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Enhanced enzymatic synthesis of amoxicillin by addition of organic cosolvents with response surface methodology

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ABSTRACT

Penicillin acylase (PA) from *Kluyvera citrophila* was employed for the synthesis of amoxicillin under kinetic control. Twelve organic cosolvents were investigated as additives for enzymatic synthesis of amoxicillin. It indicated that the reaction rate decreased with the increase of solvent's LogP. Ethylene glycol was found to be the suitable additive due to its enhancement of activity of PA and improvement of amoxicillin yield. Then reaction conditions including ethylene glycol concentration, pH, temperature, and substrate concentration were studied. Finally, a response surface methodology (RSM) was employed to obtain the optimal reaction conditions. The model showed that the interaction between temperature, 6-aminopenicillanic acid (6-APA) concentration and the molar ratio of acyl donor to 6-APA were significant. The highest yield of 92.4 % was achieved at the optimal conditions: 116 mmol l^{-1} of 6-APA, 15 °C and the molar ratio of acyl donor to 6-APA was 3.4.

KEYWORDS

Amoxicillin;
Enzymatic synthesis;
Ethylene glycol;
Penicillin acylase;
Response surface methodology;
Organic cosolvents.

INTRODUCTION

Amoxicillin is one of the most important β-lactam antibiotics which are the most used pharmaceuticals in the clinical application. After patented by Beecham in 1972, its production increased rapidly and reached 5,000 tons in 2000[1]. However, the conventional chemical process demands low temperatures (less than -30 °C), organochloride solvents and protection/deprotection of side groups, and generate great amounts of non-recyclable waste[2–4]. Thus enzymatic synthesis of β-lactam antibiotics has been receiving increasing attention as a green-chemistry alternative for the industrial production of these drugs. Because it can be performed in mild reaction conditions and less waste is produced.

Penicillin acylase (PA, also named penicillin amidase or amidohydrolase, EC 3.5.1.11), is known as the biocatalyst for hydrolyzing penicillin G or penicillin V to manufacture of 6-aminopenicillanic acid (6-APA) which is the most important medical intermediate in the β-lactam antibiotics industry. It was first employed in the synthetic direction to synthesize penicillin G by Kaufmann and Bauer[5]. Since then, cefaclor, cephalexin, cefazolin, and ampicillin have been successfully synthesized by enzymatic catalysis in the past few

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years[6–10]. Although the enzymatic synthesis of β-lactam antibiotics has been studied in academia and industry, there is only one company using the enzymatic route[11] and there are still more work to do to make them industrialization.

Enzymatic synthesis of amoxicillin was first demonstrated by Marconi et al.[12]with an *Escherichia coli* PA. Then this method was followed by Kato et al.[13] and Mikio et al.[14] with PA from *Xanthomonas citri*, *Pseudomonas melnogenum*, respectively. Later, Diender et al.[15] found that a "solid-to-solid" synthesis under thermodynamically control was not feasible, and they developed a suspension-to-suspension synthesis of amoxicillin[16]. Gonçalves et al.[17] worked on the kinetic analysis and modeling of amoxicillin synthesis with immobilized PA on highly activated agarose and proved that the substrate 6-APA bounded to enzyme before the synthesis[17,18]. Chow et al.[19]found that methanol was a better cosolvent than ethylene glycol for synthesis of amoxicillin. Very recently, one-pot enzymatic synthesis of amoxicillin was carried out successfully by Wu et al.[20]and Zhang et al.[1]. These researches and the results are good practices for enzymatic synthesis of amoxicillin.

The main obstacle in the enzymatic synthesis is the low yield and productivity. The efforts to improve the yield of amoxicillin in enzymatic synthesis with PA have been reported by using several strategies such as frozen medium[21], substrate supersatuation[4] and methanol additives[19]. Among these strategies, addition of organic solvents is a convenient method because it is easy to separate the product, improve the product yield and enhance the synthetic activity of PA[10,22,23]. The organic cosolvents in the enzymatic synthesis can shift the thermodynamic equilibrium toward the synthesis direction by reducing the water activity, altering the p*K* values of the reactant, and control of the substrate specificity. With the development of green chemistry, organic solvents in the enzymatic synthesis receive a lot of attentions[20]. In the present study, we tried to improve the yield of amoxicillin by using immobilized PA from *Kluyvera citrophila* (KcPA) because it has higher synthesis/hydrolysis ratios than other organisms such as *E. coli*, *Alcaligenes facaelis* and *Providencia rettgeri*[24]. The reaction conditions were optimized with execution of one-variable-at-a-time experiments and response surface methodology.

