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Optimization of Penicillin G Acylase Production

A. Kheirolomoom*, A. Kazemi-Vaysari[1], M. Ardjmand[2] and H. Doosty[3]

In this paper, effects of pH, carbon and nitrogen sources, inducer, penicillin G, shaking and aeration rate on penicillin G acylase (PGA, EC 3.5.1.11) synthesis by *Escherichia coli* were studied and the optimum conditions for batch fermentation process were determined. The maximum activity of the enzyme and the biomass concentration were obtained at pH range of 8.0-9.0, with 250 rpm using 5 $g.l^{-1}$ yeast extract as both carbon and nitrogen sources and 4 $g.l^{-1}$ of phenyl acetic acid as the inducer. Addition of 0.2 $g.l^{-1}$ penicillin G to the fermentation broth enhanced the enzyme production. The optimum aeration rate in the fermenter was obtained to be 0.85 vvm. The growth kinetics of PGA production was analyzed using a second-order model applied to Leudeking-Piret kinetics. The analysis of Leudeking-Piret model demonstrates that PGA production by *Escherichia coli* obeys the growth-associated kinetics.

INTRODUCTION

Penicillin G acylase (PGA, EC 3.5.1.11) is widely used in industrial processes for production of semi-synthetic penicillins, 6-aminopenicillanic acid (6-APA) and 7-amino-3-deacetoxy cephalosporanic acid (7-ADCA). Semi-synthetic penicillins are produced at acidic or neutral pH values (4.0-7.0) while 6-APA and 7-ADCA are produced at alkaline pH values (7.5-8.5). Various methods using yeast, bacterias and moulds can be utilized to produce PGA; however, PGA production using *Escherichia coli* has been considered by many researchers [1-4]. Use of recombinant *Escherichia coli* eliminates different problems encountered in PGA production, such as catabolic repression of some carbon sources like sugars, and enhances productivity of high producing mutant of *Escherichia coli* [5-10]. In most industrial processes, partially purified PGA is used in the free or immobilized form of the whole cell or enzyme to produce semi-synthetic penicillins. The immobilized form is used in industrial production of 6-APA and semi-synthetic penicillins for more than hundred batches without adding fresh enzyme [2,3]. Different techniques were applied to increase PGA production in *Escherichia coli*. The main technique considered is attaching the structural gene to a strong inducible promotor andusing the cloned DNA to produce an improved strain as well as cloning the PGA gene downstream from a strong promoter and inducing it with IPTG [11-14]. Various genetic engineering techniques have yielded a 2-150 fold increase in PGA production as reviewed and reported by Shewale and Sivaraman [1]. However, in addition to the advantages of PGA production by genetic engineering techniques, the quantitative analysis of the kinetics of PGA production is necessary to achieve the optimum conditions for PGA production by fermentation operations. In the present article, the effects of pH, shaking, protein and carbon sources, inducer and penicillin G as well as aeration rate on PGA synthesis by *Escherichia coli* are studied and the optimum conditions for the fermentation operation are determined. The growth kinetics is analyzed and a second-order model is proposed and used to verify Leudeking-Piret kinetics for PGA synthesis by *Escherichia coli*.

*. *Corresponding Author, Department of Chemical Engineering, Sharif University of Technology, Tehran, I.R. Iran.*

1. *Biochemical and Bioenvironmental Engineering Research Center, Sharif University of Technology, Tehran, I.R. Iran.*
2. *Science and Research Campus, Department of Chemical Engineering, Islamic Azad University, Tehran, I.R. Iran.*
3. *Department of Chemical Engineering, Sharif University of Technology, Tehran, I.R. Iran.*

MATERIALS AND METHODS

Chemicals

6-APA were purchased from Nacalai Tesque, Inc. (Kyoto, Japan) and penicillin G was provided by Jaber Ibn-e-Hayan pharmaceutical Co. (Tehran, Iran). All reagents were of analytical or microbiological grade. Analytical and enzyme separation and purification

chemicals were obtained from Merck AG (Darmstadt, Germany) and Sigma (St. Louis, Missouri, USA).

