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Enzymatic Synthesis of Amoxicillin via a One-pot Enzymatic Hydrolysis and Condensation Cascade Process in the Presence of Organic Co-solvents

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Abstract A cascade reaction combining the enzymatic hydrolysis of Penicillin G potassium salt (PGK) with the kinetically controlled enzymatic coupling of in situ formed 6-aminopenicillanic acid (6-APA) with *p*-hydroxyphenylglycine methyl ester (D-HPGM) to give amoxicillin as the final product by using a single enzyme has been demonstrated successfully. Ethylene glycol (EG) was employed as a component of reaction buffer to improve the synthesis yield. Reaction parameters, including different cosolvents, EG content, the loading of immobilized penicillin G acylase (IPA), and reaction temperature and time were studied to evaluate their effects on the reaction. The best result of 55.2% yield was obtained from the reaction which was carried out in the mixed media containing 40% sodium dihydrogen phosphate buffer (apparent pH 6.0) and 60% EG (*v*/*v*), with the initial concentration 150 mM and 450 mM of PGK and D-HPGM, respectively, catalyzed by 50 IU/mL IPA at 25 °C for 10 h. The IPA could be recycled for nine batches without obviously losing of catalytic activity. The important strategy will have potential application in the β-lactam antibiotics industry due to the advantages of saving the effort of isolating 6-APA, reducing usual enzymatic steps and the industrial cost of amoxicillin synthesis.

Keywords Amoxicillin · Cascade · Enzymatic synthesis · Penicillin G acylase · Penicillin G potassium salt

Abbreviation

PGK	Penicillin G potassium salt
6-APA	6-Aminopenicillanic acid
D-HPGM	*p*-Hydroxyphenylglycine methyl ester
EG	Ethylene glycol
IPA	Immobilized penicillin G acylase
PGA	Penicillin G acylase
CLEAs	Cross-linked penicillin acylase aggregates
7-ADCA	7-Aminodeacetoxycephalosporanic Acid

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HPGA	*p*-Hydroxyphenylglycine amide
HPLC	High-performance liquid chromatography
UV	Ultraviolet

Introduction

Amoxicillin is one of the major β-lactam antibiotics with much advantage such as high spectrum of activity, high solubility, high rate of absorption, and its stability under acid conditions. Nowadays, to our knowledge, almost all amoxicillin except some products of Royal DSM N.V. (DSM) are prepared in industry by chemical methods such as Dane anhydride route, which typically involve more than ten steps, low temperatures (−30 °C), and toxic organic solvents like methylene chloride and silylation reagents [1, 2]. The production of 1 kg of amoxicillin will generate 30±40 kg of non-recyclable waste. A large proportion of this non-biodegradable waste originates in the activation, coupling, and deprotection steps [2]. Therefore, enzymatic synthesis has become more interesting due to its high selectivity, specificity, and mild reaction conditions. Enzymatic synthesis of amoxicillin is currently catalyzed by penicillin G acylase (PGA) under either a kinetically controlled strategy or a thermodynamic one. Up to now, much effort has been done to improve the enzymatic synthesis of amoxicillin in order to replace chemical routes: optimization of particular reaction conditions such as pH [3], low temperature [4], or substrate supersaturation in highly condensed aqueous systems [5], use of cross-linked penicillin acylase aggregates [6], various organic cosolvents [7, 8] and multiphase system [9], and in situ amoxicillin removal with ZnSO4 [10], as well as some mathematic models on the kinetics and mechanism of reaction to improve the yield of this enzymatic synthesis [11–16]. The strategy of enzymatic synthesis drastically reduces the number of reaction steps and decreases the amount and toxicity of waste products in the chemical amoxicillin synthesis process. However, such an enzymatic method still requires further improvement in order to be used for industrial production of amoxicillin.

