield coefficient [ $Y_{PAC}$ ], which represents the unit of enzyme produced by unit cell mass, was calculated. As shown in Fig. 1A, the maximal cell productivity of about 10.68 U mg<sup>-1</sup> was obtained also in medium No. IV.

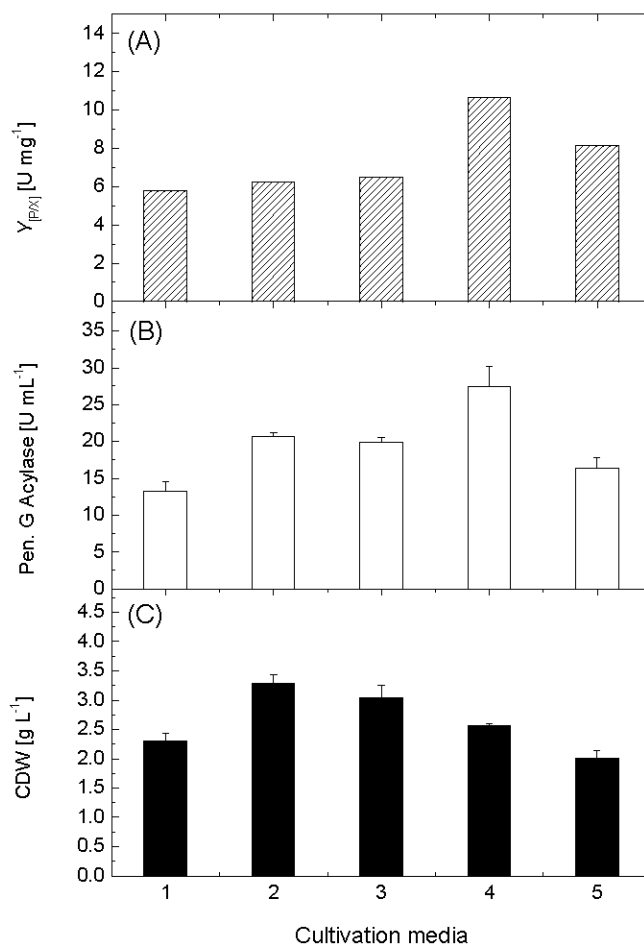


Fig. 1: Effect of different media on the cell growth and PAC production by *E. coli*.

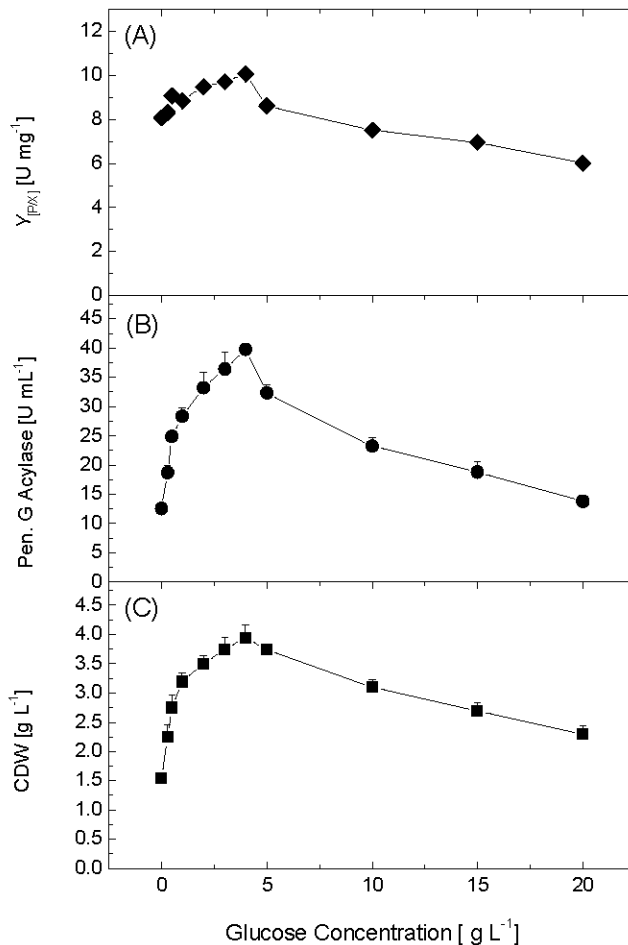


Fig. 2: Effect of the Initial Glucose Concentration (IGC) on the cell growth and PAC production by *E. coli*.

