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Article

Enzymatic resolution of epichlorohydrin catalyzed by whole cells in an organic solvent/buffer biphasic system

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ABSTRACT

The biocatalytic resolution of (*R,S*)-epichlorohydrin to (*R*)-epichlorohydrin by whole cells from a recombinant *Escherichia coli* BL21(ED3) expressing epoxide hydrolase activity in a series of organic solvent/buffer biphasic systems was studied. Isooctane was found to be the best organic solvent. The optimum volume ratio of the organic phase to the aqueous phase, reaction temperature, pH, substrate concentration, cell concentration, and reaction time were 7:3, 30 °C, 8.0, 574 mmol/L, 0.07 g/ml wet cells, and 45 min, respectively. Under the optimized conditions, a yield of 37.5% with 99.3% ee and an average productivity of 0.286 mol/(L·h) were obtained in a stirred reactor (1-L scale). Thus, the bioconversion was more efficient in the isooctane/buffer biphasic system than that in a monophasic aqueous system, where the substrate concentration and the average productivity of (*R*)-epichlorohydrin were improved by 55.2% and 98.6%, respectively.

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1. Introduction

Epichlorohydrin (ECH) is an important organic intermediate. As a promising enantiopure epoxide, (*R*)-ECH and (*S*)-ECH are important C₃ chiral building blocks for applications in medical, pesticide, chemical, material, and many other industries [1,2]. For example, (*R*)-ECH serves as a key chiral intermediate for the synthesis of angina pectoris drugs, such as metoprolol and alprenolol [3,4]. (*S*)-ECH is one of the precursors of statins, which is the highest selling cholesterol-lowering drug with global sales of \$20 billion [5].

In recent years, because of maturing technology and the adaptation of a large-scale synthesis of ECH from glycerol, which is a by-product of biodiesel, the market price of ECH has fallen. Therefore, one of the most economic ways for preparing chiral ECH is the kinetic resolution of racemic ECH. A number of researchers have reported the biocatalytic resolution of ECH by

epoxide hydrolase because of its high stereoselectivity and mild reaction conditions [6]. Choi et al. [7] reported the enantioselective hydrolysis of racemic ECH for the production of (*S*)-ECH using *Aspergillus niger* cells. (*S*)-ECH could be obtained from its racemate (60 mmol/L) with an optical purity of 100% enantiomeric excess (ee) in 20% yield. Kim et al. [8] investigated enantioselective hydrolysis for preparing (*R*)-ECH with an enantiopurity of 100% ee and a yield of 26% from 50 mmol/L racemate mixture using *Pichia pastoris* recombinantly expressing the enantioselective epoxide hydrolase from *Rhodotorula glutinis*. Woo et al. [9] demonstrated the chiral resolution of racemic ECH to prepare (*S*)-ECH using the enantioselective epoxide hydrolase from *Novosphingobium aromaticivorans* with a 99.99% ee and yield of 20.7% (theoretical, 50%) from a 500 mmol/L racemate mixture. However, because of the low substrate concentration and low yield, some improvements are still necessary prior to practical applications of

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these techniques.

Escherichia coli strain expressing epoxide hydrolase from *Agrobacterium radiobacter* AD1 was constructed in our lab in a previous study [10]. The epoxide hydrolase was highly stereoselective and could prepare (*R*)-ECH from ECH. However, the epoxide would easily undergo spontaneous hydrolysis when the reaction was performed in an aqueous system, which leads to an inefficient reaction and the production of by-products, which were toxic to the biological catalyst [11]. An organic solvent/buffer biphasic system provides a very promising alternative to overcome this shortcoming [12–15]. In this study, *E. coli* cells recombinantly expressing an epoxide hydrolase were employed as whole cell catalysts for the enantioselective resolution of ECH to prepare (*R*)-ECH in an organic solvent/buffer biphasic system. The influence of the biphasic system on the reaction is systematically discussed herein.

2. Experimental

2.1. The cultivation of whole cells and their preparation

The recombinant *E. coli* expressing the epoxide hydrolase gene (GenBank: Y12804.1) from *Agrobacterium radiobacter* AD1 was reported previously and was preserved in our lab [10]. The cultivation of recombinant *E. coli* BL21 (DE3) was described previously [16]. The cells were harvested by centrifugation (10000 r/min × 10 min, 4 °C), washed twice with physiological saline, stored at –20 °C until further use.

2.2. Bio-resolution of ECH in a biphasic system

Racemic ECH, (*S*)-ECH and (*R*)-ECH (99%) were purchased from FuChen Chemical Co, Ltd. (Xi'an, China). All other chemicals were from commercial sources and of analytical or chromatographic grade. A suitable amount of wet cells, a certain volume of phosphate buffer (0.2 mol/L, pH 8.0), and isooctane were added into a 50 ml bottle with a stopper. Then a suitable amount of racemic ECH was added and stirred (400 r/min) for 15 min. After sampling and centrifugation (12000 r/min × 2 min), ethyl acetate was added for extracting ECH for further GC analysis.

