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Journal of Molecular Catalysis B: Enzymatic 54 (2008) 13–18
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
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Efficient enzymatic synthesis of ampicillin in organic media

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Received 15 September 2007; received in revised form 6 December 2007; accepted 6 December 2007
Available online 15 December 2007

Abstract

The kinetically controlled synthesis of ampicillin with immobilized penicillin acylase (IPA) from *Escherichia coli* in fully organic medium was studied. D-phenylglycine methyl ester (D-PGM) was selected as the activated acyl donor due to its good solubility in organic solvents. A series of organic solvents with different polarity were screened and ethyl acetate was found to be the most satisfying solvent for the enzymatic synthesis of ampicillin. Remarkable catalytic activity of the IPA was retained in ethyl acetate, and high yield could be obtained. Furthermore, some significant factors that greatly affect the ampicillin synthesis process, such as substrate concentration, temperature and water content of IPA etc., were investigated in details. As a result, high yield (92.9%) and synthesis/hydrolysis (S/H) ratio (1.50) were successfully achieved under the optimum conditions.

Keywords: Ampicillin; Enzymatic synthesis; Ethyl acetate; Organic medium; Penicillin acylase

1. Introduction

Ampicillin is one of the most widely used β-lactam antibiotics and suitable for a broad spectrum of bacterial infections. The enzymatic synthesis of ampicillin from 6-aminopenicillanic acid (6-APA) and D-phenylglycine (D-PG) with penicillin G acylase from *E. coli* was first reported in 1963 [1]. In the past several decades, kinetically controlled strategy was demonstrated to be efficient in the enzymatic synthesis of ampicillin (Fig. 1). In this strategy, active acyl donor was used, such as ester or amide derivatives of D-PG, usually methyl ester (D-PGM). Many approaches have been reported to improve the efficiency of the synthesis. In aqueous medium, the effect of the substrate concentration was investigated [2–7]. It has been shown that high reactant concentrations are favorable for synthesis. Optimizations of pH [2,8–11], ionic strength [2,8,9] and temperature [9,12] were also evolved to improve the efficiency of the acyl transfer to the β-lactam nucleus.

In the course of the biocatalytic reaction, D-PGM and the enzyme formed an acyl–enzyme complex intermediate first; subsequently 6-APA and water molecules competed in the nucleophilic attack to the intermediate [13–17]. Ampicillin would be produced when 6-APA attacked the intermediate, and D-PG would be obtained when water attacked it. Namely, in the kinetically controlled strategy water acts as competing nucleophile to the acyl–enzyme intermediate so that the hydrolysis is an unavoidable problem. The reduction of water activity in the reaction medium is beneficial, since it depresses the competing hydrolytic reactions in favor of synthesis. Water activity can be conveniently depressed by using organic cosolvents. Methanol [18–20] and ethylene glycol [21,22] were employed as cosolvents of aqueous medium for higher yield and S/H. And yield further increased at low temperature in the presence of ethylene glycol [23].

It could be expected that water activity would be lower in pure organic medium than that in aqueous medium with an organic cosolvent. However, few papers describing the use of pure organic solvents have appeared in the literature, since it is commonly believed that the propensity of the organic solvents to inhibit the enzyme activity and decrease the protein stability makes their use nonviable [24,25]. In addition, it might be another reason that D-phenylglycine methyl ester hydrochloride (D-PGM·HCl) was usually used as the activated acyl donor in the past. In aqueous medium, it does not matter to use D-PGM·HCl or D-PGM as the substrate due to pH titration. But in organic medium, the enzymatic reaction cannot take place using D-PGM·HCl because of its poor solubility in organic solvents and acidity. Basso et al. [26] made D-PGM from D-PGM·HCl and

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doi:10.1016/j.molcatb.2007.12.004

