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A two-step, one-pot enzymatic synthesis of ampicillin from penicillin G potassium salt

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ABSTRACT

A two-step, one-pot synthesis of ampicillin from penicillin G potassium salt (PGK) in aqueous buffer/organic co-solvent has been achieved. Ethylene glycol (EG) was chosen as the organic co-solvent. Factors including co-solvent content, enzyme loading, reaction temperature and substrate concentration were investigated. The optimum conditions were as follow: pH 8.0 phosphate buffer solution, 50% EG (v/v), 25 °C, 100 mM PGK and 300 mM D-phenylglycine methyl ester (D-PGM), 43.2 IU/ml IPA-750. The maximum yield was 57.3% after a reaction time of 17 h. It is the first report about the synthesis of ampicillin from penicillin G potassium salt in one-pot combining the enzymatic hydrolysis and the subsequent enzymatic condensation, and the novel methodology will have important application in the β-lactam antibiotics industry.

1. Introduction

Ampicillin is one of the most widely used β-lactam antibiotics in therapy as it is suitable for a wide spectrum of bacterial infections and has a good level of activity and tolerability. Up to now, many evolutions have been acquired in the enzymatic synthesis of ampicillin and kinetically controlled strategy was demonstrated to be efficient [1–7]. Furthermore, many approaches also have been reported to improve the efficiency of the synthesis. In aqueous medium, the effect of the substrate concentration was investigated [4–9]. It has been shown that high reactant concentrations are favorable for synthesis. Synthesis of ampicillin and cephalexin in highly condensed systems in the absence of a liquid aqueous phase has also been studied [10]. Youshko and Svedas [11] reported that the space-time yield of solid-state enzymatic ampicillin synthesis was shown to be up to ten times higher compared to the homogeneous solutions and heterogeneous systems. Optimizations of pH [6,11–13], ionic strength [12,13] and temperature [13–15] were also evolved to improve the efficiency of the acyl transfer to the β-lactam nucleus.

Recently, a lot of efforts have been made in the enzymatic synthesis of β-lactam antibiotics in the presence of organic co-solvents [14,16–28] to enhance the performance of enzyme, among which ethylene glycol (EG) has been used most widely. Wei and Yang [21] found that EG was a favorable co-solvent in the synthesis of ampicillin. Synthesis of cephalexin and ampicillin in the presence of EG has been studied thoroughly by Illanes et al. [14,22–26]. Although enzymatic synthesis of β-lactam antibiotics in aqueous medium has acquired great results [9], EG exhibits some advantages as the co-solvent in the synthesis of β-lactam antibiotics.

A related concept involving the use of enzymatic cascades that subject a starting compound to a number of consecutive reactions 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. One-pot chemoenzymatic synthesis of 3′-functionalized cephalosporines (cefazolin) by three consecutive biotransformations in fully aqueous medium has been studied by Justiz et al. [29]. High yields were obtained through a careful selection of the enzyme catalyst, experimental conditions, and synthetic strategy. One-pot enzymatic synthesis of cephalexin has been established by Wegman et al. [30]. To our best knowledge, however, there is no report on the synthesis of ampicillin using one-pot method which attracted our interest.

As we known, penicillin G acylase can not only synthesize ampicillin from 6-APA and D-PGM, but also convert penicillin G potassium salt into 6-APA by hydrolysis, which has been applied at the industrial scale widely. These considerations lead us to combine the hydrolysis of PGK into 6-APA with the kinetically controlled enzymatic coupling of 6-APA with D-PGM to give ampicillin as the final product by using a single enzyme (see Fig. 1). The novel strategy can not only save the effort of isolating 6-APA, but also efficiently reduce the industrial cost of ampicillin because of the

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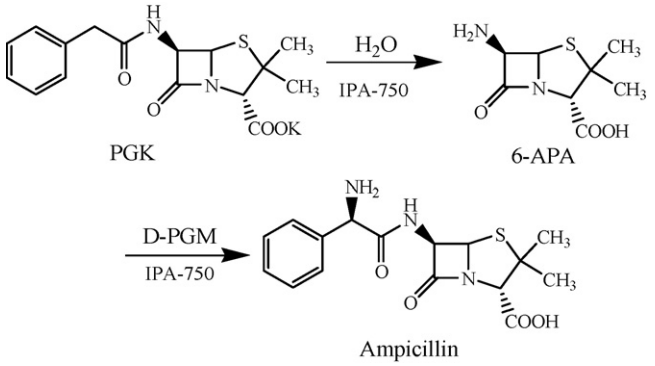

Fig. 1. Two-step, one-pot enzymatic synthesis of ampicillin.

lower price of PGK than that of 6-APA. Here, we report two-step, one-pot enzymatic synthesis of ampicillin catalyzed by IPA in aqueous buffer/organic co-solvent. After examining in detail the effects of the reaction parameters including co-solvent content, reaction time, ratio of D-PGM to PGK, temperature and enzyme loading, on the outcome of the procedure, 57.3% yield were achieved.