The superiority of this medium formula over other media may be due to the presence of glucose in medium. Since media III and media IV share the same components with exception of glucose which was completely absence in medium III. However, on using the same medium formulation with addition of more yeast extract (medium No. V), both of cell growth and PAC production reduced, but the cell productivity, calculated as  $Y_{PX}$  was less than medium IV but still higher than other media. Therefore, the medium number IV which composed of tryptone, yeast extract, sodium chloride and glucose was used for further medium optimization in the following experiments.

**Effect of Initial Glucose Concentrations (IGC) on the Production of PAC:** The aim of this experiment was to further improve of PAC production through studying the effect of different glucose concentrations on the enzyme production. Therefore, different glucose concentrations up to 20 g L<sup>-1</sup> were applied to investigate its effect on cell

growth and PAC production. Figure 2 shows the data of cell growth and PAC production after 24 h cultivations. As shown, both of cell growth and PAC production were increased proportionally by increasing glucose concentration in medium from 0 up to 4 g L<sup>-1</sup>. Further increase beyond this concentration resulted in significant reduction in both cell growth and enzyme production. The maximal cell growth and enzyme production in 4 g L<sup>-1</sup> glucose supplemented culture were about 3.95 g L<sup>-1</sup> and 39.8 U mL<sup>-1</sup>, respectively. This value of enzyme produced was more than three folds higher than those obtained in culture without glucose. The maximal enzyme specific production [ $Y_{PX}$ ] of 10.1 U mg<sup>-1</sup> was obtained also in 4 g L<sup>-1</sup> glucose supplemented culture (Fig. 2A). The reduction in both enzyme volumetric and specific production with the increase of glucose concentration beyond 4 g L<sup>-1</sup> may be attribute to the catabolic repression effect on PAC production by *E. coli* as reported by other authors [10].