Enzymatic cascades, an attractive option combining a number of reactions into a single procedure, may be more efficient than the usual stepwise approach by saving the effort of isolating intermediates and avoiding the accumulation of reactive and unstable intermediates. For example, one-pot chemoenzymatic synthesis of 3′-functionalized cephalosporines (cefazolin) by three consecutive biotransformations in fully aqueous medium was studied by Justiz and became a typical example of cascade catalysis in β-lactam chemistry [17]. High yields were obtained through a careful selection of the enzyme catalyst, experimental conditions, and synthetic strategy. Schroen et al. reported the integrated process for enzymatic synthesis of cephalexin from adipyl-7-ADCA involving the hydrolysis of adipyl-7-ADCA and the sequential synthesis of cephalexin catalyzed by glutaryl acylase and Assemblase®, as well as the removal of product with β-naphthol [18]. However, the activity of the cephalexin-synthesizing enzyme was influenced negatively. Additional, two-step one-pot enzymatic synthesis of cephalexin from D-phenylglycine nitrile has been established by Wegman employing the nitrile hydrolase-catalyzed hydration of D-phenyglycine nitrile and PGA-catalyzed acylation of 7-ADCA [19]. Cephalexin was obtained in 79% yield. To our best knowledge, however, there are few reports on the synthesis of amoxicillin with enzymatic one-pot method which has important potential application in industrial production of amoxicillin.

In those reports about enzymatic synthesis of amoxicillin mentioned above, all reaction started from 6-aminopenicillanic acid (6-APA) and an activated substrate, e.g., *p*-hydroxyphenylglycine methyl ester (HPGM) or *p*-hydroxyphenylglycine amide. It is

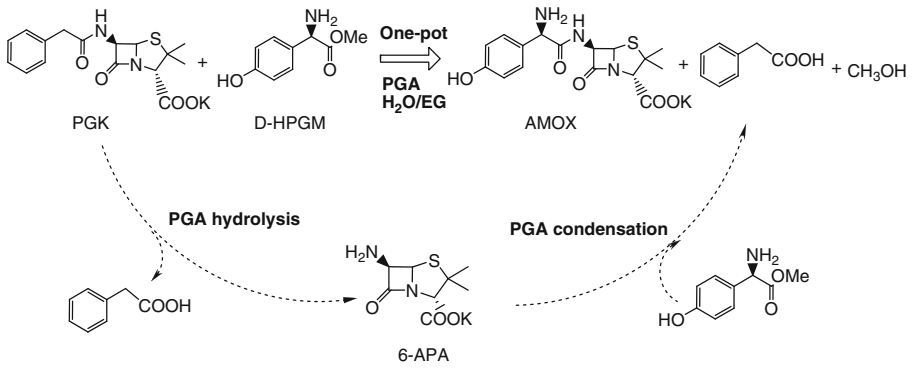
well-known that PGA cannot only convert such mentioned substrates into an antibiotic, but also hydrolyze penicillin G potassium salt (PGK) into 6-APA. In fact, most of the β-lactam nuclei, e.g., 6-APA and 7-ADCA used in the enzymatic semisynthesis of β-lactam antibiotics are produced from the hydrolysis of PGK or cephalosporin C catalyzed by PGA. These considerations lead us to combine the hydrolysis of PGK into 6-APA with the kinetically controlled enzymatic coupling of 6-APA with *p*-hydroxyphenylglycine methyl ester (D-HPGM) to give amoxicillin as the final product by using a single enzyme (see Scheme 1.). This one-pot strategy is possible to further reduce the number of steps in the enzymatic synthesis process of β-lactam antibiotic. It cannot only save the effort of isolating 6-APA, but also efficiently reduce the industrial cost of amoxicillin because of the much lower price of PGK than that of 6-APA.

In this paper, enzymatic synthesis of amoxicillin via a one-pot enzymatic hydrolysis and condensation cascade process starting from PGK was studied. After examining in detail the effects of the reaction parameters including cosolvent content, reaction time, temperature, and enzyme loading on the outcome of the procedure, the cascade reaction can be efficiently performed in the aqueous buffer/EG cosolvent, and 55.2% yield was achieved.