2. Materials and methods

2.1. Materials

Immobilized penicillin acylase (IPA-750) from *Escherichia coli* was purchased from Hunan Flag Bio-technology Co. Ltd. (China). By measuring the initial rate of penicillin G hydrolysis (5%, w/v, pH 8 and 25 °C), with declared activity was 108 IU/g. D-Phenylglycine (D-PG) was kindly donated by Zhejiang Apeloa Pharma Co. Ltd (China). PGK was purchased from Tokyo Chemical Industry Co. Ltd. (Japan). EG was analytical grade from Sinopharm Chemical Reagent Co. Ltd. (China). D-PGM·HCl was prepared according to our previous report [31]. All other reagents were analytical grade.

2.2. Synthesis of ampicillin

The two-step, one-pot enzymatic synthesis of ampicillin was carried out in vials agitated at 200 rpm in a temperature-controlled incubator shaker, using PGK and D-PGM as substrates, sodium phosphate buffer and EG as solvent. The reaction was started by adding appropriate amount of enzyme to the reaction mixture (1 ml solvent with substrates). When the reaction was completed, the reaction mixture was added to 49-fold water to ensure that substrates and products were completely dissolved. The solution was subjected to HPLC analysis.

2.3. 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 C_{18} column (150 mm × 4.6 mm). The eluent was composed of 70% (v/v) sodium phosphate buffer (20 mmol dm^{-3}, pH 6.0), 30% (v/v) methanol. The pH of the eluent solution was adjusted with phosphoric acid. The flow rate of the eluent was 1.0 ml min^{-1} and the solutes were detected by the UV detector at 214 nm. Elution times were 3.0, 3.2, 6.0 and 7.0 min for D-PG, 6-APA, ampicillin and D-PGM, respectively. Concentration of substrates and products were calculated from calibration curves using stock solutions. The yield of ampicillin was determined based on the initial concentration of PGK and expressed as a percentage. Every sample of the reactions was performed repeatedly for 3 times and the relative error was less than 5%.

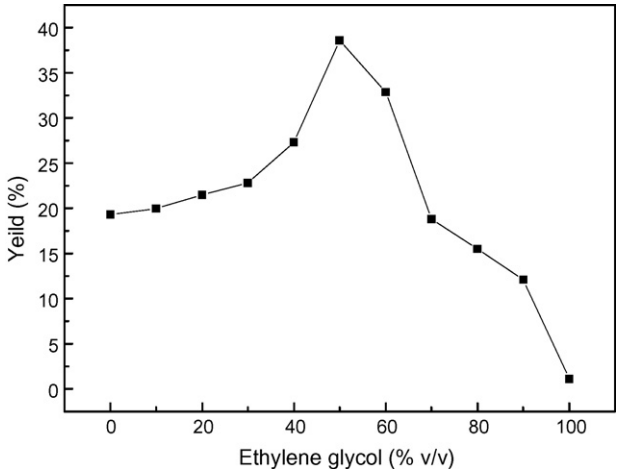

Fig. 2. Effect of co-solvent content on the one-pot enzymatic synthesis of ampicillin in aqueous buffer/EG media. *Conditions*: pH 7.0, 25 °C, 100 mM PGK, 200 mM D-PGM, IPA-750 of 43.2 IU/ml mixture, 12 h.

2.4. Operational stability of biocatalysts

The operational stability of the biocatalysts was determined by measuring the residual activity of hydrolysis at the end of one batch. After washing enzymes with phosphate buffer (pH 8.0) for 5 times and removing solvent, enzymes were added to a solution containing 5% penicillin G (w/w in phosphate buffer, pH 8.0) and the hydrolysis was carried out at 25 °C and 200 rpm for 10 min. The rate of penicillin G hydrolysis in buffer solution was measured to determine the residual enzyme activity.