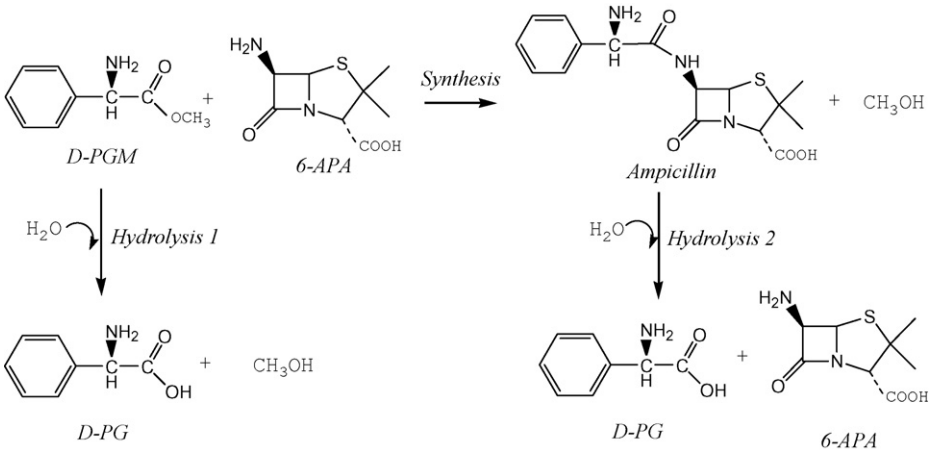

Fig. 1. Kinetically controlled synthesis of semi-synthetic ampicillin with PGA.

used it as the solvent for the enzymatic synthesis of ampicillin in the absence of a liquid aqueous phase. However, their best result was only 32% yield with S/H of 0.99 due to the serious mass transfer limitation which was induced by large viscosity of the reaction system.

Generally, organic solvents have both an inhibitory effect on the enzyme activity and a deleterious effect on the enzyme stability in deed. Nevertheless, by careful selection of organic solvents and use of stable and active enzyme derivatives, it is possible to perform effective enzymatic synthesis in organic phase. There is a significant advantage that the hydrolysis of both activated acyl donor and product can be limited markedly in organic medium.

On the other hand, water molecule is necessary for the enzymatic reaction in organic phase all the same [27]. The activity of the enzyme needs some so-called "essential water" to maintain, and there is optimum water content for the enzyme [28]. Therefore, the reaction system in organic medium cannot be absolutely anhydrous, and the water content of the system is a key variable.

In the present work, the effect of organic solvents with different log *P* was studied. It was shown that log *P* was an important factor which correlated to the synthesis yield of ampicillin but not the only factor. As a result, ethyl acetate was considered as the best organic solvent for the enzymatic synthesis of ampicillin using 6-APA and D-PGM as substrates. Further optimizations of substrate concentration, temperature and the water content of the system and so on were carried out. In the optimal conditions, efficient synthesis of ampicillin in ethyl acetate was achieved for the first time.

2. Materials and methods

2.1. Materials

Immobilized penicillin acylase (IPA-750) from *E. coli* was purchased from Hunan Flag Bio-technology Co. Ltd. (Hunan, China). Declared activity was 108 U/g, measured as initial rate of penicillin G hydrolysis (5% w/v, pH 8 and 28 °C). 6-APA and D-PG were kindly donated by North China Pharma Certical Co. (Shanxi, China) and Zhejiang Apeloa Pharma Co. Ltd. (Zhejiang, China) respectively. All other reagents were of analytical grade and dried by storing over activated 3 Å molecular sieves before use.

2.2. Preparation of D-PGM·HCl and D-PGM

Twenty-two milliliters thionyl chloride (0.3 mol) was slowly added dropwise to a suspension of 30.2 g D-PG (0.2 mol) in 220 ml methanol at 0 °C (a water–ice bath). After the reaction mixture was stirred at room temperature for 18 h, the solution was evaporated under reduced pressure at 50 °C to yield a white solid. The resulting solid was triturated with 250 ml ethyl ether, followed by filtration and washing with ethyl ether. The white powder was dried in vacuum at 50 °C to yield 40.2 g D-PGM·HCl. The yield was almost 100%.

Ten milliliters 4 M NaOH aqueous solution (40 mmol) was added to a solution of 8.06 g D-PGM·HCl (40 mmol) in 50 ml water to eliminate the HCl. Then ethyl acetate in which D-PGM·HCl was almost completely undissolved was used to extract D-PGM. The organic phase was dried with anhydrous $MgSO_4$ for 3 h and filtered. The solution was evaporated under reduced pressure at 50 °C to yield 5.62 g D-PGM, a yellow liquid. The yield was 85%.