Materials and Methods

Materials

Immobilized penicillin acylase (IPA) from *Escherichia coli* was a commercial product from Hunan Flag Biotech Co. Ltd. (Changsha, China), with declared activity of 108 U/g, which was measured using the initial rate of penicillin G hydrolysis (5% *w*/*v*, pH 8 and 25 °C). *p*-OH-phenylglycine was from Zhejiang Apeloa Pharma Co. Ltd. (Dongyang, China). PGK was purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). EG was analytical grade from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). D-HPGM was prepared as follows: thionyl chloride was added drop-by-drop to the mixture of *p*-OH-phenylglycine and methanol under continuous stirring in ice bath. After the addition, the ice bath was removed, and the mixture was stirred at room temperature overnight. Then, the reaction mixture was subjected to evaporation and *p*-OH-phenylglycine methyl ester hydrochloride (HPGM·HCl) was obtained in good yield. HPGM·HCl was then dissolved in



Scheme 1 Two-step traditional synthesis and one-pot enzymatic cascade process of amoxicillin

water and 4 M NaOH solution was added to form the precipitation of HPGM. All other reagents were analytical grade.

Enzymatic Cascade Synthesis of Amoxicillin

Enzymatic reactions of amoxicillin were carried out in vials shaken at 200 rpm in a temperature-controlled shaker. PGK and D-HPGM were used as substrates, and mixture with suitable composition of sodium phosphate buffer and EG as reaction media. The reaction was catalyzed by IPA with optimized loading. After a set reaction time, the reaction was stopped by adding excess water and filtering off the enzyme. For high-performance liquid chromatography (HPLC) analysis, the reaction mixture was added to 49-fold of water to ensure that the substrates and products were completely dissolved.

Analysis

Substrates and products were identified and analyzed by HPLC using a Shimadzu SPD-10Avp equipped with a Shimadzu SPD-10Avp UV-vis detector and a reversed-phase Agilent Zorbax SB C18 column (150×4.6 mm). The eluent was composed of 90% (*v*/*v*) 20-mM sodium dihydrogen phosphate buffer pH 4.7 and 10% (*v*/*v*) methanol at a flow rate of 1 mL/min. Concentration of substrates and products were analyzed in the UV detector at 230 nm and calculated from calibration curves using stock solutions. HPLC samples were always assayed in triplicate, differences among them never exceeding 5%. The yield of amoxicillin was determined according to the initial concentration of PGK and expressed as a percentage.

Recycling of IPA

Recycling of IPA for one-pot enzymatic synthesis of amoxicillin in buffer-EG mixture was investigated as follows. After 10 h, one batch of reaction was stopped by filtering off the reaction mixture. IPA was washed by buffer-EG mixture for five times. Solvent was removed, and IPA was used as the catalyst for the next batch of reaction.

Results and Discussion

Optimization of Enzymatic Cascade Process in Water Media

6-APA was produced through the hydrolysis of PGK by PGA in water, and most synthesis of amoxicillin reported was also performed in water. Thus, we carried out the enzymatic cascade process in full water media. In the stepwise process, the hydrolysis of PGK could be finished in less than 2 h. The hydrolysis in alkaline buffer (pH 7-8) is more efficient than that in acid media (pH 6; Fig. 1). The synthesis of amoxicillin from 6-APA and HPGM can be performed in buffer media. After optimization, yield of 53.2% could be obtained under the reaction conditions: 100 mM 6-APA and 200 mM HPGM, 10 IU/mL IPA, 25 mM Na_2HPO_4-NaH_2PO_4 buffer with pH 6.6, 35 °C and 20 h reaction time (data in detail not shown). However, the cascade process in full buffer media is unsatisfactory. For example, the cascade reaction containing 0.1 M PGK and 0.2 M HPGM gave amoxicillin yields no more than 20% after 24 h or longer time under the catalysis of 10 IU/mL IPA in buffer with pH 6-8. Even further optimization of enzyme loading, temperature or substrate ratio, had no