EXPERIMENTAL

Enzyme assay

The immobilized PA from *Kluyvera citrophila* (KcPA) was donated by Shijiazhuang Pharmaceutical Co. Ltd. (Shijiazhuang, China). The enzyme was immobilized on oxriane resin by covalent attachment and the activity was 120 ± 10 IU/g. A unit of PA was defined as the amount of enzyme required to produce 1 μmol of 6-APA per minute in 4% solution of penicillin G at pH 7.8 and 37 °C. The enzyme activity was determined by a spectrophotometric assay with *p*-dimethylamino benzaldehyde (PDAB) as a colorimetric substrate[25].

Chemicals

Amoxicillin, penicillin G potassium salt and 6-APA were donated by Shijiazhuang Pharmaceutical Co. Ltd. (Shijiazhuang, China). D-p-hydroxyl-phenylglycine methyl ester hydrocholoride (HPGME) was purchased from the Shanghai Industrial Chemical Co. Ltd. (Shanghai, China). All other reagents were of analytic grade.

Analysis

Each reactant and product was identified and analyzed by HPLC with an Agilent G1311A pump and Agilent G1315B DAD detector. An Agilent XDB C-18 column (250 mm length and 4.6 mm internal diameter, 5 μm particle size and 8 nm pore size) was used. Samples of 100 μl were taken from the reaction mixture, diluted with 900 μl of eluent and injected. The samples were eluted at 30 °C with 97.5% phosphate sodium buffer (50 mmol l^{-1}, pH 5.0) and 2.5 % acetonitrile at 1 ml min^{-1}, and monitored at 254 nm. The components were eluted in the following order: HPG (3.1 min), 6-APA (3.8 min), amoxicillin (5.3 min), and HPGME (8.9 min).

Screening of organic cosolvent

Enzymatic syntheses of amoxicillin were performed in a stirred bioreactor with jackets for water circulation to keep the temperature constant in the presence of 2% organic solvents. A pH controller was used to monitor pH values during the reaction process. The initial

reaction volume, 6-APA and HPGME concentrations, temperature and pH were 100 ml, 50 and 100 mmol l^{-1}, 25 °C and 6.0. The enzyme load and the stirring rate were 20 IU (mM of 6-APA)$^{-1}$ and 120 rpm, respectively. The maximum reaction yield was calculated by monitoring the concentration of 6-APA concentration. Enzyme stability in different organic solvents was checked by incubating immobilized KcPA in 2% organic solvents at pH 6.0, 25 °C. Aliquots were withdrawn at various time and the relative activity was determined.

Optimization with RSM

The factors such as 6-APA concentration, pH, temperature, and enzyme loading were studied by one-variable-at-a-time experiments. Based on these results, a central composite design (CCD) of RSM was employed to investigate the optimal conditions and the interaction between the three most significant factors[26]. The response surface model can reflect the influence and the interaction between the factors simultaneously. A Box-Behnken design with 3 factors (temperature, 6-APA concentration and the ratio of HPGME to 6-APA) and 3 levels was performed and there were total 15 experiments presented in TABLE 1. The three levels were assigned values of -1, 0, and +1 respectively. Statistica 7.0 software (StatSoft, Tulsa, USA) was used for experimental design and regression analysis of the variables as second-order polynomial models of the form:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=1+1}^{3} \beta_{ij} X_i X_j$$

Where Y is the predicted response, Xi is the variable, β_0 is constant, β_i is the linear effect and β_{ij} is the interaction effect. The regression analysis of experimental data was performed using statistical software. The quality of the polynomial model equation was checked statistically significant by the coefficient of the determination R^2; a p-value <0.05 was considered to indicate statistical significance.