Microorganism

The parent strain of *Escherichia coli* was provided by the Biotechnology Laboratory of the Pharmaceutical Faculty of Tehran University (Tehran, Iran). The treated strain called *E. coli* 39 demonstrated a good productivity of penicillin G acylase, β-lactamase free behavior as well as resistance to catabolic repression of carbon sources. *E. coli* 39 was seeded on nutrient agar and incubated at 25°C.

Culture Conditions and Media

Batch cultures of *E. coli* 39 were prepared in 0.100 l working volume of 0.500 l shake flasks. Shaking was carried out at 250 rpm, 25°C and pH 8.0 for 46 h. The medium composition is as follow in (w/v)%: K_2HPO_4 0.1%, KH_2PO_4 0.01% and $MgSO_4.7H_2O$ 0.02%. NaOH solution (1 M) was used for pH adjustment. In addition to the shake flasks, batch fermentation was performed in Chemap fermenter, model f0020 (Switzerland). The working volume was 6.0 l. The fermenter was equipped with pH and temperature controller and dissolved oxygen monitor. The fermenter operated at 25°C and pH 8.0 and was sparged with air at a rate of 0.85 vvm. The media temperature and pH were controlled automatically. A 10% w/v silicone solution was used to control foam.

Enzyme Isolation and Purification

Cell wall disruption was carried out to obtain penicillin G acylase. The enzyme was purified at first by a precipitation method using $(NH_4)_2SO_4$ salt and further purification by ion exchange chromatography using DEAE-cellulose column.

Enzyme Activity

The method of the p-dimethyl-aminobenzaldehyde (PDBA) was used in optimization study of penicillin G acylase production process as described in the literature [15]. One unit of enzyme activity, U, corresponds to the amount of enzyme which cleaves 1 μmol of penicillin G in 10 mM potassium phosphate buffer to 6-APA per minute at pH of 8.0 and 37°C.

Determination of Protein Content

Protein content was determined by the method of Lowry using folin phenol reagent, according to Lowry et al. [16].

RESULTS AND DISCUSSION

Characterization of PGA Activity Measurement

The effect of time on activity measurement of PGA was studied by measuring the activity of PGA at different intervals after adding penicillin G to the enzymatic solution of PGA. The results show that the activity of PGA changes linearly after addition of the substrate, i.e. penicillin G to PGA solution for the first 60 min period. Therefore, the optimum time in the activity measurements of PGA was considered as 30 min after addition of the substrate to the enzymatic solution to prevent the contamination and loss in activity of the enzyme. The effect of concentration of enzymatic solution on activity measurement was studied using PGA solutions with the following dilution ratios: 1, 1:2, 1:5, 1:10, 1:25 and 1:50, respectively. The results illustrate that the enzyme activity changes linearly for dilution ratios up to 1:2 and further increase in the ratio results in nonlinear behavior of PGA activity which is due to increase in concentration of inhibitors existing in the enzymatic solution of PGA. To decrease such an inhibition effect, the dilution ratio of 1:25 of PGA solution was used in activity measurements. Consequently, all the activity measurements of PGA were carried out at optimum conditions, i.e. dilution ratio of 1:25, pH of 8.0, 37°C and 30 min after addition of the substrate to the enzymatic solution.

Effect of Media pH and Shaking Rate (rpm)

Culture media pH values were adjusted using different buffers. For pH values between 5.0 to 8.0, phosphate buffer and pH values between 8.5 to 10.5, glycine-NaOH buffer were used. The biomass concentration and relative activity of the enzymatic solutions as a function of media pH are shown in Figure 1. For pH

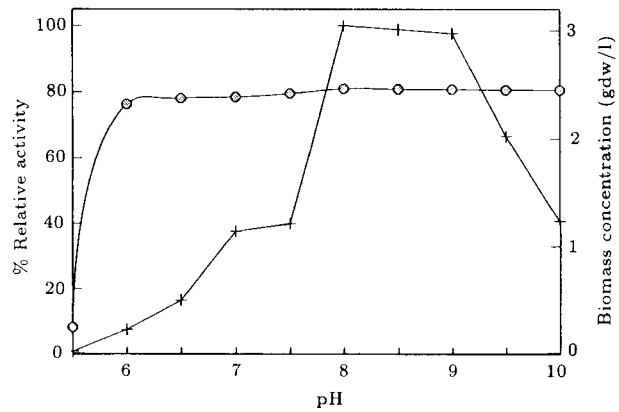

Figure 1. The relative activity of penicillin G acylase in % (+) and the biomass concentration in gdw.l^{-1} (•) as a function of pH.