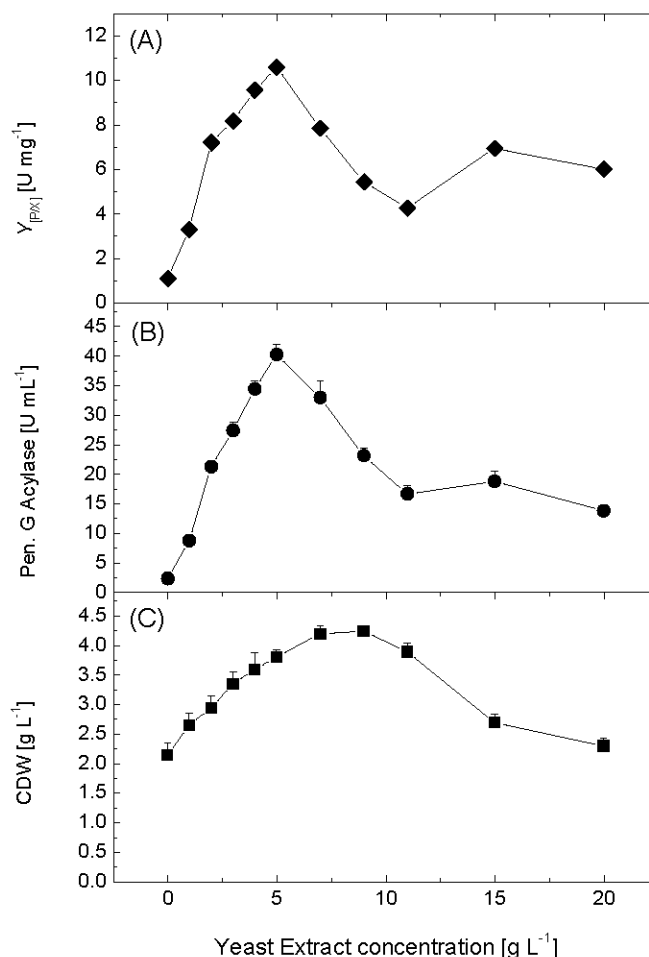


Fig. 3: Effect of the Initial Yeast Extract Concentration (IYEC) on the cell growth and PAC production by *E. coli*.

However, the repression effect of glucose on the expression of the cloned *pac* gene from *E. coli* ATCC 11105 is highly dependent on the identity of host strain. High repression effect of glucose is significant when this gene expressed homogeneously in ATCC 11105, whereas this repression effect can be overcome by heterologous expression in other *E. coli* strains [21]. Thus, it can be concluded that the glucose repression effect on the *pac* gene from *E. coli* ATCC 11105 results mostly from the classical catabolite repression mechanism. The present results are also in agreement with those obtained by other research group who found that the initial glucose concentration of only 5 g L<sup>-1</sup> is sufficient to get optimal expression of *pac* gene [21]. However, it is also worthy to note that the glucose repression effect on the penicillin acylase production has been reported not only in *E. coli* but also in many other producer strains such as *B. megaterium* [16] and *Streptomyces lavendulae* [5].

**Effect of Initial Yeast Extract Concentration (IYEC) on PAC Production:** The influence of the IYEC, as one of the key nutrients for the cell growth and PAC production, was studied. As shown in Fig. 3, the addition of yeast extract to the cultivation medium shows strong influence on both cell growth and PAC production. The cell growth increased with the increase of IYEC in the medium up to 9 g L<sup>-1</sup>. Further increase in yeast extract resulted in significant reduction in cell growth (Fig. 3A). On the other hand the maximal PAC production of 40 U mL<sup>-1</sup> was obtained in culture of 5 g L<sup>-1</sup> yeast extract. This amount of enzyme produced was about 16 folds of those value obtained in medium without yeast extract. Further increase in the IYEC resulted in a significant reduction in enzyme production. However, the maximal specific enzyme production of 10.6 U mg<sup>-1</sup> was also obtained in 5 g L<sup>-1</sup> YE culture. As shown, IYEC plays significant role in the PAC production process.

Based on its chemical composition, yeast extract is not only considered as normal organic nitrogen source but also it is an excellent source for many nutrients. It is rich with amino acids, vitamins and many low molecular weight growth factors. Therefore, yeast extract is widely used in medium formulation for the production of cell mass and the induction of different primary and secondary metabolites [22-24]. For *E. coli* cultivation, yeast extract is essential medium constituent to promote cell growth and recombinant protein production in batch and fed-batch cultures [25]. However, beside the high nutritional value of yeast extract to support cell growth and enzyme production, it also reduces the secretion of acetic acid during cell growth phase of *E. coli* and promote the utilization of acetic acid during carbon limitation [26,27]. The recent study of Pinotti *et al.* [8] showed also that the production of PAC is highly dependent on amino acid consumption. The production of PAC was increased by about 150% upon the medium supplementation with balanced amino acids solution with preferred consumption of alanine, aspartic acid, glycine, serine, arginine, threonine, lysine and glutamic acid. These all amino acids are present in yeast extract in relatively high concentrations. Moreover, the catalytic site of PAC located in the  $\beta$ -subunit, contains serine residue which is essential for enzymatic activity [28]. It has been also observed that when the medium is supplemented with high concentration of amino acids beyond certain limit, the production of PAC was decreased accordingly [8]. This supports also our results for the decrease of PAC production with the excessive addition of YE to the cultivation medium. On the other hand, the decrease of enzyme activity by the increase of the IYEC beyond 5 g L<sup>-1</sup> may be attributed to the effect of some accumulated low molecular weight enzyme inhibitors in the yeast extract when applied in high concentrations as observed by other authors [29].