TABLE 1 : CCD design and the experimental results

No.	Temperature (°C)	6-APA (mmol l^{-1})	HPGME/6-APA (mol/mol)	Yield (%)
1	-1(15)	-1(100)	0(3)	82.65
2	-1	1(140)	0	91.38
3	1(25)	-1	0	70.04
4	1	1	0	62.38
5	0(20)	-1	-1(2.5)	74.89
6	0	-1	1	60.75
7	0	1	-1	68.85
8	0	1	1(3.5)	67.15
9	-1	0(120)	-1	86.56
10	1	0	-1	64.29
11	-1	0	1	77.15
12	1	0	1	61.47
13	0	0	0	74.48
14	0	0	0	75.29
15	0	0	0	74.38

RESULTS AND DISCUSSION

Selection of organic cosolvent

High .concentration of organic solvents does not enhance the PA-catalyzed reaction rate[27], so we selected 12 organic solvents with different log P values for the enzymatic synthesis of amoxicillin at 2% (v/v) solvent concentrations (TABLE 2). The relative reaction rate of amoxicillin synthesis (υ/υ_0) depended highly on the property of the organic solvents which is shown in TABLE 2. The υ/υ_0 roughly correlated with the solvent hydrophobicity. For example, the reaction rate was lower in high LogP value organic solvents than high ones. The maximum yield was also affected significantly by the presence of 2% organic cosolvents. This is similar to the synthesis of ampicillin and pivampicillin[28,29]. However, there was no much influence on the activity of immobilized penicillin acylase before and after addition of organic cosolvents as shown in TABLE 2. Among these organic solvents, addition of ethylene glycol and glycerol would enhance the yield of amoxicillin; and the other solvents decreased the yield. When the 6-APA concentration was 50 mmol l^{-1}, the yields of amoxicillin were 48.4%, 29.4%, 24.0% and 45% in the presence of 2% ethylene glycol, methanol, DMF and glycerol, respectively. It is known that the active site of PA is extremely hydrophobic because hydrophobic amino acid residues such as Met, Phe and Ile lined in the binding pocket[30]. The alignment of neucleotide sequence of PA genes from *E.coli* and *K.citrophila* revealed that

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TABLE 2 : Effect of organic solvents on the synthesis of amoxicillin, relative activity of PGA and relative reaction rate; data presented in the table were the average of three experiments.

Solvent (2%)	Log *P*	υ/υ_0 (%)[a]	Y/Y_0 (%)[b]	Relative activity of PA (%)
Glycerol	-3.04	102	110	105
Ethylene glycol	-1.43	100	118	110
Dimethyl sulfoxide	-1.35	78	91	95
Dimethylformamide	-1.00	48	87	94
Methanol	-0.77	89	102	98
Acetonitrile	-0.34	57	88	90
Acetone	-0.24	32	82	90
2-Propanol	0.27	24	90	96
Ethyl acetate	0.67	30	80	95
1-Butanol	0.79	12	85	94
Ethyl ether	0.85	45	75	92
Triethylamine	1.58	66	89	101

The log*P* values of the solvents were calculated using the hydrophobic fragment method developed by Rekker and Kort (1979); [a]The reaction rate was determined in the presence of 2% (v/v) solvents (υ) and normalized to that obtained in phosphate buffer ($\upsilon_0 = 7.6\times10^{-2}$ µmol min^{-1} (IU-PA)$^{-1}$).; [b]Relative maximum yield: maximum yield in water-cosolvent mixture (*Y*) normalized to that obtained in phosphate buffer ($Y_0 = 41\%$).