Samples were analyzed using a GC-14A gas chromatograph (Shimadzu, Japan) equipped with an FID detector and a chiral chromatographic column BGB-175 (30 m × 0.25 mm × 0.25 m). The temperatures of the injector and flame ionization detector were set to 220 °C. The column oven temperature was maintained at 90 °C for 7 min. The carrier gas was He (1.6 ml/min), the flow rate was 1.6 ml/min, and the split ratio was 1:40. The retention times were 5.3 min for (*R*)-ECH and 5.6 min for (*S*)-ECH.

The molar yield (*Y*), average productivity (*Y*₁), and ee were calculated according to the residual ECH. The molar yield was determined from $Y = c/c_0 \times 100\%$, where *c*₀ and *c* denote the beginning and the ending molar concentration of ECH, respectively. Average productivity was calculated from $Y_1 = c/t$, where *c* and *t* denote the molar concentration of (*R*)-ECH and the end of the reaction time, respectively. ee was calculated from $ee =$

$(c_R - c_S)/(c_R + c_S) \times 100\%$, where *c*_R and *c*_S denote the molar concentration of (*R*)-ECH and (*S*)-ECH, respectively.

3. Results and discussion

3.1. Selection of organic solvents

Generally, organic solvents have toxic effects on biological cells and enzymes, which result in decreased biological activities. Six kinds of hydrophobic organic solvents (isobutanol, isopropanol, cyclohexane, isooctane, *n*-heptane, and *n*-hexane) were tested as the organic phase for the biphasic system, and their effects on the resolution of (*R,S*)-ECH to (*R*)-ECH catalyzed by whole cells from a recombinant *E. coli* system were studied.

The ability of a solvent to sufficiently dissolve the substrate is an important factor to be considered when selecting it [17]. The solubility of ECH in the six tested organic solvents was investigated in our study. The solubility values (20 °C) of ECH were 22.1, 18.5, and 19.0 g/100 g solvent in isooctane, *n*-heptane, and *n*-hexane, respectively, and it was miscible with isobutanol, isopropanol, and cyclohexane. Thus, compared to its low solubility in an aqueous system (6.58), organic solvents are more efficient for dissolving ECH. The second criterion that must be considered when selecting an organic solvent is its biocompatibility. As shown in Fig. 1, various organic solvents had different effects on the yield and ee. Isobutanol and isopropanol were miscible with ECH, however, the ee of (*R*)-ECH was almost zero when either was used as the reaction medium. The reason may be due to the toxicity of these solvents on the epoxide hydrolase, which decreased the overall enzymatic catalytic activity. The molar yields were much lower for cyclohexane, *n*-heptane, and *n*-hexane although the ee values were higher. It is important to note that the molar yield and ee were both higher when using isooctane as the reaction medium, which indicated that isooctane was biocompatible with the epoxide hydrolase. Bottalla et al. [18] and Gong et al. [11] also found that isooctane was biocompatible with epoxide hydro-

Fig. 1. Effects of various organic solvents on the yield and enantiomeric excess (ee) of the biocatalytic resolution of (*R,S*)-ECH for the production of (*R*)-ECH in the biphasic system using recombinant *E. coli* cells expressing epoxide hydrolase. Reaction conditions: 30 ml organic solvent, 127.6 mmol/L ECH, 0.01 g/ml of *E. coli* cells, 30 °C, 400 r/min.

Fig. 2. Effect of isooctane volume ratio on the initial reaction rate of the biocatalytic resolution of (*R,S*)-ECH. Reaction conditions: 30 ml biphasic solution containing different volumes of isooctane and buffer (0.2 mol/L, pH 8.0), 127.6 mmol/L ECH, 0.01 g/ml of *E. coli* cells, 30 °C, 400 r/min.

lases expressed by *Bacillus megaterium* ECU1001 and *Aspergillus niger*. Thus, isooctane was selected as the organic solvent for the biphasic system.