**Media Osmotic Pressure and PAC Production:** The aim of the present experiment was to study the influence of osmotic stress (both of hypo- and hyperosmotic stress) on the cell growth and PAC production by *E. coli*. The osmotic pressure was adjusted by the addition of different concentrations of sodium chloride to the cultivation medium during preparation. Different media were prepared with wide range of osmotic pressure ranged from 62 upto 1910 mOsmol Kg<sup>-1</sup>. However, it was not possible to prepare a media of osmotic pressure below 62 mOsmol kg<sup>-1</sup> since it was composed of the rest of medium components without sodium chloride.

As shown in Fig. 4, the increase of medium osmotic pressure from 62 upto about 360 mOsmol kg<sup>-1</sup> increased both of cell growth and enzyme production significantly. The maximal cell growth of 3.7 g L<sup>-1</sup> concomitant with the highest PAC volumetric production of about 40 U mL<sup>-1</sup> was obtained in medium of 360 mOsmol kg<sup>-1</sup>. Further increase in medium osmolarity resulted in a significant reduction in both cell growth and PAC production. On the other hand, the maximal specific enzyme production of 11.4 U mg<sup>-1</sup> was obtained in medium of 207 mOsmol Kg<sup>-1</sup>. In all media of different osmotic pressures ranged from 62 upto 1310 mOsmol kg<sup>-1</sup>, the specific PAC production was between 8.3 to 11.4 U mg<sup>-1</sup>. This indicates that the high fluctuation in the volumetric enzyme production curve (Fig. 4B) in media within this range was mainly due to the differences in cell growth rather than cell productivity. However, further increase in medium osmolarity beyond 1310 mOsmol kg<sup>-1</sup> decreased also the specific PAC production significantly. In general, the role of abiotic stress such as osmotic stress on the cell growth and different gene expression is very critical in almost all types of living cells as recently reviewed by Sharfstein and his coworkers [30]. The increased PAC production by the increase of osmotic pressure from 62 to 360 mOsmol kg<sup>-1</sup> may be attributed to better cell growth as well as the fact that the increase in osmotic pressure increases the enzyme excretion from the periplasmic membrane to the cultivation medium. This excretion process for PAC may be improved by osmotic chock [31]. Higher osmotic stress inhibits the cell growth and PAC production accordingly. Thus, cell cultivation under optimum osmotic pressure of 360 mOsmol kg<sup>-1</sup> is necessary to increase both of cell growth, enzyme production and excretion to the cultivation medium.

**Time Course of Cell Growth and Enzyme Production with Optimized and Un-optimized Media in Shake Flask Cultures:** Two parallel experiments were conducted to study the kinetics of cell growth and PAC production before and after optimization of medium composition in shake flask cultures. As shown in Fig. 5, cells grew exponentially with different rates in both cultures. The rate of cell growth in the optimized medium was about 0.25 g L<sup>-1</sup> h<sup>-1</sup>, this value was almost double of those obtained in medium before optimization. However, not only the growth rate was higher but also significant reduction in exponential growth phase from 24 to only 19 h was observed when cells grew in optimized medium. The maximal cell mass obtained after medium optimization was about 4.25 g L<sup>-1</sup>, this value was higher by about 52%