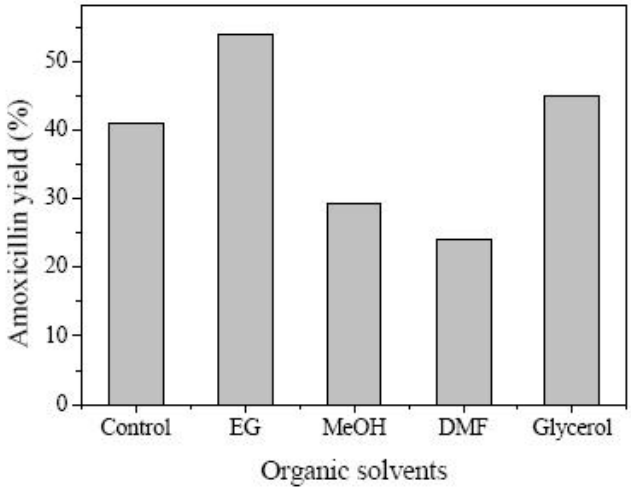

Figure 1 : Effect of cosolvents on the yield of amoxicillin. Temperature 25 °C, pH 6.5, 50 mmol l^{-1} 6-APA, 100 mmol l^{-1} HPGME, enzyme 20 IU per mmol 6-APA.

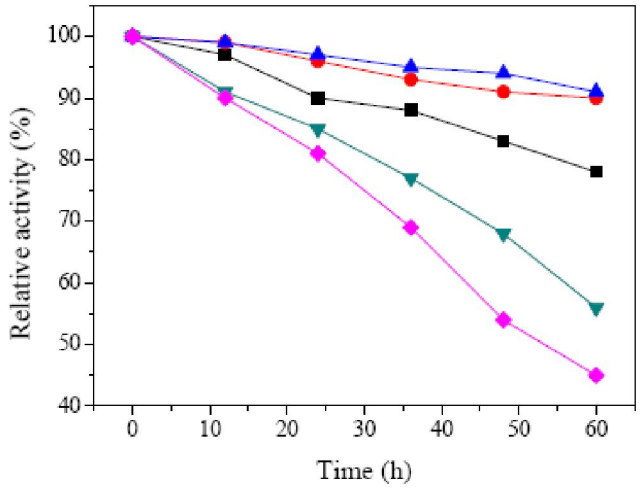

Figure 2 : Effect of cosolvents on the stability of KcPA. Sqaure: phosphate buffer without solvents; circle: 2% ethylene glycol; uptriangle: 2% glycerol; down triangle: 2% methanol; diamond: 2% DMF. Conditions: 20 °C, 100 mmol l^{-1} pH 6.5 phosphate buffer.

more than 83% homology and they have common ancestral origin[31]. The hydrophobic interactions between the active site of the enzyme and organic solvents showed important role in the enzymatic reactions. It was concluded that KcPA had high selectivity to amoxicillin in the presence of ethylene glycol although Chow et al.[19]reported that methanol was better than ethylene glycol when an *E. coli* PA was employed.

Enzyme stability

The enzyme stability is another important factor for selection of organic cosolvent. Ethylene glycol, DMF, methanol and glycerol were chosen to test the stability of KcPA. There were 91%, 56%, 45% and 92% residual activity obtained after 24 hours incubation in the 2% ethylene glycol, DMF, methanol and glycerol, respectively (Figure 2). Phosphate buffer (0.1 M, pH 6.5) was used as the control to make a comparison, and

there was 81% remained activity. Compared to the PAs from *E. coli*, *A. facaelis*, *P. rettgeri*, KcPA had similar stability in the ethylene glycol, but higher stability in other organic solvents[10,27,29]. The results suggested that ethylene glycol and glycerol protected the enzyme against inactivation. The solvents with higher logP are stronger denaturants than lower ones. Thus, ethylene glycerol was chosen as the suitable additive to synthesize amoxicillin by consideration of amoxicillin yield and the enzyme stability.

One-variable-at-a-time experiments

According to the previous report, factors including the concentrations of ethylene glycol, temperature, pH, the ratio of HPGME to 6-APA, and the substrate concentration, were investigated as below.

Effect of ethylene glycol concentration on the synthesis of amoxicillin

Several reports investigated the effect of ethylene glycol on the synthesis of β-lactam antibiotics and found that the synthesis yield increased[10,29,32,33]. Ethylene glycol, as a soft solvent, not only stabilizes the nonionic substrate which is the only acceptable form for PA[23], but also represses the hydrolysis of HPGME. In this study, the yield was varied with the concentrations of ethylene glycol as shown in Figure 3. The maximum yields of amoxicillin were 49%, 51%, 52.3%, 53.9%, 48.3% and 43.1% in the presence of 5%, 10%, 20%, 30%, 40% and 50% ethylene glycol, respectively. However, addition of ethylene glycol will increase the viscosity of the reaction mixture and decrease the reaction rate because of the increased diffusional limitation[10]. Therefore, the time to reach maximum yield was increased when the concentration of ethylene glycol increased.