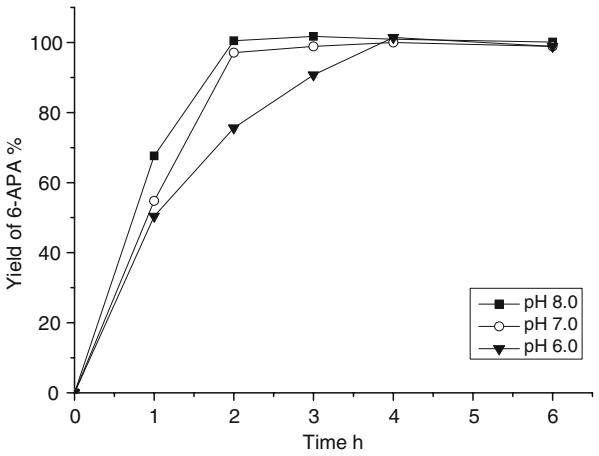


Fig. 1 Effect of pH on the enzymatic hydrolysis of PGK

obvious improvement. The yields of stepwise process were higher than that of one-pot reaction. The phenylacetic acid formed from the hydrolysis of PGK, which acted as a strong competitive inhibitor of PGA, was one of the causes [20]. The results of the cascade reaction in water media are far from satisfactory. It promoted us to change the idea that buffer media is ideal because it is not only suitable to PGA hydrolysis reaction but also to PGA synthesis reaction. Continuing our interest in nonaqueous enzymology [8, 21], we transfer our emphasis to buffer-cosolvent mixed media or organic media where PGA catalyzed the cascade reaction.

Selection of Positive Cosolvents

It is well-known that enzyme activity can be well-preserved in some suitable organic solvent and great development has been achieved in nonaqueous enzymology [22]. Recently, there are some reports concerning enzymatic synthesis of β-lactam antibiotics in organic media or water and cosolvent mixture [23–31]. The presence of organic solvents can enhance the performance of enzyme, at the same time depress the hydrolysis of products obviously, and thus high synthesis yield can be expected. Among these cosolvents, ethylene glycol was used most widely and exhibited some advantages as a cosolvent in the synthesis of β-lactam antibiotics. Illanes et al. [28–30] and Wei [31] have studied thoroughly the synthesis of cephalexin and ampicillin in the presence of EG. However, there is no report about the use of cosolvents in the enzymatic one-pot synthesis of amoxicillin, and the efficiency should be studied.

We selected several usual organic solvents as the cosolvents, and determined the yields in mixed media with different composition after 12 h or 20 h reaction time. The results are shown in Fig. 2. When the contents of cosolvents were low (20%, *v*/*v*), except tert-pentanol, there was no great difference about the effect on the amoxicillin yields (20 h) among the selected cosolvents. The yields in these cosolvent mixtures were similar to that in pure water media. However, hexane had the better improvement in the reaction rate than others, and the yield of 12 h was close to that of 20 h. Unexpectedly, tert-pentanol was fully negative to the reaction, and the yield was much lower than that in the buffer, whatever the content of tert-pentanol. Although, the reaction of amoxicillin synthesis from 6-APA and HPGM could be performed smoothly in anhydrous tert-pentanol, the mixture of tert-