3.2. Effect of volume ratio of the organic and aqueous phase

The volume ratio of the organic and aqueous phase was defined as V_o/V_a (V/V), where V_o is the volume of organic solvent and V_a is the volume of the aqueous phase. A change in V_o/V_a is thought to affect the interfacial area of the biphasic system, and in turn, the biological catalytic rate [19]. In our study, the effect of V_o/V_a on the reaction was explored with the initial reaction rate being the study target. As shown in Fig. 2, the initial reaction rate increased as the volume of isooctane increased. In addition, there was a sharp decline in reaction rate when the volume ratio was over 70%. Generally, once in contact with the organic solvent, enzymes and living cells always experience some degree of denaturation and deactivation. In the biphasic system, most of the substrate ECH existed in the organic phase, therefore, its low concentration in the aqueous phase led to a decrease in toxic and inhibition effects on the cells. However, an excess of organic solvent will disrupt the water film on the enzyme surface and the enzyme activity will be reduced or even lost [20]. According to the change of the initial reaction rate, 7:3 (V/V) was determined to be the optimum V_o/V_a in our study.

The partition coefficients of ECH and the byproduct 3-chloro-1,2-propanediol (MCH) in the isooctane/buffer biphasic system are shown in Table 1. In the range of tested V_o/V_a , the partition coefficients of ECH improved rapidly with an in-

Table 1

Partition coefficients (*K*) of (*R,S*)-ECH and (*R,S*)-MCH in the two-phase system.

V_o/V_a	<i>K</i> (ECH)	<i>K</i> (MCH)
1:9	1.152	0.001
3:7	1.434	0.001
5:5	1.774	0.001
7:3	1.824	0.001
9:1	1.987	0.001

Fig. 3. Effect of temperature on the biocatalytic resolution of (*R,S*)-ECH. Reaction conditions: 21 ml isooctane, 9 ml buffer (0.2 mol/L, pH 8.0), 127.6 mmol/L ECH, 0.01 g/ml of *E. coli* cells, 400 r/min.

crease of isooctane volume while those of MCH were almost zero. This indicates that the organic solvent not only provided the interface of the biphasic system but was also used as the solvent to extract the product (*R*)-ECH effectively while leaving MCH in the aqueous phase. Thus, the use of an isooctane/buffer biphasic system could simplify the separation and purification of the product (*R*)-ECH.

3.3. Effect of resolution temperature

Reaction temperature is a key parameter in bioprocesses. It can significantly influence the activity, enantioselectivity, and stability of a biocatalyst as well as the reaction equilibrium [21]. Changes in the catalytic activity of the whole cells were studied from 20 to 40 °C. As shown in Fig. 3, when the temperature was gradually increased from 20 to 30 °C, the initial reaction rate and ee significantly increased, and both reached a maximum at 30 °C. With further increasing in temperature, the initial reaction rate and ee began to decrease rapidly. The increase of temperature facilitates the binding of ECH by the enzyme while the collision probability between the enzyme and substrate will be increased. Both effects will result in an increase of reaction rate. Too high temperature causes damage to the spatial structure of the enzyme and decreases the activity of the cells, thus leading to decreases of ee and reaction rate. Therefore, 30 °C was determined to be the optimum reaction temperature, which was in accordance with that of epoxide hydrolase found by Woo et al. [9] and Lee et al. [22].

ECH in the aqueous phase is unstable and prone to spontaneous hydrolysis resulting in a low yield of (*R*)-ECH [23]. The spontaneous hydrolysis rates of ECH in an aqueous phase system and a biphasic system were compared. As shown in Fig. 4, the spontaneous hydrolysis rate of ECH was 7.1% at 30 °C in the aqueous phase system and 4.9% in the biphasic system. As the temperature increased, the differences of the spontaneous hydrolysis rate between the two systems became more obvious. For example, the hydrolysis rate was 10.1% in the aqueous phase system while it was 7.2% in the biphasic system at 40 °C. The spontaneous hydrolysis rate in the isooctane/buffer bipha-

Fig. 4. Spontaneous hydrolysis rate of ECH in two different systems. Aqueous phase system: 30 ml buffer (0.2 mol/L, pH 8.0), 255.1 mmol/L ECH, 60 min, 400 r/min. Biphasic system: 21 ml isooctane, 9 ml buffer (0.2 mol/L, pH 8.0), 255.1 mmol/L ECH, 60 min, 400 r/min.

sic system was obviously lower than in the aqueous phase system, which indicates that the biphasic system could inhibit the spontaneous hydrolysis of the substrate and improve the yield of the chiral product.

3.4. Effect of buffer pH

It is well known that a catalytic reaction usually occurs in the aqueous phase, which is also called the catalyst surplus phase. Variations in pH can alter the ionic state of the reaction substrates and enzymes as well as the enzymatic activity [24]. The effect of the pH of the aqueous buffer on the catalytic reaction rate was investigated at pH = 7.0–9.0 in the biphasic system.

As shown in Fig. 5, the initial reaction rate and ee increased gradually with pH reaching the maximum at pH 8.0, and then decreased dramatically. This indicates that the cells had a maximum catalytic activity at pH 8.0, in accordance with that of epoxide hydrolase previously reported [8,25,26]. Accordingly, pH 8.0 was determined to be optimum for further experiments.