The purity of D-PGM·HCl and D-PGM were more than 98% and 95% respectively by HPLC analysis.

2.3. Synthesis of ampicillin

All syntheses of ampicillin were carried out in vials agitated at 200 rpm in a temperature-controlled incubator shaker, using 6-APA and D-PGM as substrates. The reaction was started by adding appropriate amount of enzyme to the reaction mixture (1 ml solvent with substrates). When the reaction was over, 19 ml water was added to dissolve all the substrates and products. The solution was subjected to HPLC analysis. The yield of ampicillin was determined based on the initial concentration of 6-APA and expressed as a percentage, and the S/H ratio was equal to the molar ratio of ampicillin/D-PG. Every sample of the reactions was performed repeatedly for 3 times and the relative error was less than 5%.

Table 1
Enzymatic synthesis of ampicillin in pure organic solvents

Entry	Solvent	Log P[a]	Yield (%)	Entry	Solvent	Log P	Yield (%)
1	Propanetriol	−3.0	2.1	17	Pyridine	0.71	0.0
2	Ethylene glycol	−1.4	0.3	18	Methyl acetate	0.16	1.1
3	Dimethyl sulfoxide	−1.3	0.6	19	Ethyl formate	0.27	1.2
4	Dioxane	−1.1	0.1	20	Ethyl acetate	0.68	37.6
5	Lactic acid	−0.73	0.0	21	Butyl acetate	1.7	14.8
6	Acetonitrile	−0.33	1.9	22	Ethyl butyrate	1.8	17.2
7	Acetone	−0.23	0.2	23	Ethyl oenanthate	3.4	24.0
8	Methanol	−0.76	0.1	24	Ethyl caprate	4.9	34.3
9	Ethanol	−0.24	0.1	25	Ethyl ether	0.85	25.6
10	Chlorethyl alcohol	−0.17	0.1	26	Isopropyl ether	1.5	30.4
11	Isopropanol	0.07	0.6	27	Acetophenone	1.8	20.0
12	*n*-Butyl alcohol	0.82	1.8	28	Dichloromethane	1.2	2.8
13	*n*-Octyl alcohol	2.9	5.0	29	Trichloromethane	2.0	3.4
14	*tert*-Butyl alcohol	0.47	2.4	30	Tetrachloromethane	3.0	18.9
15	*tert*-Pentyl alcohol	1.0	7.7	31	Cyclohexane	3.2	10.0
16	Tetrahydrofuran	0.49	0.1	32	*n*-Hexane	3.5	7.5

Conditions: 100 mM 6-APA, 200 mM D-PGM, 1 ml organic solvent, 100 mg IPA-750, 25 °C, 24 h.
[a] Laane [30].

2.4. Dehydration of IPA-750

There was a rather large amount of water in IPA-750 itself. The initial water content of IPA-750 was measured to be 57% by lyophilization. In other words, the ratio of the weight of the water to the dry weight of the IPA-750 was 133%. In order to investigate the effect of the water content on the reaction, we got a series of enzymes which had different water content by dehydrating IPA-750 with the same original enzyme activity (100 mg IPA-750 per portion) by spontaneous evaporation at 4 °C (the keeping temperature of the enzyme) in the fridge for different time.

One unit of activity was defined as the amount of IPA that hydrolyzed 1 mmol of penicillin G at 25 °C and pH 8 in 0.2 M phosphate buffer.

2.5. 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 with 70% (v/v) sodium phosphate buffer (20 mmol dm^{-3}, pH 6.0), 15% (v/v) methanol and 15% (v/v) acetonitrile. The pH of the eluent solution was adjusted to 6.0 with phosphoric acid. The flow rate of the eluent was 1.0 cm^3 min^{-1} and the solutes were detected by the UV detector at 214 nm.