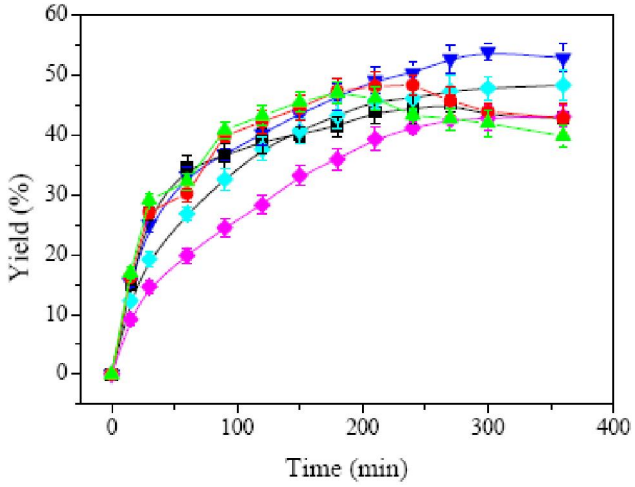

Figure 3 : Effect of ethylene glycol concentration on the enzymatic synthesis of amoxicillin. Square: 20% ethylene glycol; circle: 30% ethylene glycol; uptriangle: 40% ethylene glycol; diamond: 50% ethylene glycol. Reaction conditions: 50 mmol l^{-1} 6-APA, 100 mmol l^{-1} HPGME, 20 °C, pH 6.5, enzyme loading 20 IU per mmol 6-APA.

Effect of temperature and pH on the synthesis of amoxicillin

Temperature and pH are important factors in the enzymatic catalysis reactions[34]. The yield of amoxicillin at 15, 20, 25, 30 and 35 °C are shown in Figure 4A. The maximum yield decreased from 58.7% to 31.2% with the temperature increased from 15 °C to 35 °C. This is similar to the previous results on synthesis of cefaclor[23]. The productivity at 20 °C (0.67mM h^{-1}) was 37% higher than that at 15 °C, so we chose 20 °C as the optima. Enzymatic syntheses of amoxicillin were performed from a pH range from 6.0 to 7.0 (Figure 4B). The highest yield (53.9%) was achieved at pH 6.5, which is similar with the synthesis of cefaclor[10,23,29] and cephalexin[35–37]. The reason probably is that the acetic pH is favorable for the protonation of the β-lactams' nucleus[3].

Effect of substrate concentration and ratio on the synthesis of amoxicillin

The concentration of 6-APA varied from 25 mmol l^{-1} to 150 mmol l^{-1} and the results are shown in Figure 4C. The yield of amoxicillin was 65% when the 6-APA concentration was 125 mmol l^{-1} and the ratio of HPGME to 6-APA was 2. When the 6-APA concentration was higher than 125 mmol l^{-1}, the yield decreased with the increase of 6-APA concentration. This is probably because high concentration of nucleophile shows an inhibitory effect and reduced the rate of antibiotic synthesis, similarly to the ampicillin synthesis[7]. When the concentration of 6-APA was 125 mmol l^{-1}, the yield of amoxicillin increased from 49% to 74.8% with increasing the molar ratio of HPGME to 6-APA from 1 to 3 (Figure 4D). The yield decreased with the ratio increased to 3 because the hydrolysis of HPGME was serious. Therefore, it can be concluded that optimal HPGME concentration improved the conversion of 6-APA and the ratio of HPGME to 6-APA has an impor-