Fig. 5. Effect of pH on the biocatalytic resolution of ECH. Reaction conditions: 21 ml isooctane, 9 ml buffer (0.2 mol/L, pH 8.0), 127.6 mmol/L ECH, 0.01 g/ml of *E. coli* cells, 30 °C, 400 r/min.

Fig. 6. Effect of cell concentration on the biocatalytic resolution of (R,S)-ECH. Reaction conditions: 21 ml isooctane, 9 ml buffer (0.2 mol/L, pH 8.0), 574 mmol/L ECH, 30 °C, 400 r/min.

3.5. Effect of cell concentration

Cell concentration is directly related to enzyme quantity and catalytic efficiency. Based on the same substrate concentration, if the higher the cell concentration, the faster the reaction rate. However, excessive biomass affects the mass transfer [27]. In our study, the cell concentration was altered while keeping the substrate concentration constant. Figure 6 shows that the ee, yield, and initial reaction rate all increased with increasing cell concentration with the maximum at 0.07 g/ml cells. At higher cell concentrations, the initial reaction rate started to decrease although the yield and ee were not significantly affected. These results indicated an overdose of catalyst. Taking into account the cost and efficiency of the process, a cell concentration of 0.07 g/ml was determined to be optimum for further studies.

3.6. Effect of substrate concentration

The substrate concentration is an important factor in kinetic resolution because a high concentration is toxic to cells and enzymes while a low concentration leads to a low reaction rate [28]. In the isooctane/buffer biphasic system, the effect of substrate concentration on the reaction was investigated with ee values as the target. As shown in Fig. 7, the biocatalytic resolution of (R,S)-ECH to (R)-ECH by whole cells was achieved with ee values of more than 99% when the substrate concentration was increased from 382.7 to 574 mmol/L. When the substrate concentration was further increased, the ee values decreased to 46.2% at 637.8 mmol/L and 18.9% at 765.4 mmol/L. These results might be explained by the fact that the substrate ECH is a non-natural substrate with poor biocompatibility, which is toxic to the cells at higher concentrations and influences the catalytic activity and enantioselectivity of the biocatalyst. Thus, the optimum concentration of ECH was determined to be 574 mmol/L.

3.7. Bio-resolution of ECH in a mechanically stirred reactor

Fig. 7. Effect of substrate concentration on the biocatalytic resolution of (*R,S*)-ECH. Reaction conditions: 21 ml isooctane, 9 ml buffer (0.2 mol/L, pH 8.0), 0.07 g/ml of *E. coli* cells, 30 °C, 90 min, 400 r/min.

According to the optimized conditions, 1 L of reaction liquid (isooctane/phosphate buffer (pH 8.0) volume ratio = 7/3) containing 0.07 g/ml wet cells and 574 mmol/L racemic ECH was added into a 2 L mechanically stirred reactor and subjected to continuous sampling and testing at 30 °C. As shown in Fig. 8, the cells showed a high stereoselectivity for ECH. The hydrolysis conversion rate of (*S*)-ECH into MCH was significantly faster than that of (*R*)-ECH. After terminating the reaction at 45 min, the concentration of (*R*)-ECH was 215 mmol/L, the molar yield was 37.5%, the ee was 99.3%, and the average productivity was 0.286 mol/(L·h). Compared with the aqueous monophonic system, the substrate concentration of ECH in the isooctane/buffer biphasic system was increased from 369.9 to 574 mmol/L, an improvement of 55.2%, as well as the average productivity of (*R*)-ECH increased from 0.144 to 0.286 mol/(L·h), an improvement of 98.6%.

Table 2 summarizes the biological methods for the bio-resolution of ECH published in the last several years. As shown in Table 2, the reported biological separation reactions are all in an aqueous monophonic system or an organic phase, and generally, the substrate concentration and productivity are not high. Woo et al. [9] reported the bio-resolution of ECH using epoxide hydrolase from recombinant *N. aromaticivorans* in an aqueous monophonic system at a high substrate concentration, 500 mmol/L, but the yield of (*S*)-ECH was only 10.7%. Lee et al. [22] carried out bio-resolution of ECH using an epoxide hydrolase from *R. glutinis* in an aqueous monophonic system with a

Fig. 8. Time course of biocatalytic resolution of (*R,S*)-ECH in the two phase system in a mechanically stirred reactor. Reaction conditions: 0.7 L isooctane, 0.3 L buffer (0.2 mol/L, pH 8.0), 574 mmol/L ECH, 0.07 g/ml of *E. coli* cells, 30 °C, 50 min, 400 r/min.

relatively high yield of (*S*)-ECH of 28.5%, but the substrate concentration was only 20 mmol/L. Therefore, compared with the existing reports, this study suggests that a biphasic whole-cell catalysis system could improve the concentration of substrate and yield of chiral ECH.