2.6. Downstream process

Because of the very low solubility of 6-APA, D-PG and ampicillin in ethyl acetate, the mixtures were filtered washing by ethyl acetate when the reactions were over. The liquid containing the unreacted D-PGM could be reused, whereas the solid, including the immobilized enzyme, the reaction product ampicillin, and the unreacted β-lactam nucleus 6-APA, were recovered by selective filtration (to separate IPA-750) and selective precipitations at different pH [4].

3. Results and discussion

3.1. Selection of organic solvents

Generally, organic solvents strongly affected the enzymatic reaction. In order to improve the activity of IPA, some conventional organic solvents with different polarity were screened and the results were shown in Table 1. As shown in Table 1, in relatively polar solvents, having a log $P < 0.5$, the reactions were very poor and the yield was less than 2.5%; while in solvents having a log $P > 0.5$, the yield was quite variable. The reason for the low reaction activity in polar solvents might be that the essential water on the surface of the enzyme was easily deprived by polar solvents. However, there is no direct relationship between enzyme activity and log P, although solvent polarity was a significant parameter for the enzymatic synthesis.

Moreover, as seen from Table 1, in the organic solvents whose molecular structures were similar (entries 10–13, 14–15, 18–24, 25–26, 28–30), a trend was shown that the yield increased as the increase of log P. In these solvents, esters represented relatively good effects. Therefore, we screened different esters such as methyl acetate, ethyl formate, ethyl acetate, butyl acetate, ethyl butyrate, ethyl oenanthate and ethyl caprate for the enzymatic synthesis. As a result, the optimal solvent was ethyl acetate, with the highest yield of 37.6%. Subsequently, the conditions of the enzymatic synthesis of ampicillin in ethyl acetate were optimized.

3.2. Initial substrate concentration

6-APA is almost totally undissolved in ethyl acetate (less than 0.1 mg/ml at 25 °C by HPLC), in which the liquid

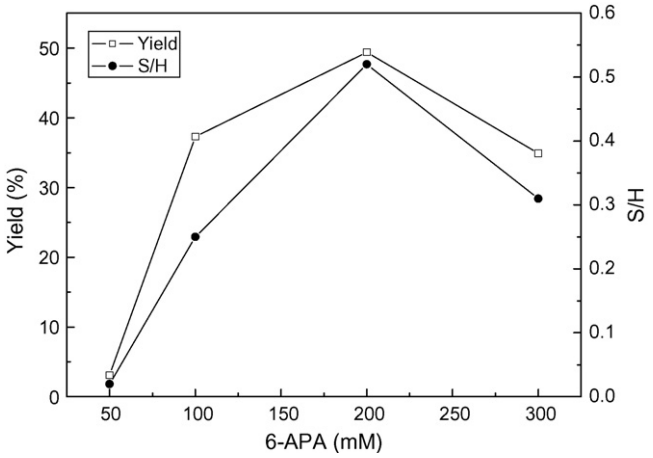

Fig. 2. Effect of initial substrate concentration. Conditions: 25 °C, the D-PGM/6-APA ratio of 2, IPA-750 of 100 mg/ml ethyl acetate, 24 h.

D-PGM dissolved very well. Therefore, the reaction system was a "solution–precipitate" system actually. In order to investigate the influence of the substrate concentration, we measured the yield and S/H ratio of the enzymatic ampicillin synthesis in pure ethyl acetate with different initial concentrations.

Youshko et al. have reported that penicillin acylase-catalyzed ampicillin synthesis via acyl group transfer in aqueous solution was highly dependent on the initial substrate concentration and that higher initial synthesis/hydrolysis rates ($V_{Amp}/V_{D\text{-}PG}$) could be obtained at higher nucleophile concentration [4,7]. In non-aqueous cases, as we can see from Fig. 2, only low synthetic yield and poor S/H ratio were obtained at low nucleophile concentration (50 mM 6-APA). This could be explained that 6-APA in the reaction system at low concentration was less nucleophilic than water in IPA so that heavy hydrolysis of D-PGM and the product ampicillin occured. 6-APA has been reported to be a mixed inhibitor in the hydrolysis of D-PGM. For larger substrates as ampicillin, 6-APA would be a competitive inhibitor [29]. So the increase of the initial 6-APA concentration provided more competitive nucleophilic agent and higher synthetic yield and S/H were obtained. However, it is quite interesting to note that excessive substrates, such as 300 mM 6-APA and 600 mM D-PGM, have contrary effects on the yield and S/H ratio. This may be mainly caused by the inefficiency of substrate pervasion at high concentration. Therefore, the optimal substrate concentration was 200 mM 6-APA and 400 mM D-PGM.