values between 6.0 to 9.5, the biomass concentration slightly changes and no considerable amount of biomass can be found for pH values less than 6.0. The maximum production of PGA occurs at pH of 8.0. Strict care has to be taken to keep the media pH equal to or more than 8.0, since for pH values less than 8.0, a sharp decrease in the activity of PGA is observed (Figure 1).

The biomass concentration and enzyme activity for different time intervals of fermentation process are shown in Figure 2. The maximum growth rate appears after 15-20 h of fermentation process, when *Escherichia coli* growth at log-phase occurs (Figure 2). The enzyme activity and biomass concentration for different rpm values are measured and shown in Figures 3 and 4, respectively. The experimental results demonstrate that the maximum activity of PGA as well as biomass concentration were obtained at 250 rpm. Increasing rpm values results in increase of shear force and, hence, decrease in activity and biomass concentration.

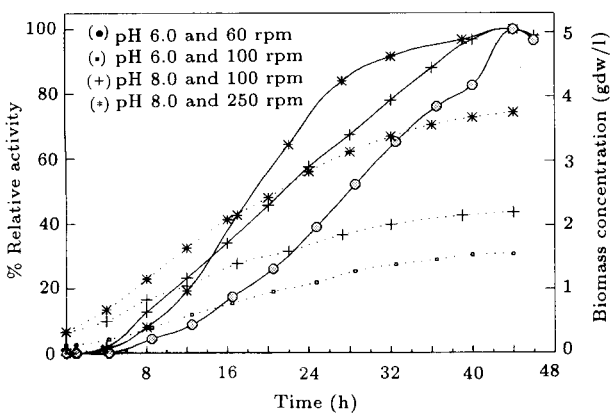

Figure 2. The relative activity of penicillin G acylase in % (— curves) and the biomass concentration in gdw.l^{-1} (.... curves) as a function of the cultivation time (h).

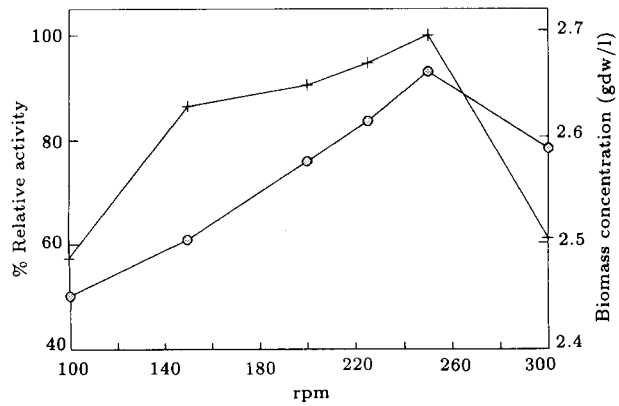

Figure 3. The relative activity of penicillin G acylase in % (+) and the biomass concentration in gdw.l^{-1} (•) as a function of shaking speed in rpm.

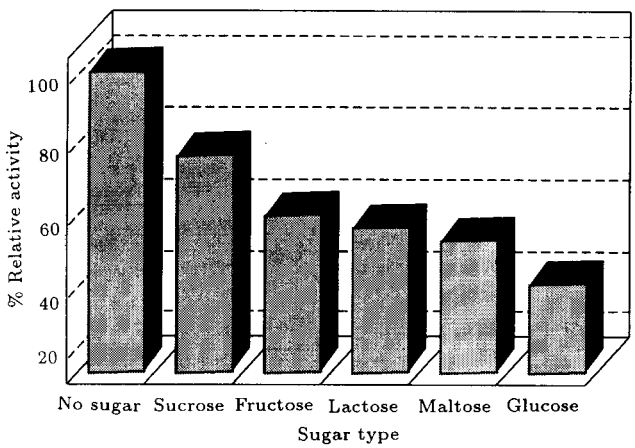

Figure 4. The effect of sugar source on the relative activity of penicillin G acylase (%).