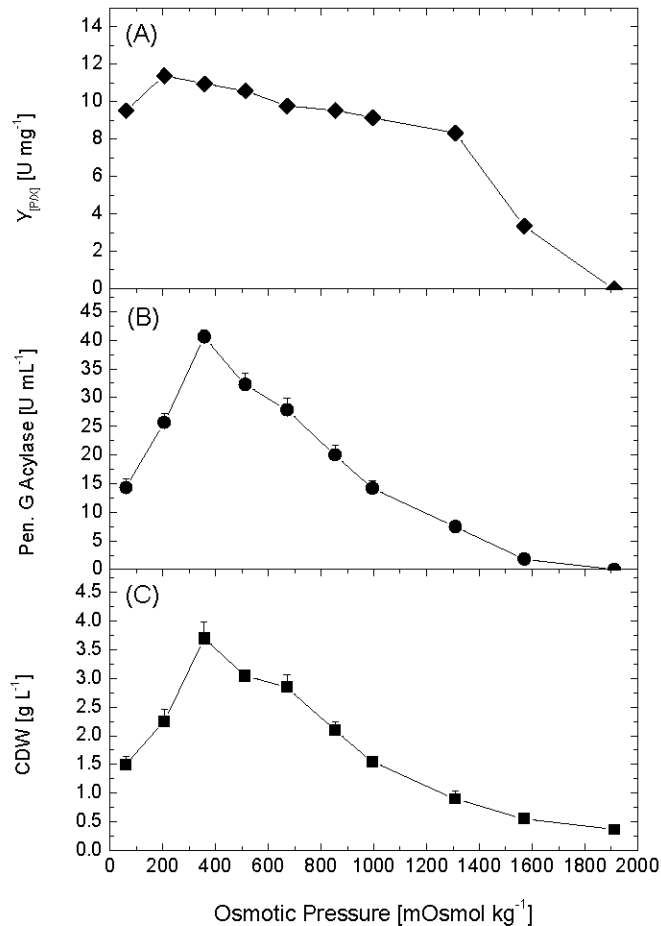


Fig. 4: Effect of medium osmotic pressure on the cell growth and PAC production by *E. coli*

than those obtained in medium before optimization. On the other hand, the new medium formula increased the volumetric PAC production by 42% reaching maximal volumetric enzyme production of 40 U mL<sup>-1</sup> after 19 h. The specific PAC production [ $Y_{PXX}$ ], reached its maximal value of about 10.2 U mg<sup>-1</sup> after only 13 hours and kept more or less constant during the rest of exponential growth phase and decreased slightly thereafter. However, in case of initial medium before optimization, the maximal specific PAC production was almost the same as in optimized medium after 21 h.

The pH profiles of both cultures were almost the same. The pH dropped during the growth phase from 7.5 to about 4.5. The decrease of pH is due to the acid production during the production phase. The medium pH kept more or less constant as the cell entered the stationary phase and increased gradually thereafter. The increase of pH may be due to the production of ammonia in culture as a result of cell lysis during the decline phase.

The pH reached about 5.2 at the end of cultivation in both cultures. Thus we can conclude that the new medium formula improved the PAC production process in two ways. First, it increased the volumetric PAC by about 42%. This increase was mainly through the increase of cell growth rather than the increase in cell productivity. Second, it reduced the production time from 24 h to only 19 h.

**Semi-Industrial Production of PA Using in Situ Sterilizable 15 L Stirred Tank Bioreactor:**

After medium optimization in small scale, production of PAC was scaled up to 15-L *in situ* sterilizable stirred tank bioreactor. The use of this type of bioreactor was important since it sterilizes the medium under the same conditions as those applied in large scale bioreactors in regard to the heating and cooling time. During sterilization, agitation was set at 50 rpm to improve the sterilization efficiency and heat transfer. Heating was performed by indirect steam