3.3. Molar ratio of D-PGM/6-APA

In aqueous medium, the increase of the D-PGM/6-APA ratio can enhance the conversion of 6-APA. Fig. 3 illustrates that there is the same effect in organic medium. When the molar ratio increased from 0.5 to 2, the yield of ampicillin and S/H were both improved significantly. But when the molar ratio was 3, the S/H was lower than that at the molar ratio of 2, due to more hydrolysis of D-PGM. Thus, the optimal molar ratio of D-PGM/6-APA was 2.

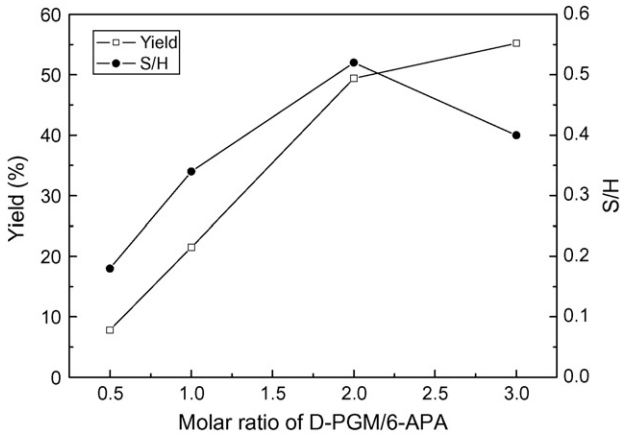

Fig. 3. Effect of the molar ratio of D-PGM/6-APA. Conditions: 25 °C, 200 mM 6-APA, IPA-750 of 100 mg/ml ethyl acetate, 24 h.

3.4. Concentration of IPA-750

The concentration of the enzyme markedly impacts the reaction in organic medium. As shown in Fig. 4, at 200 mM 6-APA and 400 mM D-PGM, there was an apparent lack of enzymatic activity for the synthesis when the concentration of IPA-750 was 50 mg/ml solvent, whereas the hydrolysis was aggravated badly when the IPA concentration was more than 200 mg/ml solvent.

As a result, the condition of 200 mM 6-APA, 400 mM D-PGM and 100 mg IPA-750/ml solvent, was regarded as an appropriate initial condition for the enzymatic synthesis of ampicillin in ethyl acetate, with the yield of 49.5% and the S/H of 0.52.

3.5. Effect of temperature

In aqueous medium, temperature is a key variable for the enzymatic synthesis and low temperature is more beneficial [23]. So the influence of the reaction temperature in organic medium was also studied. The time courses of the reactions in ethyl acetate at five temperatures were shown in Fig. 5.

At 50 °C, there were almost no reactions of the synthesis and hydrolysis. It was implied that the activity of the enzyme would

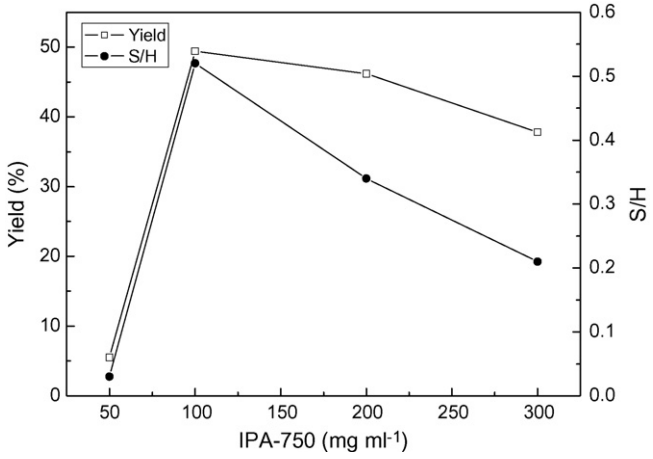

Fig. 4. Effect of IPA-750 concentration. Conditions: 25 °C, 200 mM 6-APA, 400 mM D-PGM, 24 h.