Effect of Culture Media Composition

The synthesis of PGA in *Escherichia coli* is induced by phenylacetic acid (PAA) and its salts. However, it is catabollicaly repressed by glucose, fructose, lactose and other carbon sources. Although, PAA is an inducer for PGA production, it is also a growth inhibitor at concentrations more than a defined optimum value [17,18]. Due to the catabolic repression effect, special carbon sources have to be used in PGA production. The original culture media introduced in the materials and methods section of the present article contains no sugar. To determine the effect of different sugars, glucose, fructose, saccarose, lactose and maltose solutions (2 g.l^{-1}) were sterilized and added to the original culture media, respectively. After 40 h the enzyme activity was measured, the results of which are shown in Figure 4. The maximum enzyme activity was observed when Yeast Extract (YE) was used as carbon source and no sugar was added to the media (Figure 4). When a special sugar was used as a carbon source for PGA production by *E. coli* 39, the maximum and minimum activities were obtained for sucrose and glucose, respectively. However, a special genetically engineered species of *Escherichia coli* may demonstrate alternate behavior.

Corn Steep Liqour (CSL), YE and peptone were used to study the effect of nitrogen source on PGA activity. The experimental results show that for the fermentation broths containing 5 g.l^{-1} of CSL, YE and pepton, respectively, the maximum activity appears when YE was used as the carbon source (Figure 5). This is due to the low sugar content of YE which catabollicaly represses the synthesis of PGA by *Escherichia coli*. The effect of YE concentration on PGA activity was shown in Figure 6. The maximum activity of PGA was obtained when 5 g.l^{-1} of YE was used as the source of both carbon and nitrogen.

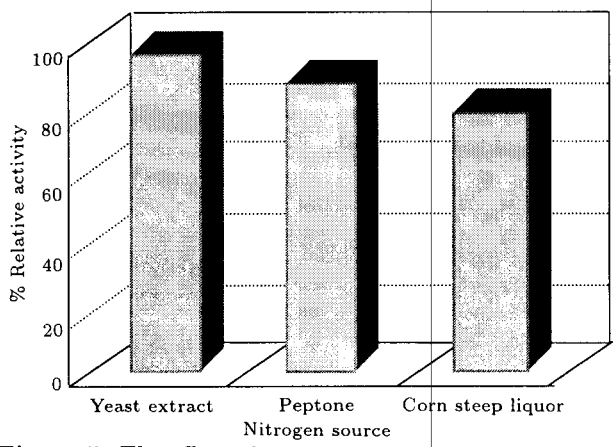

Figure 5. The effect of nitrogen source on the relative activity of penicillin G acylase (%).

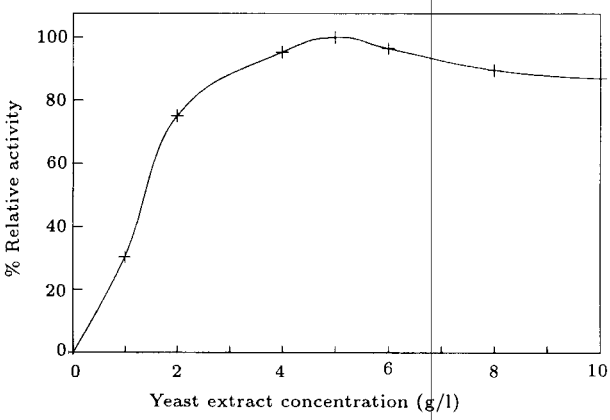

Figure 6. The effect of yeast extract concentration ($g.l^{-1}$) on the relative activity of penicillin G acylase (%).

Effect of PAA Concentration

PAA and its salts are PGA inducers. The effect of PAA on PGA activity was studied using PAA solutions of 0, 1, 2, 3, 4, 5 and 7 $g.l^{-1}$, respectively. The results are depicted in Figure 7 at 25°C, pH of 8.0 and 250 rpm. The optimum concentration of PAA was found to be 4 $g.l^{-1}$. The inhibitory effect of PAA on PGA synthesis appears for PAA concentrations more than the optimum value, i.e. 4 $g.l^{-1}$.