3. Results and discussion

3.1. Effect of EG on the synthesis of ampicillin

For enzymatic synthesis of β-lactam antibiotics under kinetic control, the yield of product may increase in the presence of EG [14,21–26]. Both Illanes and Fajardo [22,23,25] and Wei and Yang [21] reported that EG inhibited the hydrolysis of both the ampicillin and D-PGM, so the synthesis yield of the ampicillin could be improved. Illanes et al. [14,24–26] also achieved great success in the synthesis of cephalexin in the presence of EG.

So we tried to synthesize ampicillin in one-pot in aqueous buffer/EG co-solvent. The effect of solvent content on the one-pot enzymatic synthesis of ampicillin has been studied. As shown in Fig. 2, the yield of ampicillin varied from 19.3% (in the absence of co-solvent) to 38.6% at 50% (v/v) co-solvent concentration, then the yield decreased with the further increase of EG content. This may be attributed to the following reasons: 1. The hydrolysis of PGK was inhibited at low water concentration. 2. Enzyme activity was also inhibited at high organic solvent concentration. Thus, 50% (v/v) EG in the reaction mixture was selected as the optimal co-solvent concentration in the following experiments.

3.2. Influence of immobilized enzyme loading on the synthesis of ampicillin

As shown in Fig. 3, the concentration of the IPA-750 has a substantial influence on the one pot synthesis of ampicillin. Increased enzyme loading from 32.4 UI/ml to 43.2 UI/ml leads to the increased yield of ampicillin by more than 50%. However further increase of IPA-750 concentration at the same reaction conditions surprisingly results in a suppressed ampicillin synthesis: with enzyme loading 64.8 IU/ml yield falls down nearly to the level observed with 32.4 UI/ml. According to our observation, 43.2 IU IPA-750/ml was

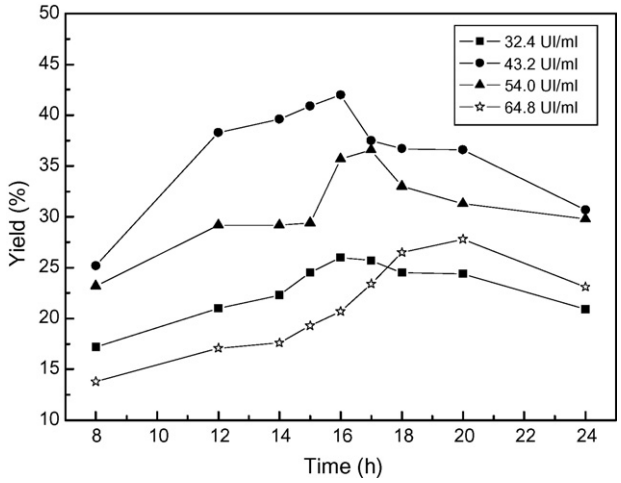

Fig. 3. Time course of the one-pot enzymatic synthesis of ampicillin in different concentration of IPA-750 in aqueous buffer/EG media. *Conditions*: pH 7.0, 50% (v/v) EG, 25 °C, 100 mM PGK, 200 mM D-PGM.

regarded as an appropriate initial concentration for the one-pot enzymatic synthesis of ampicillin in 50% (v/v) EG, with the yield of 42.0 %.

3.3. Effect of pH on the synthesis of ampicillin

The hydrolysis of PGK could be affected by the pH. Thus, sodium phosphate buffers of different pH were investigated for the reaction. As reported [30], at pH 8.0, the initial rate of PGK hydrolysis was improved. This observation led us to consider the influence of pH on the one-pot enzymatic synthesis of ampicillin. The time course of such reactions is shown in Fig. 4, unexpectedly, the pH had few effects on the reaction time when the maximal yield was achieved. However, the yield of ampicillin was increased while pH varied from 6.0 to 8.0. The effects of pH on the one-pot synthesis of ampicillin were performed as the same results of former researches: yield decreased at low pH, which has been predicted for the kinetically controlled synthesis of β-lactam antibiotics in aqueous medium [32] and has been proven experimentally for the synthesis of ampicillin in the presence of co-solvents [22]. As a result, the reaction reached the maximal yield in 52.8% at 17 h when the pH was 8.0.