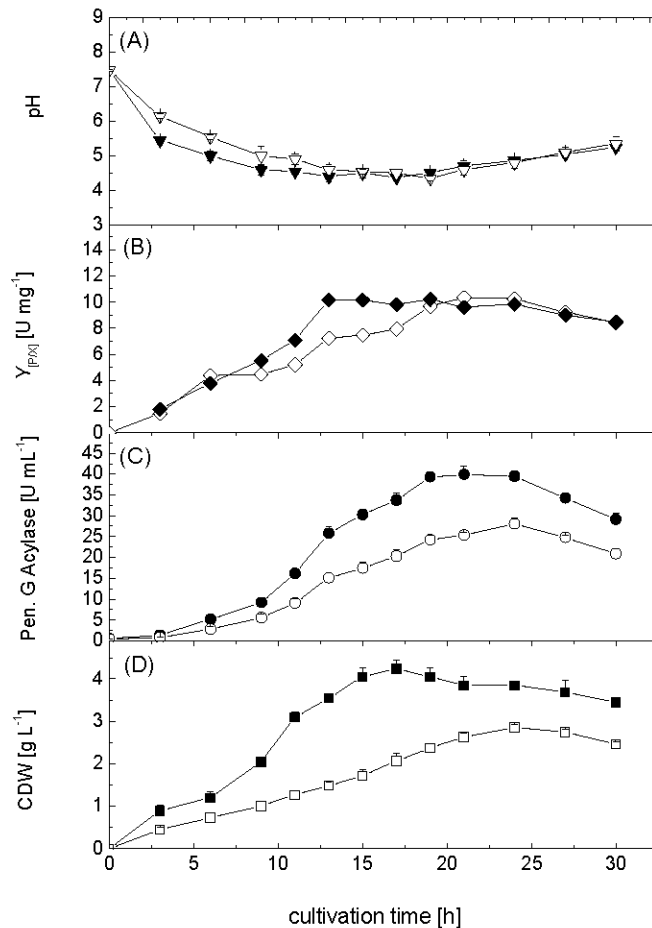


Fig. 5: Kinetics of cell growth and PAC production in shake flasks cultures. Closed and opened symbols represent the results of optimized and unoptimized medium, respectively

injection to the double jacket of the bioreactor. After 30 min. sterilization cycle, the bioreactor was cooled rapidly by chilled water of 5°C. This rapid heating and cooling process protects the medium ingredient and decreases the degradation of heat sensitive components during sterilization.

The medium composition and cultivation parameters were almost the same of those in the optimized shake flask cultures. Fig. 6 shows the kinetics of cell growth, PAC production and the changes in pH and DO during cell cultivations in the pilot scale bioreactor. Cells grew exponentially with high rate of about 0.9 g L<sup>-1</sup> h<sup>-1</sup> reaching the maximal cell growth of about 3.6 g L<sup>-1</sup> after only 4 hours. The cell density in culture was kept more or less constant thereafter. During the growth phase, both of pH and DO curves dropped rapidly. The pH value reached about 6 after 4 hours and kept more or less constant for the rest of cultivation time, whereas, the DO reached its

lowest value of about 14% after 5 hours and increased gradually as the cell entered the stationary phase.

On the other hand, the PAC production increased gradually in the culture with rate of 5.37 U mL<sup>-1</sup> h<sup>-1</sup> and reached its maximal value of 53.6 U mL<sup>-1</sup> after 10 hours. After the enzyme production phase, PAC concentrations decreased rapidly in culture with rate of 3.64 U mL<sup>-1</sup> h<sup>-1</sup>. This degradation rate was almost double of those obtained in the corresponding shake flask cultures under the same cultivation conditions. The higher enzyme degradation rate in bioreactor culture was due to the increased aeration and agitation which reflect negatively on enzyme activity and account for enzyme degradation as well.

In general, Table 1 summarizes the differences in the kinetic parameters between shake flask and bioreactor cultures. As shown, medium optimization increased the cell mass and specific growth rate as well, whereas