Effect of Penicillin G on PGA Production

Solutions of the original culture media containing 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, 1 and 1.5 $g.l^{-1}$ of penicillin G were prepared and PGA activity in the fermentation broth was measured after 40 h. The optimum concentration of penicillin G, at which maximum activity of PGA occurs, was found to be 0.2 $g.l^{-1}$ (Figure 8). Due to the antibacterial effect, higher concentrations of penicillin G causes decrease in PGA activity as shown in Figure 8. The time of adding penicillin G to the culture media also affects the PGA activity. This was studied using 0.2 $g.l^{-1}$ solutions of penicillin G in the fermentation broth. The activity of PGA was measured for time intervals of 0 to 35 h, shown in Figure 9. The maximum activity is observed when penicillin G was added to the culture 8 h after

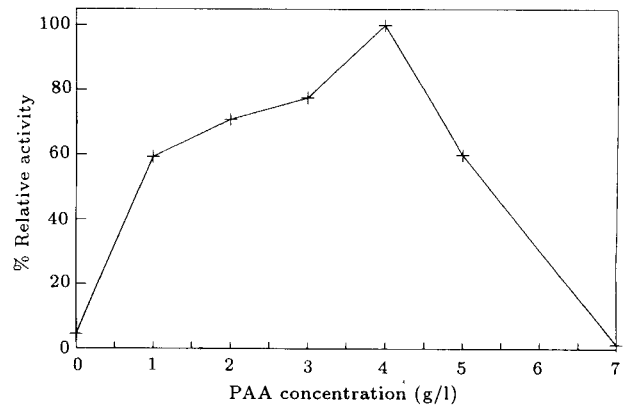

Figure 7. The effect of phenyl acetic acid concentration ($g.l^{-1}$) on the relative activity of penicillin G acylase (%).

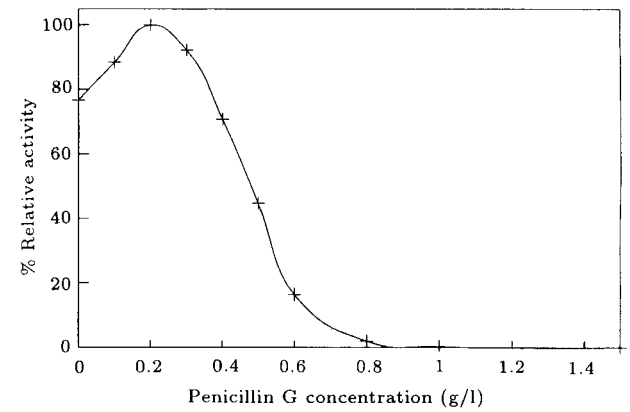

Figure 8. The effect of penicillin G concentration ($g.l^{-1}$) on the relative activity of penicillin G acylase (%).

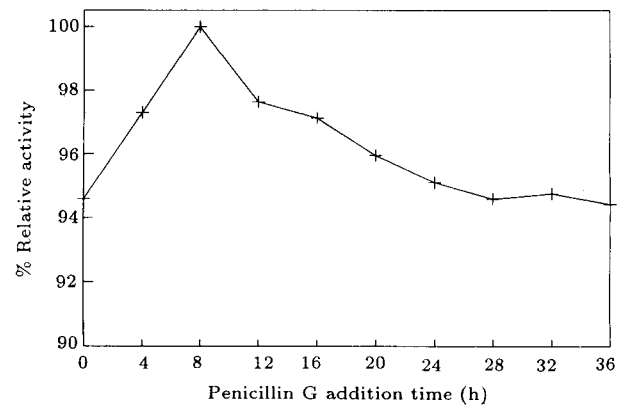

Figure 9. The effect of penicillin G addition time (h) on the relative activity of penicillin G acylase (%).