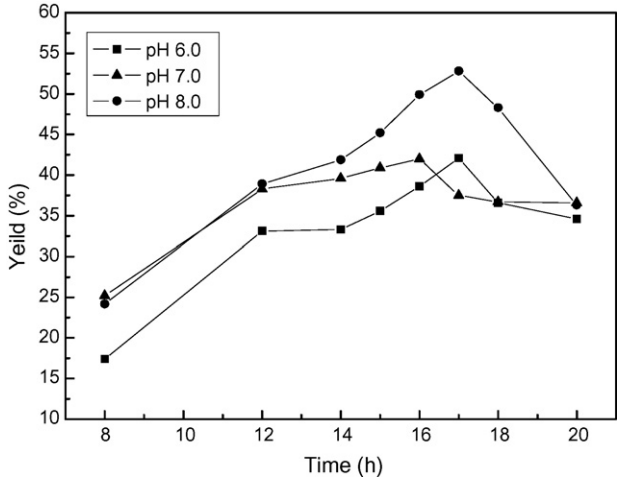

Fig. 4. Time course of the one-pot enzymatic synthesis of ampicillin as a function of pH in aqueous buffer/EG media. *Conditions*: 50% (v/v) EG, 25 °C, 100 mM PGK, 200 mM D-PGM, IPA-750 of 43.2 IU/ml mixture.

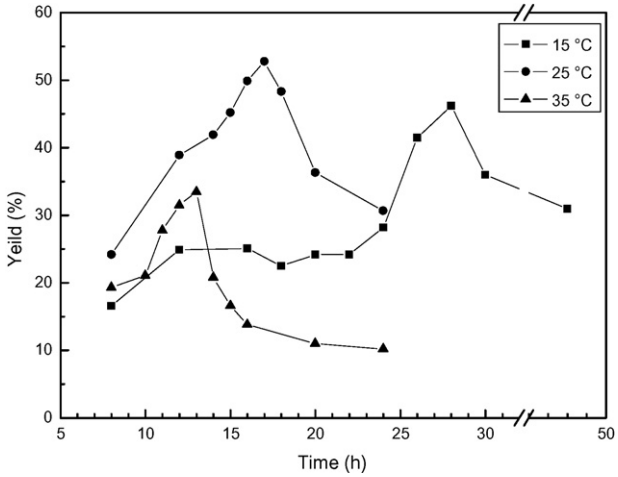

Fig. 5. Time course of the one-pot enzymatic synthesis of ampicillin at different temperatures in aqueous buffer/EG media. *Conditions*: pH 8.0, 50% (v/v) EG, 100 mM PGK, 200 mM D-PGM, IPA-750 of 43.2 IU/ml mixture.

3.4. Effect of temperature on the synthesis of ampicillin

Temperature is a key variable for the enzymatic synthesis. Low temperature is usually more beneficial to enzymatic synthesis of β-lactam antibiotics in aqueous medium [25]. The time courses of the one-pot enzymatic synthesis of ampicillin at different temperatures were shown in Fig. 5.

At 15 °C, the synthesis rate was very low. Thus, it took a long reaction time of 28 h to approach the maximum yield. Then, the yield of the ampicillin decreased because the product was hydrolyzed by the enzyme. However, it took much less time (12 h) to reach the equilibrium at 35 °C, because the activity of the enzyme was improved at higher temperature, while lower yield was obtained due to the enhanced hydrolysis of ampicillin and D-PGM. Compared with those data at 15 °C and 35 °C, the maximal yield was gained at 25 °C after 17 h. Therefore, 25 °C was considered as the optimum temperature for the reaction with the yield of 52.8%.

3.5. Molar ratio of D-PGM/PGK at different initial substrate concentration

Using excess D-PGM is a drawback in the kinetically controlled synthesis of β-lactam antibiotics. However, it has proven to be necessary to attain high yield, mainly because penicillin acylase can also hydrolyze D-PGM to the side product D-PG [26] as an esterase. Therefore, the molar ratio of D-PGM to PGK at different concentrations of PGK was investigated. As shown in Table 1,

Table 1
Effect of molar ratio of D-PGM/PGK at different initial substrate concentration in the one-pot enzymatic synthesis of ampicillin in aqueous buffer/EG media. *Conditions*: pH 8.0, 50% (v/v) EG, 25 °C, IPA-750 of 43.2 IU/ml mixture, 17 h.