4. Conclusions

The biocatalytic resolution of (*R,S*)-ECH to (*R*)-ECH by whole cells of a recombinant *Escherichia coli* expressing epoxide hydrolase in organic solvent/buffer biphasic systems was studied. In an isooctane/buffer biphasic system, the enzymes from the whole cells could catalyze the bio-resolution of (*R,S*)-ECH to (*R*)-ECH with high efficiency and stereoselectivity. Isooctane can effectively improve the substrate concentration in the reaction system because the substrate ECH is soluble in it and this helps to suppress the non-enzymatic hydrolysis of the substrate. Under the optimized conditions in a stirred reactor (1-L scale), the substrate concentration was 574 mmol/L with a yield of 37.5%, a product ee of 99.3%, and an average productivity of 0.286 mol/(L·h). Compared with other published processes, the substrate concentration and the yield are improved in our method. However, as organic solvents are usually toxic to biological catalysts and are harmful to the environment, future studies will include efforts at recycling the organic solvent, as well as developing a continuous biphasic reaction system in

Table 2

The comparison of kinetic resolution of known epoxide hydrolases toward racemic ECH.

Enzyme source	Reaction system	ECH concentration (mmol/L)	ee (%) / Isomer	Yield (%)	Reference
Duck liver	aqueous	n.i.	86.1/(<i>S</i>)	n.i.	[26]
Immobilized <i>A. niger</i>	aqueous	n.i.	99/(<i>S</i>)	26	[29]
<i>R. glutinis</i>	aqueous	50	100/(<i>R</i>)	26	[8]
Recombinant <i>E. coli</i>	aqueous	127.6	99/(<i>R</i>)	18	[16]
<i>N. aromaticivorans</i>	aqueous	500	99.9/(<i>S</i>)	10.7	[9]
<i>R. glutinis</i>	organic	20	99/(<i>R</i>)	28.5	[22]
<i>A. niger</i> Spps	organic	60	100/(<i>S</i>)	20	[7]
<i>A. niger</i> ZJB-09173	organic	153.6	98/(<i>S</i>)	18.5	[30]
Recombinant <i>E. coli</i>	biphasic	574	99.3/(<i>R</i>)	37.5	this study

n.i.: No information.

Graphical Abstract

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Enzymatic resolution of epichlorohydrin catalyzed by whole cells in an organic solvent/buffer biphasic system

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An isooctane/buffer biphasic system is useful for dissolving the substrate epichlorohydrin (ECH) and suppressing its non-enzymatic hydrolysis. This biphasic system could prove useful for improving the concentration of substrate and yield of (R)-ECH.

order to improve the efficiency of solvent usage and reduce the amounts of organic solvents employed.

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有机溶剂/缓冲液两相体系中全细胞催化拆分环氧氯丙烷

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摘要: 研究了有机溶剂/缓冲液两相体系中重组大肠杆菌*E. coli* BL21 (DE3)全细胞水解动力学拆分外消旋环氧氯丙烷制备(R)-环氧氯丙烷的过程. 结果表明, 最适反应条件为: 最适有机溶剂异辛烷与缓冲液的体积比7:3, 最适缓冲液pH 8.0, 底物浓度574 mmol/L, 全细胞加入量0.07 g湿菌体/ml溶液, 温度30 °C. 在此条件下于1 L反应器中反应45 min, (R)-环氧氯丙烷的摩尔产率、光学纯度和时空产率分别达到37.5%, 99.3% ee和0.286 mol/(L·h). 与单一水相体系相比, 异辛烷/缓冲液两相体系中底物浓度和(R)-环氧氯丙烷时空产率分别提高了55.2%和98.6%.

关键词: 环氧化物水解酶; 全细胞; 手性环氧氯丙烷; 生物拆分; 有机溶剂/缓冲液双相体系

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1. 前言

环氧氯丙烷(ECH)是一种重要的有机合成中间体, 其单一对映异构体(*R*)-ECH和(*S*)-ECH均是非常重要的三碳手性合成砌块, 在医药、农药、化工和材料等领域有非常广泛的应用^[1,2]. 例如, (*R*)-ECH是合成治疗心绞痛类药物美托洛尔和阿普洛尔等的关键手性中间体^[3,4], (*S*)-ECH是目前临床上用量最大、全球销售额达200亿美元的他汀类降血脂药物的起始原料之一^[5].