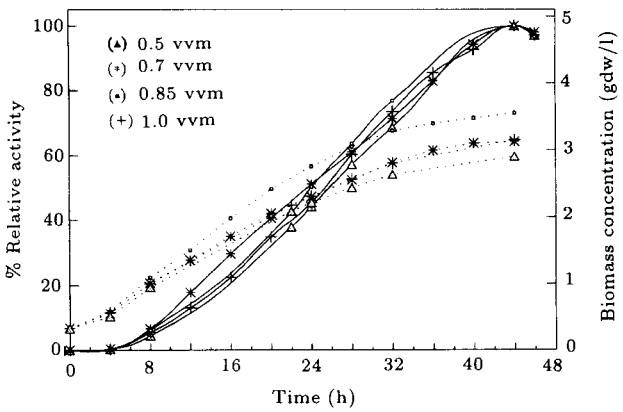

Figure 10. The relative activity of penicillin G acylase in % (— curves) and the biomass concentration in gdw.l^{-1} (.... curves) as a function of cultivation time (h) at different aeration rates.

the start of the fermentation process, i.e., when the cell growth passed the lag phase and PGA production was established.

Effect of Aeration Rate on PGA Production

The batch fermenter described in the materials and methods section of the present article was used to study the effect of dissolved oxygen concentration on PGA activity and biomass concentration. Air was sparged at the rate of 0.5, 0.7, 0.85 and 1 vvm, and PGA activity as well as biomass concentration were measured for the time intervals between 0 to 44 h of the fermentation process (Figure 10). The optimum aeration rate was found to be 0.85 vvm at which the maximum activity of PGA and maximum biomass concentration were obtained.

Modeling Growth Kinetics for PGA Production

Successive second-order polynomials were fitted to the experimental data points to determine the biomass concentration and PGA activity as a function of time. The second-order polynomial is provided by the following equation:

$$y_i(t) = a_i + b_i t + c_i t^2, \quad i = 1, 2, \tag{1}$$

where $y_1(t)$ and $y_2(t)$ are biomass concentration in gdw.l^{-1} and PGA activity in U.ml^{-1}, respectively. The constant values a_i, b_i and c_i are obtained through the regressions of y_1 and y_2 data points versus cultivation time, t. The instantaneous specific growth rate, μ, can be calculated using the following equation:

$$\mu = \frac{1}{y_1(t)} \cdot \frac{dy_1(t)}{dt} = \frac{b_1 + 2c_1 t}{a_1 + b_1 t + c_1 t^2}. \tag{2}$$

The specific production rate of PGA, ν, can be calculated by:

$$\nu = \frac{1}{y_1(t)} \cdot \frac{dy_2(t)}{dt} = \frac{b_2 + 2c_2 t}{a_1 + b_1 t + c_1 t^2}. \tag{3}$$

The regression results for $y_1(t)$, $y_2(t)$, μ and ν are as follows (Figures 11 and 12):

$$y_1(t) = 0.207843 + 0.138953\ t - 0.001299\ t^2, \tag{4}$$

$$y_2(t) = -0.080133 + 0.037697\ t - 4.798061 \times 10^{-5}\ t^2, \tag{5}$$

$$\mu = \frac{0.138953 - 0.002598\ t}{0.207843 + 0.138953\ t - 0.001299\ t^2}, \tag{6}$$

$$\nu = \frac{0.037697 - 9.596122 \times 10^{-5}\ t}{0.207843 + 0.138953\ t - 0.001299\ t^2}. \tag{7}$$

The model of Leudeking-Piret kinetics was used to determine whether PGA synthesis obeys growth associated or non-growth associated behavior [18]. The

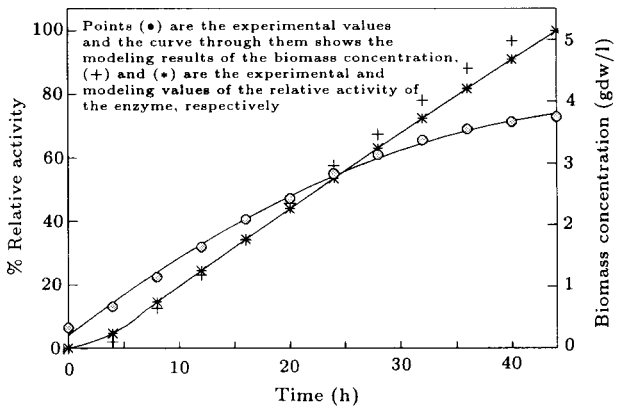

Figure 11. Biomass concentration (gdw.l^{-1}) and the relative activity of penicillin G acylase (%) as a function of cultivation time (h).