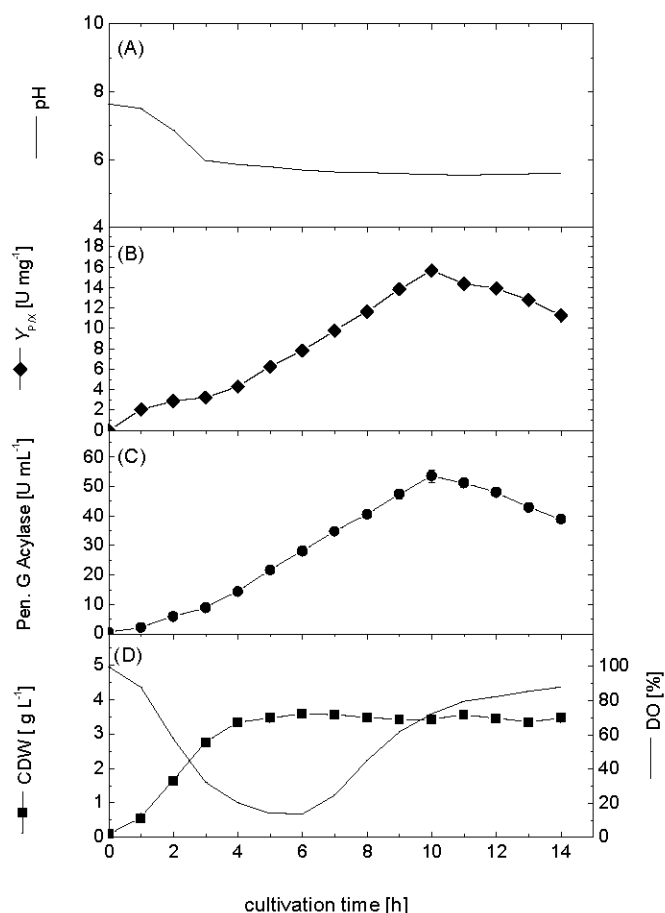


Fig. 6: Kinetics of cell growth and PAC production by *E. coli* in 15-L stirred tank bioreactor using optimized medium

Table 1: Comparison between the kinetics of cell growth and PAC production in different production scales

Parameters	Shake Flask		Bioreactor
	Non-optimized medium	Optimized medium	Optimized medium
Growth Parameters			
$X_{max}$ [g L <sup>-1</sup> ]	2.800	4.250	3.600
$dx/dt$ [g L <sup>-1</sup> h <sup>-1</sup> ]	0.120	0.250	0.900
$\mu$ [h <sup>-1</sup> ]	0.083	0.119	0.500
Production Parameters			
$P_{max}$ [U mL <sup>-1</sup> ]	28.150	39.900	53.650
$Q_p$ [U mL <sup>-1</sup> h <sup>-1</sup> ]	1.250	2.070	5.370
$-Q_p$ [U mL <sup>-1</sup> h <sup>-1</sup> ]	1.200	1.730	3.640
$Y_{PDX}$ [U mg <sup>-1</sup> ]	10.230	10.360	15.660

**Abbreviations:**  $X_{max}$ : maximal cell dry weight;  $dx/dt$ : growth rate;  $\mu$ : specific growth rate;  $P_{max}$ : maximal enzyme production;  $Q_p$ : enzyme production rate;  $-Q_p$ : enzyme degradation rate;  $Y_{PDX}$ : enzyme yield coefficient [U] enzyme produced / [g] cell dry weight

the specific enzyme production in both cultures was almost the same. Thus the significant increase in volumetric enzyme production from 28.15 to 39.90 U mL<sup>-1</sup> after medium optimization was mainly due the improvement of cell growth. On the other hand, the cell growth in bioreactor culture was characterized by higher specific growth rate than those obtained in shake flask by about 4.5 folds. However, the obtained cell mass in bioreactor cultures was less due to the termination of growth after only 4 hours. The higher growth rate in bioreactor compared to shake flask was due to the better mixing and enhanced oxygen transfer coefficient in the bioreactor culture which is usually reflected positively on cell growth and enzyme productivity especially for aerobic microorganisms like *E. coli*. [32,33]. Although the cell mass in bioreactor culture was less than in shake flask one, both of volumetric and specific enzyme production were higher. This indicates that the improved cultivation conditions in term of better mixing and oxygen transfer in culture, were reflected positively on the production process.

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