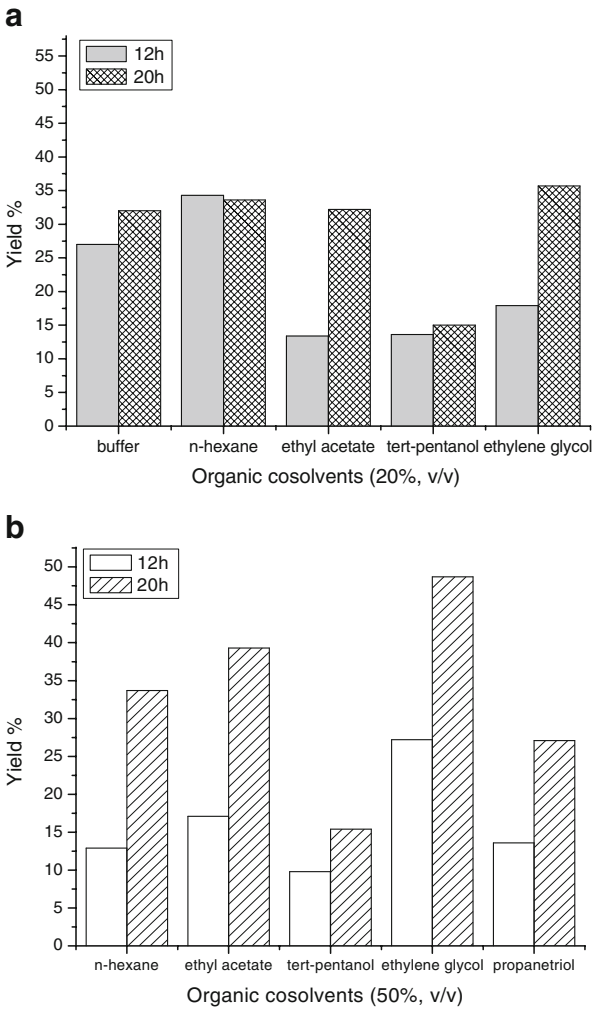


Fig. 2 Effect of organic cosolvents on the enzymatic cascade synthesis of amoxicillin. Reaction conditions: 150 mM PGK and 450 mM HPGM, 25 °C and 50 IU/mL IPA. Reaction time was 12 h or 20 h. **a** 20% content of solvents (*v*/*v*); **b** 50% content of solvents (*v*/*v*)

pentanol/buffer with 20% or 50% tert-pentanol were unfavorable toward the synthesis of amoxicillin [see Fig. 1 of ref. 8]. Herein, the bad result of tert-pentanol may be attributed to the unfavorable effect of tert-pentanol upon the activity of PGA toward the amoxicillin synthesis and PGK hydrolysis. Figure 2b had the similar phenomenon as to Fig. 2a. EG showed the best result and the yield can be improved. Thus, we choose EG as a suitable cosolvent for the further optimization.

Optimization of EG Concentration in Buffer-EG Cosolvent Mixture

The effect of EG content on the enzymatic cascade synthesis of amoxicillin was shown in Fig. 3. The yield of amoxicillin increased from 35% (100% buffer) to 56% (60% EG concentration), then it decreased with the further increase of EG content. The yield in pure EG media was less than 10%. The increase of yield with the EG content increasing was

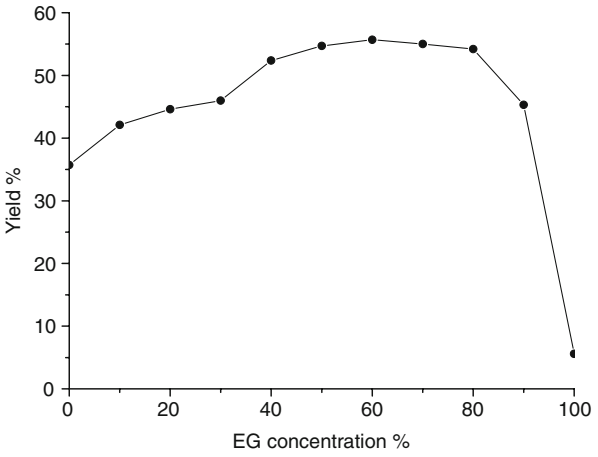


Fig. 3 Effect of ethylene glycol concentration on the enzymatic cascade synthesis of amoxicillin. Reaction conditions: 150 mM PGK and 450 mM HPGM, 25 °C and 50 IU/mL IPA, 16 h, pH 6.0 phosphate buffer with different EG concentration (*v*/*v*)

caused by the reduction of water activity and the incidental suppression of amoxicillin hydrolysis. However, in the full EG media, the hydrolysis of PGK could not carry out efficiently, and this result led to the decrease of amoxicillin yields. Enzyme activity was also inhibited at high organic solvent concentration. For enzymatic synthesis of β-lactam antibiotics under kinetic control, Illanes [28–30] and Wei [31] both reported that EG inhibited the hydrolysis of ampicillin and D-PGM, thus the synthesis yield of the ampicillin could be improved. Sixty percent (*v*/*v*) EG in the reaction mixture was selected as the optimal cosolvent content in the further experiments.