Initial concentration of PGK (mM)	Molar ratio of PGK (mM)/D-PGM (mM)	Yield (%)
50	1/1	8.2
	1/2	11.6
	1/3	17.2
	1/4	19.7
100	1/1	20.9
	1/2	52.8
	1/3	57.3
	1/4	58.6
150	1/1	23.4
	1/2	37.5
	1/3	47.9
	1/4	52.7

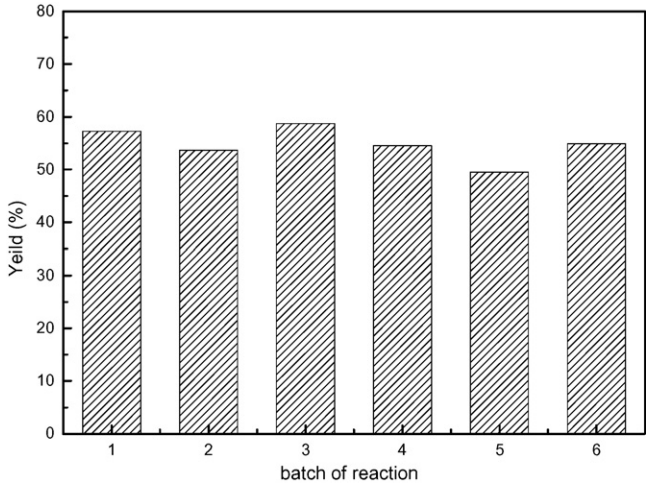

Fig. 6. Synthesis of ampicillin in one-pot under sequential batch with IPA-750. *Conditions*: pH 8.0, 50% (v/v) EG, 25 °C, 100 mM PGK, 300 mM D-PGM.

low yields were obtained at 50 mM PGK with different ratios of D-PGM to PGK. When the initial concentration of PGK was 100 mM, the yield increased from 20.9% to 57.3% when the molar ratio of D-PGM to PGK changed from 1/1 to 1/3. However, there was no evident increase in the yield as the ratio varied from 1/3 to 1/4. It showed that 3 multiple D-PGM was enough for the reaction. The similar results could be observed under the 150 mM initial concentration of PGK. But compared with the former, the yields of ampicillin were brought down possibly because of the lack of enzyme.

In conclusion, 100 mM PGK and 300 mM D-PGM was considered as the optimum substrate concentration for the reaction with the yield of 57.3%.

3.6. Optimal conditions for two-step, one-pot enzymatic synthesis of ampicillin

According to the above results, the optimal reaction conditions were obtained. The two-step, one-pot enzymatic synthesis of ampicillin was carried out in the reaction media being composed of 50% EG and 50% phosphate buffer with pH 8.0 at 25 °C, the initial concentration of PGK and D-PGM were 100 mM and 300 mM, respectively. 43.2 IU/ml IPA-750 was added and reaction time was 17 h. Yield of product was up to 57.3%.

3.7. Repeated utilization of IPA

Repeated utilization of IPA-750 for one-pot enzymatic synthesis of ampicillin in buffer–EG mixture has been investigated. The reaction mixture was filtered after the reaction was completed. IPA-750 was washed with buffer–EG mixture for 5 times. After solvent was removed, IPA was reused as the catalyst for the next batch of reaction. Reactions were carried out in buffer–EG mixture at 25 °C, initial concentration of PGK and D-PGM were 100 mM and 300 mM respectively. 43.2 IU/ml IPA-750 was added and reaction time was 17 h.

As seen from Fig. 6, no mechanical losses were produced since the reaction was conducted in a closed system with full retention of the biocatalyst particles in 6 batches. The result successfully demonstrated the feasibility of the one-pot two-step enzymatic synthesis of ampicillin and provided an attractive protocol for enzymatic synthesis of penicillins from penicillin G salts.

4. Conclusions

A two-step, one-pot enzymatic synthesis of ampicillin in phosphate buffer–EG system has been demonstrated for the first time. D-Phenylglycine methyl ester (D-PGM) and penicillin G potassium salt (PGK) were used as the substrates. EG were employed to improve the synthesis yield. Reaction parameter, including co-solvent content, reaction time, ratio of PGM to PGK, temperature and enzyme loading were studied to evaluate their effect on the reaction. The best result of 57.3% yield was obtained at 25 °C, 43.2 IU/ml IPA-750 under 3/1 D-PGM molar excess, after a reaction time of 20 h.

The novel strategy can not only save the effort of isolating 6-APA, but also efficiently reduce the industrial cost of ampicillin because of the lower price of PGK than that of 6-APA, which showed a great potential in industry. Further studies about the yields, isolating the product and reducing the excess of acyl donor are being conducted in our laboratory.

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