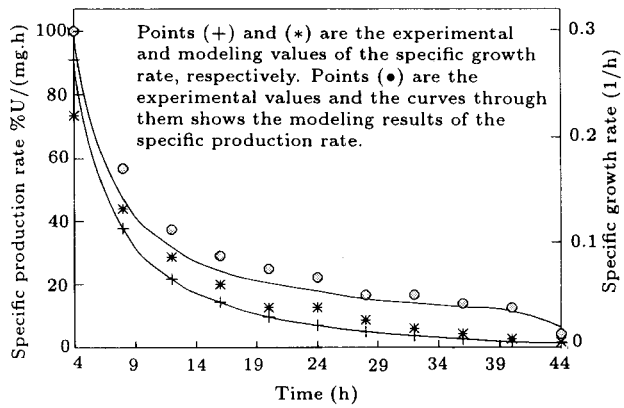

Figure 12. The specific production rate (%U.mg^{-1}.h^{-1}) and the specific growth rate (h^{-1}) as a function of the cultivation time (h).

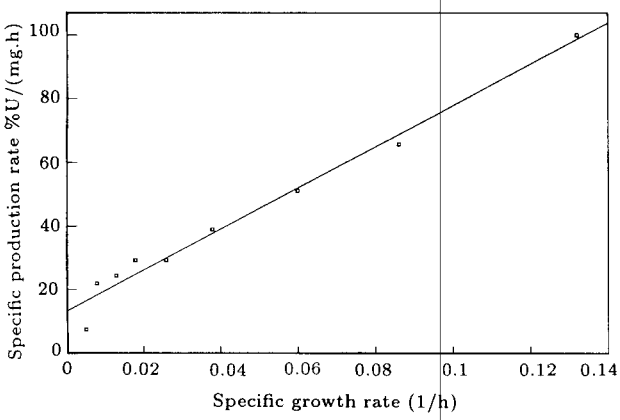

Figure 13. The specific production rate (%U.mg^{-1}.h^{-1}) versus the specific growth rate (h^{-1}) values.

relation between specific growth rate, μ, and specific PGA production rate, ν, can be expressed linearly by Leudeking-Piret model:

$$\nu = \alpha\mu + \beta. \tag{8}$$

The parameters α and β were determined through linear regression of ν versus μ data points. The regression results in Figure 13 illustrate that the value of α is about 50 times greater than β, i.e., PGA synthesis has been carried out through a growth associated fermentation process.

CONCLUSIONS

The experimental results show that the maximum PGA production occurs at pH range 8.0-9.0 and for pH values less than 8.0, a sharp decrease in PGA activity is observed. The best results are gained when YE was used as both nitrogen and carbon sources, with an optimum concentration of 5 g.l^{-1} that yields maximum activity of PGA and biomass concentration. Furthermore, optimum PGA activity as well as biomass concentration are obtained when 4 g.l^{-1} PAA was used as PGA inducer. Experimental results demonstrate that adding penicillin G to the fermentation broth enhances the enzyme synthesis by *Escherichia coli* and the optimum time for adding penicillin G was determined to be 8 h after the initiation of the fermentation operation. The optimum aeration rate for fermentation was obtained when air sparged at the rate of 0.85 vvm through the fermentation broth. Successive second-order polynomials were successfuly fitted to the experimental data points to determine PGA activity and biomass concentration. Leudeking-Piret kinetics was used for analysis of PGA synthesis kinetics by determining the model parameters, for which, the results obey the growth associated kinetics.

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NOMENCLATURE

a_i, b_i, c_i	regression constant values defined by Equation 1
t	time (h)
$y_1(t)$	biomass concentration (gdw.l^{-1})
$y_2(t)$	penicillin G acylase activity (U.ml^{-1})

Greek Symbols

α, β	Leudeking-Piret constant parameters
μ	specific growth rate (h^{-1})
ν	specific production rate (h^{-1})

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