近年来, 随着生物柴油副产物甘油合成ECH技术的日益成熟及规模化应用, ECH市场价格持续走低. 因此, 以外消旋ECH为原料制备手性ECH已成为非常经济有效的途径之一. 同时, 由于生物拆分法具有立体选择性强和反应条件温和等优点^[6], 因此不少研究者报道了利用生物催化拆分法制备光学纯的ECH. Choi等^[7]采用来自黑曲霉(*Aspergillus niger*)的环氧化物水解酶催化拆分ECH制备(*S*)-ECH, 底物浓度60 mmol/L, 产率为20%, 对映体过量值(ee)为100%. Kim等^[8]利用胶红酵母(*Rhodotorula glutinis*)的重组环氧化物水解酶催化拆分ECH制备(*R*)-ECH, 底物浓度50 mmol/L, (*R*)-ECH产率为26%, ee值为100%. Woo等^[9]利用来自海洋新鞘氨醇杆菌(*Novosphingobium aromaticivorans*)的环氧化物水解酶催化拆分ECH制备(*S*)-ECH, 底物浓度500 mmol/L, (*S*)-ECH产率为20.7%, ee值大于99%. 由于底物浓度偏低, 且产率普遍不高, 离实际应用尚有距离.

本实验室前期构建了一株表达放射形农杆菌(*Agrobacterium radiobacter*) AD1环氧化物水解酶基因的重组大肠杆菌BL21 (DE3)工程菌^[10], 该菌株表达的重组环氧化物水解酶具有高立体选择性, 能有效催化ECH的不对称水解合成(*R*)-ECH. 但由于环氧化物对生物催化剂有毒害作用, 且化学性质活泼, 在单一水相中易发生自发水解反应, 导致反应效率不高^[11], 而应用有机溶剂/缓冲液两相体系能够克服这一缺点^[12-15]. 本文研究了以重组大肠杆菌BL21 (DE3)全细胞为生物催化剂, 在有机溶剂/缓冲液两相体系中催化拆分ECH不对称水解反应制备(*R*)-ECH, 系统探讨了两相体系对反应的影响

规律.

2. 实验部分

2.1. 全细胞的培养及制备

重组大肠杆菌*E. coli* BL21 (DE3)菌株由本实验室保存^[10], 培养方法见文献[16]. 获得的菌体用生理盐水悬浮洗涤后, 于10000 r/min离心10 min收集菌体, 于-20 °C储存.

2.2. 两相体系中生物催化拆分ECH反应

ECH, (*S*)-ECH和(*R*)-ECH (99%)标准品为西安福晨化学有限公司产品. 其他材料均为市售色谱纯或分析纯试剂.

在50 ml带塞转化瓶中, 加入适量湿菌体和一定体积的磷酸盐缓冲液(0.2 mol/L, pH 8.0)与异辛烷, 在适当条件下磁力搅拌(400 r/min) 15 min, 加入适量外消旋ECH, 继续搅拌. 定时取样, 离心(12000 r/min × 2 min)加入800 μl乙酸乙酯萃取ECH, 取200 μl加入含800 μl乙酸乙酯的EP管中进行稀释, 加入适量无水硫酸钠干燥, 供气相色谱分析.

气相色谱分析采用日本岛津GC-14A型气相色谱仪, 手性色谱柱BGB-175 (30 m × 0.25 mm × 0.25 μm), 氢离子火焰检测器. 检测条件: 进样器温度220 °C, 检测器温度220 °C, 柱温90 °C, 保留7 min, 载气为He (1.6 ml/min), 分流比1:40. (*R*)-ECH和(*S*)-ECH的保留时间分别为5.3和5.6 min.

根据剩余ECH计算摩尔产率(*Y*)和时空产率(*Y_t*), 产物的光学纯度由ee值表征, 计算式如下: $Y = c/c_0 \times 100\%$, 式中 c_0 和 c 分别为反应起始和反应结束时ECH的摩尔浓度; $Y_t = c/t$, 式中 c 和 t 分别为(*R*)-ECH的摩尔浓度和反应结束的时间; $ee = (c_R - c_S)/(c_R + c_S) \times 100\%$, 式中 c_R 和 c_S 分别为(*R*)-ECH和(*S*)-ECH的摩尔浓度.