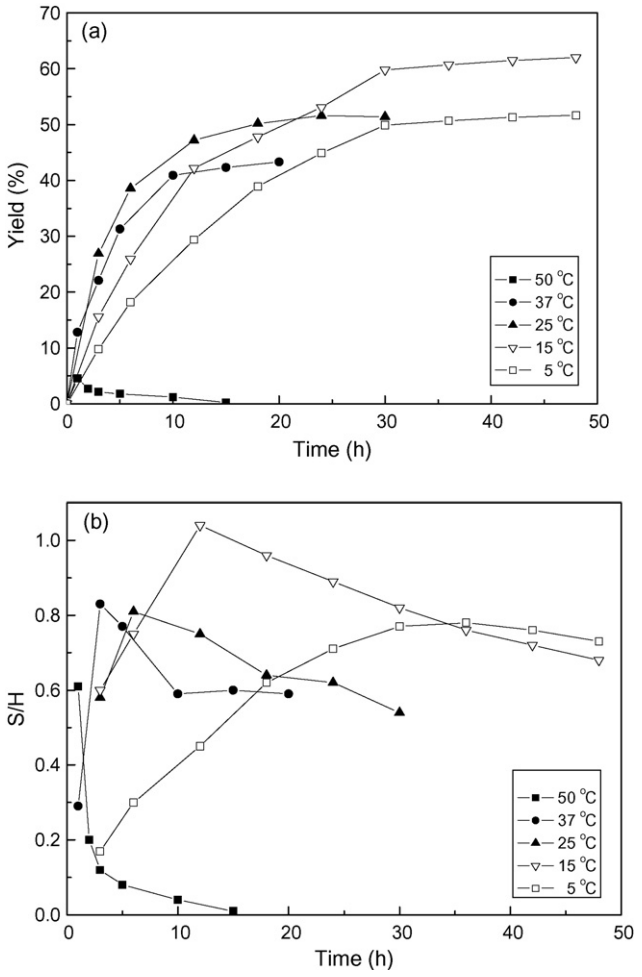

Fig. 5. Time courses of the yield (a) and S/H ratio (b) of ampicillin synthesis in ethyl acetate at different temperatures with 200 mM 6-APA, 400 mM D-PGM and IPA-750 of 100 mg/ml solvent.

decrease badly if the temperature was too high. At 37 °C, the yield increased with time increasing, and approached the equilibrium at 10 h. But the IPA activity was still restrained partially. At 25 °C, higher yield was obtained than at 37 °C. Compared with those obtained at 25 °C, both of the yield and S/H ratio increased but the reaction velocity decreased at 15 °C. When temperature was lower, such as 5 °C, the yield was close to that at 25 °C with higher S/H, but the reaction velocity was much lower. It was suggested that the velocities of the synthesis and hydrolysis both fell at low temperature, and the hydrolysis was depressed more significantly than the synthesis.

In conclusion, 15 °C was considered as the optimum temperature for the yield and S/H, but was not as good as 25 °C for the reaction velocity. The optimal reaction time was 30 h at 15 °C with the yield of 59.8% and the S/H of 0.82, while it was 12 h at 25 °C with the yield of 47.2% and the S/H of 0.75.

3.6. Influence of the water content of IPA

In organic medium, the enzyme still needs certain amount of water which is called "essential water" to maintain its activated conformation [27,28]. On the other hand, excess water

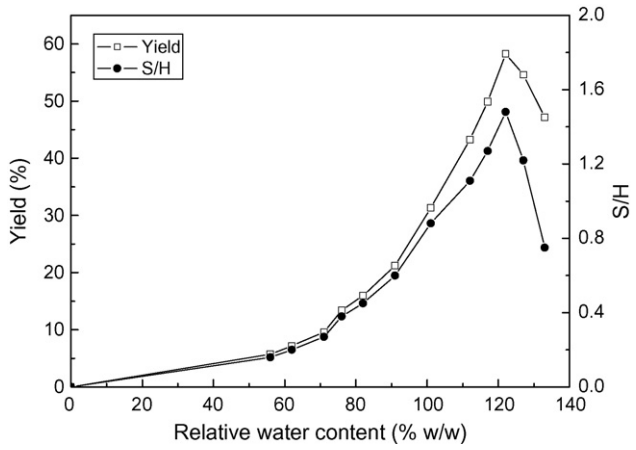

Fig. 6. Influence of the water content of the enzyme. Conditions: 25 °C, 200 mM 6-APA, 400 mM PGM, 100 mg IPA-750/ml solvent, 12 h.

content would favor the hydrolytic side reactions and depress the synthetic reaction.