Effect of IPA Catalyst Loading on Cascade Process

Enzyme loading shows a significant influence on the cascade synthesis of amoxicillin in buffer/EG mixture (Fig. 4). Low synthesis yield was obtained when enzyme loading was small. This may be attributed to the lack of the catalysts amount, and the inhibitory effect of

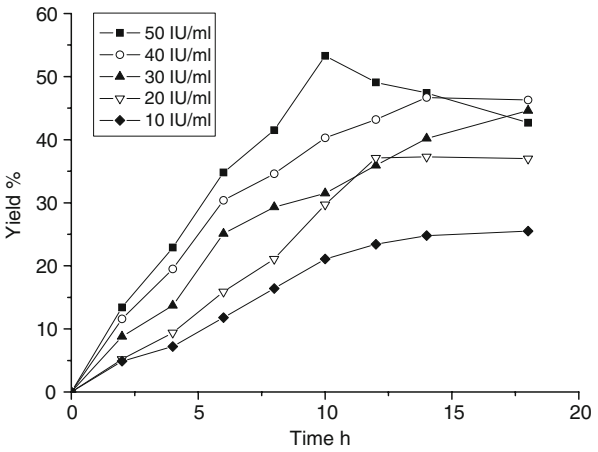


Fig. 4 Effect of enzyme loading on the enzymatic cascade synthesis of amoxicillin. Reaction conditions: 150 mM PGK and 450 mM HPGM, 25 °C and 10-50 IU/mL IPA, reaction media containing 40% pH 6.0 phosphate buffer and 60% EG (*v*/*v*)

organic solvent or phenylacetic acid hydrolyzed from PGK on the PGA [32]. Increasing the enzyme loading provided much enzyme for the synthesis, and the maximum yield of amoxicillin was improved accordingly. However, when the amount of PGA was up to 50 IU/mL, the reaction yield decreased obviously after a long reaction time. This can be explained by the heavy hydrolysis of the amoxicillin and the D-HPGM at the presence of excess enzyme. PGA loading of 50 IU/mL can also save the reaction time, at the same time provide high yields. Continuing the increase of PGA loading could not provide the better results because the reaction system became thick and the substrates dispersion was inefficient, and undesirable results came out. According to the results, 50 IU/ml PGA loading was selected for the further optimization.

Effect of Temperature on Cascade Process

Reaction temperature plays an important role in enzymatic synthesis of antibiotics [4, 26], producing opposite effects on enzyme activity and inactivation rate, and usually low temperature is more beneficial [30]. The effects of temperature on this cascade process were shown in Fig. 5. At 25 °C, the synthesis rate was low so that it needed a long time of 10 h to obtain the maximum yield. Among the three different temperatures, the yield at 25 °C was the best. It was mainly due to the decrease of the rate of product hydrolysis at low reaction temperature. However, the reaction at 35 °C was faster than those at other temperatures because of the high activity of PGA near its optimal temperature. Higher or lower temperatures than 35 °C induced the decreasing of reaction rate, and it needed more time to reach the equilibrium. Compared with 35 °C and 50 °C, the maximal yield was gained at 25 °C after 10 h. Therefore, 25 °C was considered as the optimum temperature with the yield of 52.8%.

Optimal Conditions for Enzymatic Cascade Synthesis of Amoxicillin

On the base of the above optimization, we obtained the optimal reaction conditions for PGA catalyzed cascade synthesis of amoxicillin. Reaction was carried out in the mixture media containing 40% pH 6.0 phosphate buffer and 60% EG (*v*/*v*) at 25 °C for 10 h, 50 IU/mL IPA-