3. 结果与讨论

3.1. 有机溶剂的选择

一般而言, 有机溶剂对生物细胞(酶)有一定的毒害作用, 导致生物细胞(酶)的活性下降. 本文选用6种不同

疏水性的有机溶剂(异丁醇、异丙醇、环己烷、异辛烷、正庚烷、正己烷)作为两相反应体系的有机相,考察了不同有机溶剂对环氧氯丙烷溶解能力和对重组大肠杆菌BL21全细胞催化外消旋环氧氯丙烷不对称水解反应的影响。

对底物高的溶解能力是筛选有机溶剂时需考虑的一个重要因素^[17]。实验测定了6种有机溶剂对环氧氯丙烷在的溶解能力。结果表明,ECH在异辛烷、正庚烷和正己烷中的溶解度(20 °C)分别达到22.1, 18.5和19.0 g/100 g溶剂,与异丁醇、异丙醇和环己烷互溶。水相中ECH溶解度(6.58)较低,而有机溶剂则对ECH均具有较高的溶解能力。除了对底物的溶解性,有机溶剂的生物相容性是有机相筛选时需考虑的另一个重要因素。如图1所示,当异辛烷作为有机相时,产物的摩尔产率和ee值较高。这可能是因为异辛烷生物相容性好。尽管环己烷和正庚烷有较高的ee值,但是产物的摩尔产率低。另外,以异丁烷和异丙醇为溶剂时,ECH的ee值几乎为零,使用正己烷时,ECH的ee值和产物摩尔产率均较低。这可能是因为这些溶剂对重组*E.coli* BL21 (DE3)表达的环氧化物水解酶的毒害作用较大,影响了其催化活性。Bottalla等^[18]和Gong等^[11]也发现,异辛烷对巨大芽孢杆菌和黑曲霉环氧化物水解酶具有良好的生物相容性。因此,本文选择异辛烷作为两相体系中的有机溶剂。

3.2. 两相体积比的影响

两相系统中有机溶剂与水相的体积比 V_o/V_a 影响反应的原因被认为是, V_o/V_a 的变化会改变两相界面面积,从而影响生物催化的速率^[19]。实验以初始反应速率为目标,考察了 V_o/V_a 对反应的影响,结果见图2。可以看出,随着异辛烷含量的增加,初始反应速率逐渐增大,但当异辛烷体积比增大到70%后,初始反应速率迅速下降。一般来说,由于有机溶剂普遍具有一定毒性,酶或活细胞一旦与之接触,总会出现一些变性和失活的现象。在有机相/水体系中,底物ECH大部分存在于有机相中,其在水相中的浓度较低,减少了底物对菌体细胞的毒害与抑制作用。另一方面,随着 V_o/V_a 比逐渐增大,过量的有机溶剂会夺取酶表面的水膜,使酶活力降低甚至失去活性^[20]。因此,根据初始反应速率的变化情况,最适 V_o/V_a 为7:3。

ECH和副产物3-氯-1,2-丙二醇(MCH)在异辛烷/缓冲液双相体系中的分配系数见表1。可以看出,在所考察的 V_o/V_a 值范围内,环氧氯丙烷在两相中的分配系数随着异辛烷含量的增加而迅速提高,而副产物MCH的分配系

数均趋于零。这说明有机溶剂不仅提供了两相界面,而且可以作为萃取产物的溶剂,及时有效地将产物(R)-ECH从水相中萃取出来,而副产物MCH则主要分配在水相中。因此,采用异辛烷/缓冲液两相体系可简化产物(R)-ECH的分离纯化过程。

3.3. 反应温度的影响

在酶促反应中,温度对催化剂的活性、立体选择性和稳定性以及反应平衡都会产生影响^[21],因此本文考察了20~40 °C时全细胞催化环氧氯丙烷的活性变化,结果见图3。当温度由20 °C逐渐升至30 °C时,初始反应速率和ee值明显增大,并在30 °C达到最大值。当温度超过30 °C时,初始反应速率和ee值开始迅速降低。温度的升高使酶的活性中心发生适度的变化,有利于酶分子与底物分子的结合,同时也使两者的碰撞结合几率增加,因此使细胞催化反应速率增加。过高的温度(30 °C)导致酶空间结构的破坏,菌体活性下降,导致反应速率和ee值下降。这与Woo等^[9]和Lee等^[22]报道的环氧化物水解酶最佳反应温度相一致。因此,最佳反应温度为30 °C。

ECH在水相中不稳定,易发生自发水解反应,使得酶催化拆分ECH的产率大大降低^[23]。本文比较了水相和两相反应体系中ECH的自发水解率,结果见图4。可以看出,30 °C时水相中的水解率为7.1%,而在两相体系中为4.9%,且随着温度升至40 °C,水相中的水解率达10.1%,而在两相体系中仅为7.2%。由此可见,两相体系的应用可以抑制底物的自发水解反应,从而提高单一手性产物的产率。

3.4. 缓冲液pH值的影响

在两相反应体系中,催化反应通常发生在被称为催化剂富余相的水相中,水相缓冲液的pH值不仅改变了反应中底物和带有电荷的酶的离子状态,而且还会影响酶的催化活性^[24]。本文考察了pH 7.0~9.0的缓冲液对全细胞催化ECH反应速率的影响,结果见图5。可以看出,当缓冲液pH由7.0逐渐升至8.0时,初始反应速率和ee值明显增加。pH超过8.0时,两者开始迅速下降。这表明pH = 8.0时细胞具有最大的催化活力。这与多数文献结果相符^[8,25,26]。因此,全细胞催化反应的最适pH可确定为8.0。