As shown in Fig. 6, the yield increased with the increase of the relative water content (defined as the ratio of the water weight per 100 mg IPA-750 to the dry weight), reaching the top point at the relative water content of 122%. However, the further increase of relative water content led to a rapid decline of synthetic yield on the contrary. The S/H ratio had the same trend as the yields. It can be concluded that the optimum relative water content was 122%, namely water content of 55%, with the yield of 58.3% and the S/H of 1.48. This suggested that the majority of the water in IPA-750 was the essential water and the other minority would actively take part in the hydrolytic reactions. Therefore, when the relative water content decreased from 133 to 122%, both the yield and S/H ratio were improved observably, owing to the water which competed in the nucleophilic attack to the acyl–enzyme complex intermediate with 6-APA decreased evidently. However, when more water was deprived, there would be not enough water to retain the enzyme activity, depressing both of the synthesis and hydrolysis, so the yield and S/H ratio decreased rapidly. When IPA-750 was lyophilized completely, the enzymatic reactions were totally restrained.

3.7. Optimal conditions

On the base of the above optimizations, we investigated the ampicillin synthesis in ethyl acetate at 15 °C with the IPA-750 whose water content was 55% (Table 2). All the reaction time was 30 h.

Results reported in Table 2 show that the enzymatic synthesis reaction was improved greatly by the increase of the

Table 2
Enzymatic synthesis of ampicillin in ethyl acetate at 15 °C

Entry	6-APA (mM)	D-PGM (mM)	IPA-750 (mg)	Yield (%)	S/H
1	200	400	100	66.2	1.65
2	300	600	100	57.0	1.49
3	300	600	200	82.2	1.58
4	300	600	300	92.9	1.50

substrate concentration at 15 °C. Higher concentration of D-PGM induced higher conversion of 6-APA (namely the yield of ampicillin), with a slight decrease of S/H. And the optimal amount of the enzyme for the reaction should increase as the increase of substrate concentration. The optimal reaction conditions (entry 4) were 300 mM 6-APA, 600 mM D-PGM and 300 mg IPA-750/ml ethyl acetate at 15 °C for 30 h. Both of the highest yield and S/H ratio, 92.9 and 1.50% respectively, were obtained under the optimal conditions.

4. Conclusions

This work demonstrated that enzymatic reaction in organic medium could be an effective approach for ampicillin synthesis with high yield and S/H value. We have studied 32 organic solvents with different polarity. It could be concluded that the enzymatic synthesis of ampicillin would be effective in relatively apolar organic solvents, among which ethyl acetate was the best. High substrate concentration and appropriate water content of IPA could significantly improve the yield and S/H ratio, while 15 °C was regarded as the optimal temperature. Moreover, the downstream process would be simpler than that in aqueous medium owing to the direct reuse of the acyl donor.

Acknowledgements

We gratefully acknowledge the Zhejiang Provincial Science and Technology Council (project no. 2005C11023-03) for financial support.