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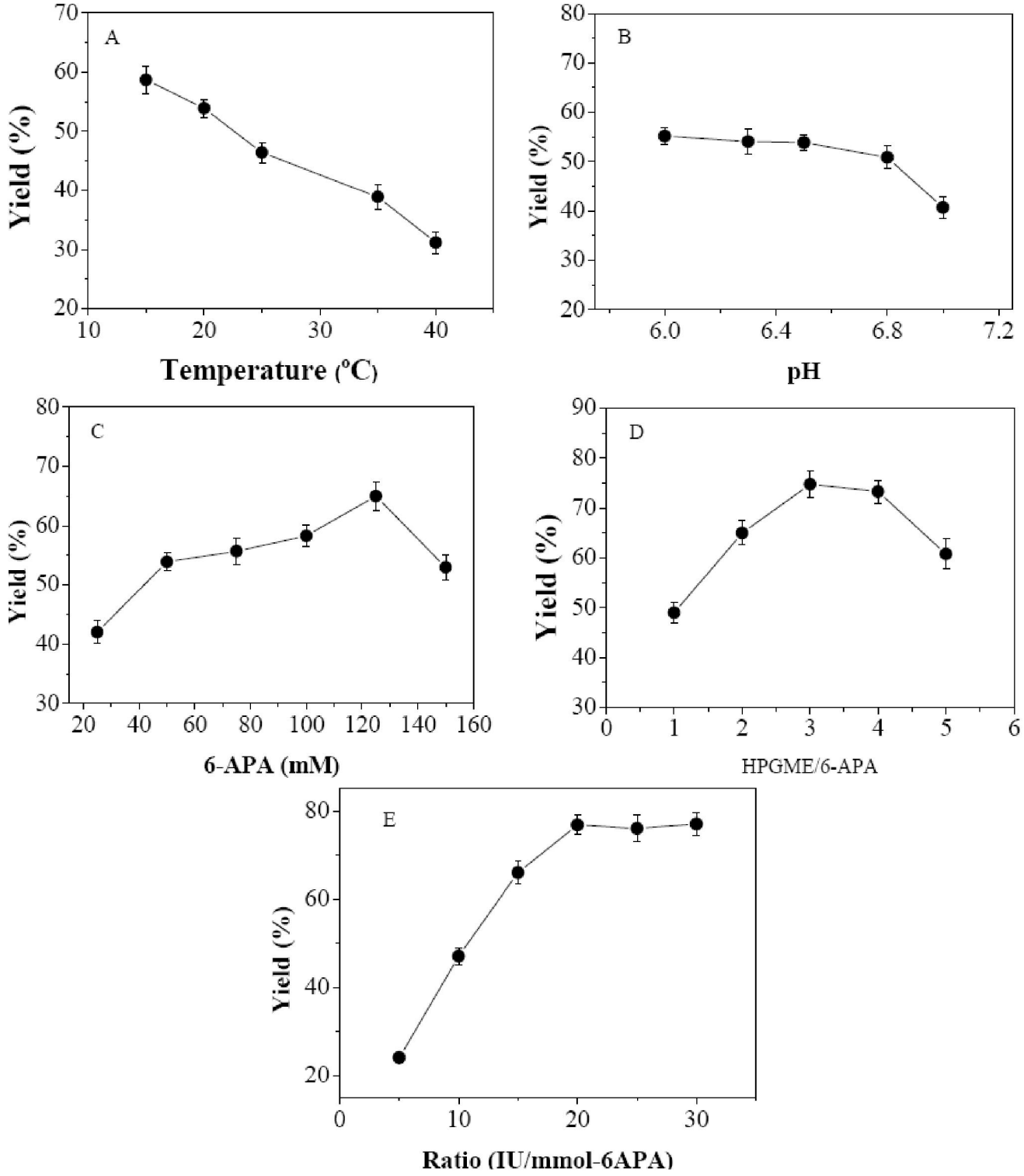

Figure 4 : Effects of temperature (A), pH (B), 6-APA concentration (C), ratio of HPGME to 6-APA (D) and ratio of enzyme to substrate (E) on the synthesis of amoxicillin. Reaction conditions: (A) 50 mmol l^{-1} 6-APA, 100 mmol l^{-1} HPGME, pH 6.5, 30% ethylene glycol, enzyme loading 20 IU per mmol 6-APA; (B) 50 mmol l^{-1} 6-APA, 100 mmol l^{-1} HPGME, 20 °C, 30% ethylene glycol, enzyme loading 20 IU per mmol 6-APA; (C) ratio of 6-APA to HPGME was 2, 20 °C, pH 6.0 30% ethylene glycol, enzyme loading 20 IU per mmol 6-APA; (D)125 mmol 6-APA, pH 6.0, 25 °C, 30% ethylene glycol, enzyme loading 20 IU per mmol 6-APA; (E) 125 mmol l^{-1} 6-APA, 375 mmol l^{-1} HPGME, 20 °C, pH 6.0, 30% ethylene glycol. All the yields were the maximum yield in 6 h reaction time.

tant influence on the synthesis of amoxicillin.