3.5. 菌体浓度的影响

菌体浓度的大小直接与酶量相关,因而菌体浓度直接影响催化效率:菌体浓度越高,催化相同浓度底物的催化反应速率就越大,但菌体过量又会影响传质作用^[27]。因此,本文在保持底物浓度固定的条件下考察了菌体浓度对反应的影响,结果见图6。可以看出,当菌体

浓度小于0.07 g/ml时,随着细胞用量的增加,ee值、反应产率和初始反应速率明显增大;当菌体浓度高于0.07 g/ml时,初始反应速率开始减少,而ee值和反应产率基本保持不变,说明此时催化剂已经过量.因此,为了拆分反应能够经济有效的进行,确定细胞浓度为0.07 g/ml.

3.6. 底物浓度的影响

高底物浓度一般会对细胞或酶产生毒害作用,影响其活性,但底物浓度过低则影响反应效率^[28].本文在异辛烷/缓冲液两相体系中,以ee值为目标,考察了底物浓度对反应的影响,结果如图7所示.可以看出,当底物ECH浓度从382.7逐渐升至574 mmol/L时,菌体均能将ECH拆分完全,(R)-ECH的ee值均可达99%以上;当浓度高于574 mmol/L时,(R)-ECH的ee值明显降低,当底物浓度为637.8和765.4 mmol/L时,(R)-ECH的ee值分别仅为46.2%和18.9%.这主要是由于底物ECH是非天然化合物,其生物相容性较差,高浓度ECH会对细胞产生毒害作用,影响环氧化物水解酶全细胞催化的活性和立体选择性.因此,为了使此拆分反应能够有效进行,确定最优ECH浓度为574 mmol/L.

3.7. 机械搅拌反应器中催化拆分ECH的反应进程曲线

按上述优化条件,在2 L机械搅拌反应器中加入1 L反应液(异辛烷/ pH 8.0磷酸盐缓冲液体积比7:3)、0.07 g/ml菌体和574 mmol/L (R,S)-ECH,反应温度30 °C,搅拌,连续取样并分析检测.如图8所示,菌体对ECH表现出良好的立体选择性,对(S)-ECH的转化速率明显快于(R)-ECH,水解产物为MCH.45 min后终止反应,此时(R)-ECH的浓度达到215 mmol/L,摩尔产率为37.5%,ee值为99.3%,时空产率达到0.286 mol/(L·h).与单一水相体系相比,异辛烷/缓冲液两相反应体中底物ECH浓度由

369.9增至574 mmol/L,提高了55.2%,(R)-ECH时空产率由0.144增至0.286 mol/(L·h),提高了98.6%.

表2归纳了相关文献中生物法拆分ECH的反应结果.由表可见,已报道的生物拆分反应均在单一水相或有机相中进行,底物浓度和产率普遍不高.Woo等^[9]在单一水相体系中利用*Novosphingobium aromaticivorans*重组环氧化物水解酶催化拆分ECH,底物ECH浓度较高,达500 mmol/L,但(S)-ECH产率仅为10.7%.Lee等^[22]在单一有机相中利用*Rhodotorula glutinis*环氧化物水解酶拆分ECH,尽管(R)-ECH产率可达28.5%,但其底物浓度仅为20 mmol/L.可见,本文采用的两相全细胞催化体系中底物浓度和手性环氧氯丙烷产率均较高.

4. 结论

系统研究了有机溶剂/缓冲液两相体系中重组大肠杆菌*E.coli* B21全细胞催化环氧氯丙烷(ECH)不对称水解反应,考察了各相关因素对该反应的影响.在异辛烷/缓冲液两相反应体系中,全细胞酶能高效率、高立体选择性地催化水解(S)-ECH,得到(R)-ECH.异辛烷对底物的溶解能力较强,生物相容性较好,故可有效提高反应体系中的底物浓度,并抑制底物的非酶水解,从而提高了底物浓度和时空产率.在最优反应条件下,该反应的底物浓度、摩尔产率、时空产率和产物的ee值分别为574 mmol/L, 37.5%, 0.286 mol/(L·h)和99.3%.与已有文献报道相比,本方法有效提高了底物浓度和产率.然而,由于有机溶剂通常对生物催化剂有一定的毒害作用,且大量有机溶剂的使用易造成环境污染.因此,本课题组正致力于有机溶剂的回收再利用以及连续化两相反应的研究,以期提高溶剂使用效率,减少有机溶剂用量.