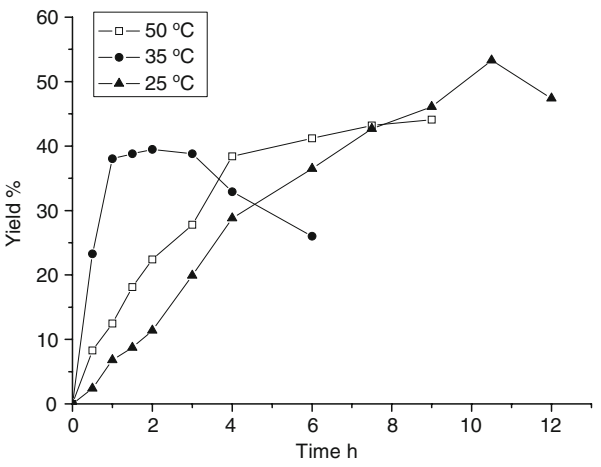


Fig. 5 Effect of temperature on the synthesis of amoxicillin. Reaction conditions: 150 mM PGK and 450 mM HPGM, 50 IU/mL IPA, reaction media containing 40% pH 6.0 phosphate buffer and 60% EG (*v*/*v*), 25 °C, 35 °C and 50 °C

750 was added, and the initial concentration of PGK and D-HPGM were 150 mM and 450 mM, respectively. Yield of product was up to 55.2%.

Recycling of IPA

Repeated utilization of IPA for enzymatic cascade synthesis of amoxicillin in buffer-EG mixture was investigated. The results were shown in Fig. 6. There was no mechanical loss of IPA catalyst since the reaction was conducted in a closed system with full retention of the biocatalyst particles in nine batches. The results implied that the PGA was stable during the nine-batch reaction process and it remained almost consistent activity. According to the linear fit results of the decrease tendency during the repeated utilization of IPA, the half-life value of PGA in this reaction system will be about 310 h. The high half-life value of PGA can permit more repeated utilization in the one-pot synthesis of amoxicillin, thus improve the economics of industrial application.

Conclusions

This work demonstrated that enzymatic cascade synthesis of amoxicillin in buffer/EG mixture media starting from penicillin G potassium salt and *p*-OH-phenylglycine methyl ester could be an effective approach for enzymatic synthesis of β-lactam antibiotics. Ethylene glycol was chosen as the best organic cosolvent after screening. Factors including EG content, IPA loading, reaction temperature, and time were optimized. The best result of 55.2% yield was obtained from the reaction in the system containing 40% pH 6.0 phosphate buffer and 60% EG (*v*/*v*), 50 IU/mL IPA, 150 mM PGK and 450 mM D-HPGM, at 25 °C after a reaction time of 10 h. The IPA could be reused for nine batches without significant losing of catalytic activity. The novel protocol for enzymatic synthesis of amoxicillin will have potential application in industrial production of β-lactam antibiotics. However, further studies are still required to increase the yields, reduce the excess of acyl donor, and improve the synthesis efficiency. A rational redesign of PGA combining high hydrolysis activity with high synthesis activity, and proper immobilization protocols are hopeful to solve this problem.

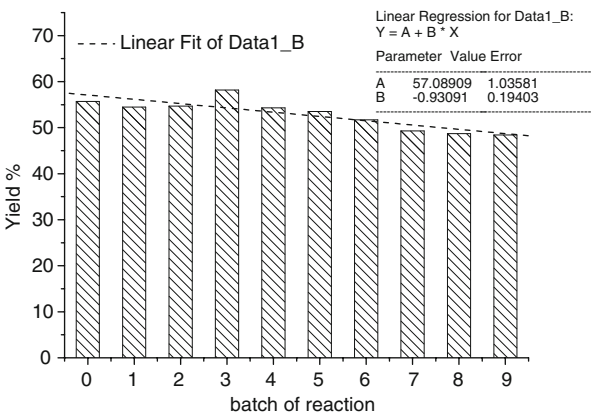


Fig. 6 Repeated utilization of IPA for cascade synthesis of amoxicillin. Reaction conditions: 150 mM PGK and 450 mM HPGM, 50 IU/mL IPA, reaction media containing 40% pH 6.0 phosphate buffer and 60% EG (*v*/*v*), 25 °C, 10 h

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