Another important factor influence the enzymatic reactions is the ratio of enzyme to substrate. The enzyme loading was ranged from 5 to 30 U per mmol of 6-APA and the other conditions were fixed (As shown in Figure 4E). When enzyme loading reached 20 U per mmol of 6-APA, the maximum conversion was obtained. The maximum yield decreased with the enzyme loading increased when the enzyme loading was higher than 20 U per mmol 6-APA. This is probably because the hydrolysis of amoxicillin and HPGME was more serious in the presence of excess enzyme.

RSM optimization and validation

According to the above-mentioned preliminary experiments, temperature, 6-APA concentration and the ratio of HPGME to 6-APA were chosen as the variables to design RSM optimization. The levels were chosen based on preliminary experiments, as follows: temperature (15-25 °C), 6-APA (100-140 mmol l^{-1}), and the molar ratio of HPGME to 6-APA (2.5-3.5), as shown in TABLE 1. The maximum yield of amoxicillin was 91.38% (No. 2 in TABLE 1) and the lowest yield was 60.75% (No. 6). The ANOVA analysis was performed go give the quadratic model as below:

$$Y = 74.94 - 9.94X_1 + 3.86\,X_2 - 1.62\,X_3 - 4.09\,X_1X_2 + 2.64\,X_1X_3 + 2.90\,X_2X_3 + 3.08X_1^2 - 7.7\,X_2^2 + 5.82\,X_3^2$$

The value of coefficient determination (R^2=0.995) indicated that the model was significant and adequate to represent the actual relationship between the response and the variables. The ANOVA gave a very low *p*-value ($p < 0.0001$) for the regression model, confirming the model is appropriate for amoxicillin production. The interactions between any two factors were significant ($p < 0.05$) which suggested that the effects of all the tested factors on amoxicillin yield were response surface effects rather than simple linear correlations. It will not reflect the true value if we treat the factors separately. In order to get a better understanding of the interactions between the variables, RSM surface plots were given in Figure 5. All the 3D plots exhibited significant quadratic surface rather than linear plane. From the model, it is easy to find that 6-APA concentration, interaction between temperature and the molar ratio of HPGME to 6-APA, temperature and 6-APA concentration, and the square of molar ratio of 6-APA to acyl donor have positive effect on the synthesis yield. The