References

[1] M.A. Wegman, M.H.A. Janssen, F. van Rantwijk, R.A. Sheldon, Adv. Synth. Catal. 343 (2001) 559.
[2] M.I. Youshko, G.G. Chilov, T.A. Shcherbakova, V.K. Svedas, Biochim. Biophys. Acta: Proteins Proteomics 1599 (2002) 134.
[3] B.Y. Golobolov, I.L. Borisov, V.M. Belikov, V.K. Svedas, Biotechnol. Bioeng. 32 (1987) 866.
[4] M.I. Youshko, L.M. van Langen, E. de Vroom, H.M. Moody, F. van Rantwijk, R.A. Sheldon, V.K. Svedas, J. Mol. Catal. B: Enzym. 10 (2000) 509.
[5] M.I. Youshko, V.K. Svedas, Biochemistry-Moscow 65 (2000) 1367.
[6] M.I. Youshko, L.M. van Langen, E. de Vroom, F. van Rantwijk, R.A. Sheldon, V.K. Svedas, Biotechnol. Bioeng. 73 (2001) 426.
[7] M.I. Youshko, H.M. Moody, A.L. Bukhanov, W.H.J. Boosten, V.K. Svedas, Biotechnol. Bioeng. 85 (2004) 323.
[8] V. Kasche, B. Galunsky, Biochem. Biophys. Res. Commun. 80 (1982) 1215.
[9] A. Schwartz, D. Steinke, M.R. Kula, C. Wandrey, Biotechnol. Appl. Biochem. 12 (1990) 188.
[10] S. Ospina, E. Barzana, O.T. Ramirez, A. Lopez-Munguia, Enzyme Microb. Technol. 19 (1996) 462.
[11] M.I. Youshko, L.M. van Langen, E. de Vroom, F. van Rantwijk, R.A. Sheldon, V.K. Svedas, Biotechnol. Bioeng. 78 (2002) 589.
[12] L.M. van Langen, E. de Vroom, F. van Rantwijk, R.A. Sheldon, FEBS Lett. 456 (1999) 89.
[13] H.J. Duggleby, S.P. Tolley, C.P. Hill, E.J. Dodson, G.D. Dodson, P.C.E. Moody, Nature 373 (1995) 264.
[14] C.E. McVey, M.A. Walsh, G.G. Dodson, K.S. Wilson, J.A. Brannigan, J. Mol. Biol. 313 (2001) 139.
[15] W.B.L. Alkema, C.M.H. Hensgens, E.H. Kroezinga, E. de Vries, R. Floris, J.M. van der Laan, B.W. Dijkstra, D.B. Janssen, Protein Eng. 13 (2000) 857.
[16] E.M. Gabor, E.J. de Vries, D.B. Janssen, Enzyme Microb. Technol. 36 (2005) 182.
[17] M.P.A. Ribeiro, A.L.O. Ferreira, R.L.C. Giordano, R.C. Giordano, J. Mol. Catal. B: Enzym. 33 (2005) 81.
[18] M.G. Kim, S.B. Lee, J. Mol. Catal. B: Enzym. 1 (1996) 181.
[19] M.G. Kim, S.B. Lee, J. Mol. Catal. B: Enzym. 1 (1996) 201.
[20] R. Fernandez-Lafuente, C.M. Rosell, J.M. Guisan, Enzyme Microb. Technol. 23 (1998) 305.
[21] A. Illanes, A. Fajardo, J. Mol. Catal. B: Enzym. 11 (2001) 587.
[22] D.Z. Wei, L. Yang, J. Chem. Technol. Biotechnol. 78 (2003) 431.
[23] C. Aguirre, P. Opazo, M. Venegas, R. Riveros, A. Illanes, Process Biochem. 41 (2006) 1924.
[24] V.V. Mozhaev, Y.L. Khmelnitsky, M.V. Sergeeva, A.B. Belova, N.L. Klyachko, A.V. Levashov, K. Martinek, Eur. J. Biochem. 184 (1989) 597.
[25] C.M. Rosell, M. Terreni, R. Fernandez-Lafuente, J.M. Guisan, Enzyme Microb. Technol. 23 (1998) 64.
[26] A. Basso, P. Spizzo, M. Toniutti, C. Ebert, P. Linda, L. Gardossi, J. Mol. Catal. B: Enzym. 39 (2006) 105.
[27] A.M. Klibanov, Trends Biochem. Sci. 14 (1989) 141.
[28] J.S. Dordic, Biotechnol. Progr. 8 (1992) 259.
[29] V. Kasche, U. Haufler, R. Zöllner, Hoppe-Seyler's Physiol. Chem. 365 (1984) 1435.
[30] C. Laane, S. Boeren, K. Vos, C. Veeger, Biotechnol. Bioeng. 30 (1987) 81.