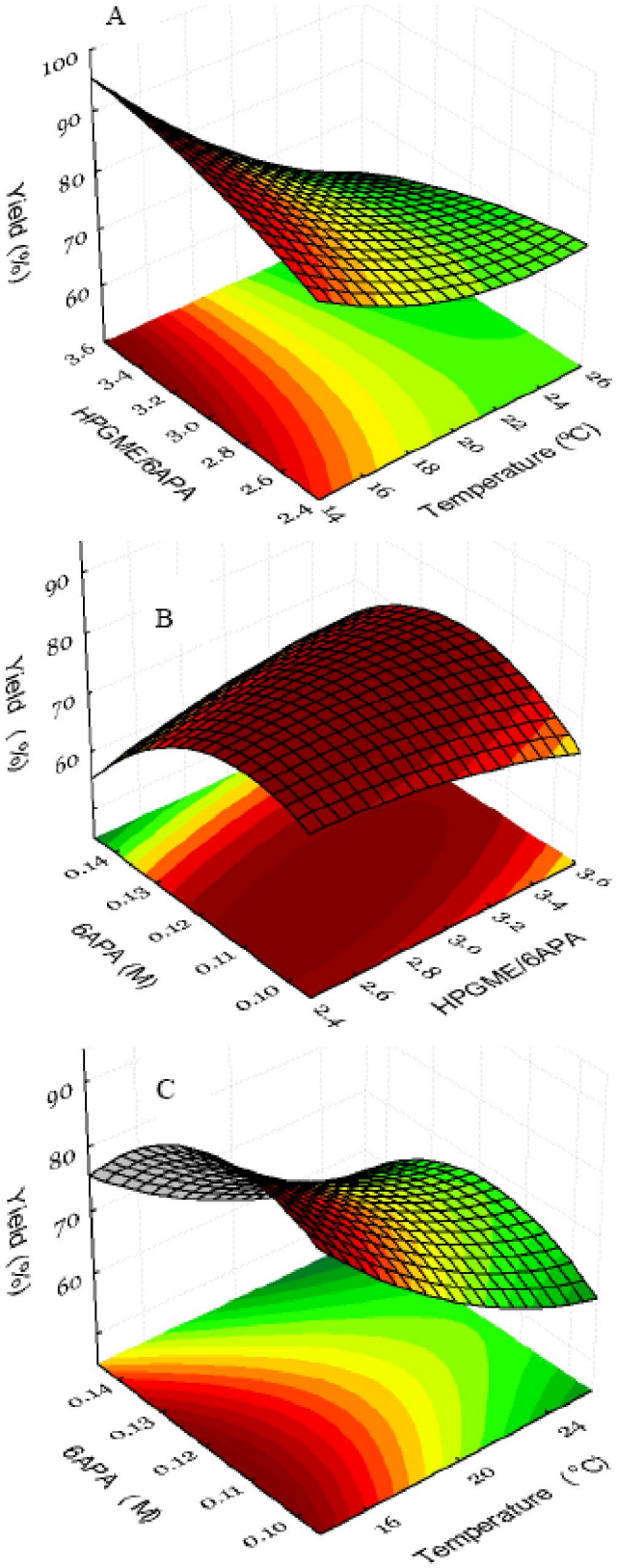

Figure 5 : 3D response surface plot for the effects of temperature, 6-APA concentration and the ratio of HPGME to 6-APA on the yield of amoxicillin. (A) at 6-APA 120 mmol l^{-1}; (B) at 20 °C; (C) when HPGME/6-APA was 3.

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others have the negative effects. The model predicted the highest yield was 93.1% under the optimal conditions: 116 mmol l^{-1} 6-APA, temperature 15 °C and the ratio of HPGME to 6-APA was 3.4. We validated the optimal conditions three times and the yield of 92.4±2.4% was achieved. This is the highest yield in the enzymatic synthesis of amoxicillin with PA. The yield is higher than that previous reports such as 88% with addition of *tert*-pentanol[38], 91% with supersatuation solution[4], and 38% in frozen media[39].

ABBREVIATIONS AND NOMENCLATURE

6-APA	6-aminopenicillanic acid
HPG	p-Hydroxyphenylglycine
HPGME	p-Hydroxyphenylglycine methyl ester
PA	penicillin acylase
υ	Reaction rate (μM amoxicillin (IU PA×min)$^{-1}$)
υ_0	Initial reaction rate (μM amoxicillin (IU PA×min)$^{-1}$)
ρ	productivity (mM $h^{-1}l^{-1}$)
Y	Yield = amoxicillin produced/initial 6-APA (%)

CONCLUSIONS

Immobilized PA from *K. citrophila* was employed as the biocatalyst to synthesize amoxicillin under kinetic control. Firstly, effect of organic solvents on the enzymatic synthesis of amoxicillin was investigated. The reaction rate decreased roughly correlated with the increased solvent's LogP. Ethylene glycol was chosen as the optimal cosolvent from 12 organic solvents for both the enhancement of amoxicillin production and improvement of PA activity. After one-variable-at-a-time experiments, three factors including temperature, 6-APA concentration and the ratio of HPGME to 6-APA were chosen as variables for further optimization by statistical methodology. The RSM model gave the highest yield of amoxicillin with 93.1% validated with values as 92.4±2.4% at the optimal conditions: pH 6.5, 15 °C, 116 mmol l^{-1} 6-APA and the ratio of HPGME to 6-APA was 3.4. The improved production conditions described here will help the industrialization of enzymatic synthesis amoxicillin.

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