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Kinetic Resolution of (*R*,*S*)-1-chloro-3-(1-naphthyloxy) -2-Propanol in an Immobilized Lipase Bioreactor

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Abstract: A bioreactor with *Pseudomonas cepacia* lipase (PCL) immobilized on the inner wall was conveniently prepared by adding lipase powder and the right amount of water to a conical flask and keeping it with mouth open in an incubator shaker at 37°C and 170 rpm for more than 10 h. The bioreactor was employed on resolution of (*R*, *S*)-1-chloro-3-(1-naphthyloxy)-2-propanol by catalyzing transesterification of it with vinyl acetate. It was showed that the wall-PCL behaved an excellent catalytic activity being 10 folds of native PCL in terms of conversion, and high enantioselectivity E = 110. And also the depressed activity of immobilized PCL owing to frequently use in organic phase could be reactivated easily by again shaking bioreactor under the aforesaid conditions after adding water. The enhanced activity was attributed to the simulation on interfacial activation mechanism of lipases at water/oil interface.

Keywords: Lipase, Activity, Enantioselectivity, Immobilization, *Pseudomonas Cepacia* Lipase, Bioreactor

1. Introduction

The chirality of molecules plays an important role in their biological activities. In most cases, bioactive substances consisted of one or more optical centers, such as drugs, insecticides, flavors and fragrances, only one of the enantiomers contributes to the biological activity. For example, β-adrenergic blocking agents (so called β-blockers) related to aryloxypropylamines (e.g. propranolol and atenolol), the therapeutic effect resides mainly in (S)-enantiomer, which is 130 times of (R)-enantiomer [1]. Therefore, the preparation of optically enriched (S)-enantiomer is important. Consequently, there are many investigations on the preparation approaches of (S)-propranolol, which can be summarized as follows: (i) enzymatic resolutions, (ii) microbial catalysis, (iii) asymmetric synthesis, and (iv) chemoenzymatic methods. Among the enzymatic resolutions, either the optical purity of intermediate obtained by resolution was lower [2], or disadvantages were found in multisteps, low overall yields and use of hazardous and expensive reagents, etc, and lastly, noncompatibility with the existing industrial process for racemic propranolol (Figure 1) [3, 4]. New developed microorganism-catalyzed deoxidization ways was not yet desired for that not only the much microorganism were consumed during process, but also the ee value of intermediates was commonly not high [5, 6]. In terms of yield and optical purity of product, sharpless's asymmetric synthesis was obviously prior to other ways [7, 8], but from the point of view environment friend, the more green way should be further explored. However, the direct resolution of propranolol was not successful [9] or gave lower enantioselectivity [10]. So a practical chemoenzymatic synthesis of (S)-propranolol was developed for industrial purpose in 1991 [11]. This method could be compatible with the existing industrial process and also gave the good resolution results of key intermediate (R, S)-1-chloro-3-(1-naphthyloxy)-2-propanol, which can be obtained in a single step from 1-naphthol and epichlorohydrin, as well as can be converted easily to propranolol. In this process, PCL displayed high enantioselectivity in nonaqueous medium, whereas the catalytic activity of this lipase was very low, which induced that either vast lipase was consumed or reaction time must be prolonged greatly. It may be just this reason so that the further evolvement of this chemoenzymatic method has not been found to date. Then the critical work about this practical chemoenzymatic synthesis should be that

the enhancement of enzyme activity, which just is what we want to do in this article.

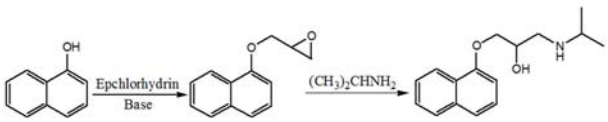

Figure 1. *Industrial production of (±)-Propranolol [11].*

As we all know, the excellent interfacial activity behaved by lipases at water/oil (organic phase) is unique physicochemical character of them [12, 13], but it is different for them in organic medium where only low catalytic activity was remained [14, 15]. On activity of lipases, both of experimental investigations [14] and computer simulations [16] focused that diffusional limitations and conformational changes of enzyme molecules mainly correlated with their catalysis. From discussion on the interfacial structure of lipases [17], it can be apprehended that the diffusional limitations of lipases are decreased by water phase and their active sites locating in hydrophobic amino acid regions are exposed out by organic phase. So lipases show higher catalytic activity at interface. PCL was one of those lipases most frequently used by organic chemist in resolution of racemates [18], and as common lipases its active site locates in hydrophobic regions and has a 'lid' on it [19, 20]. Whereupon a better way to enhance enzyme activity should be that simulate the interfacial activity in organic medium. Our performance is to immobilize PCL on the inner wall of reactor to obtain a kind of wall-immobilized PCL bioreactor, which is used to resolve the intermediate (R, S)-1-chloro-3-(1-naphthyloxy)-2-propanol (Figure 2).

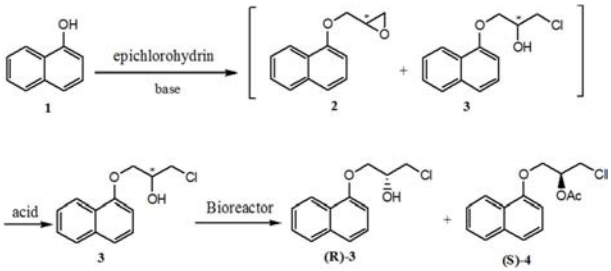

Figure 2. *Synthesis and resolution of (R, S)-1-chloro-3-(1-naphthyloxy)-2-propanol.*

2. Materials and Methods

2.1. Materials

The PCL powder (bought from Amino, Japan) was used without further purification. 1-naphthol, epichlorohydrin and vinyl acetate etc. used for synthesis or resolution were analytical grade.

2.2. Measurement and Analysis

The water content (2%) of PCL was measured using a Karl Fischer coulometer (Metrohm KF-756, Switzerland). Optical rotation was measured with a WZZ-1S digital automatic polarimeter (China). To refer literature [21], the enzyme activities in organic medium were estimated by comparison of reaction conversions obtained by using gas chromatography (GC) analysis with a Shimazu GC-14B instrument (Alltech, Japan) and a FID as the detector. A capillary column (Econo-CAP SE54.30m×0.32mm×0.25μm) was used to effect separation. Detector conditions: inj. 300°C, detec. 290°C, procd. at 210°C for 1 min, from 210°C to 260°C in 2°C/min, at 260°C for 2min. The kinetic resolutions were carried out in an incubator shaker (HZQ-X100, constant temperature by air, China) at 37°C and 170 rpm.

2.3. Synthesis of (R,S)-1-Chloro-3-(1-Naphthyloxy)-2-Propanol 3

A solution of l-naphthol (28.8g, 0.2 mol), epichlorohydrin (78 mL, 1 mol), and pyridine (1.6 mL, 0.02 mol) was stirred at ambient temperature (25-35°C) in a 300 mL conical flask until GC/TLC analysis showed completion (more than 24 h). Removal of excess epichlorohydrin and pyridine under reduced pressure around 95°C yielded 46.5 g of a crude mixture of **2** and **3** (ratio 23:77 by GC). The crude mixture was stirred with ethanol (20 mL) and concd HCl (10mL) for 0.5 h below 5°C. After ambient temperature attained, $CHC1_3$ (100mL) and water (100 mL) was added successively and layers were separated. Extraction of the aqueous layer again with $CHC1_3$ (100 mL) followed by washing of the combined organic layers with water (50 mL), drying, and removal of solvent yielded 47g (around 99%) of crude **3,** which was not filtrated by a silica gel column and used directly as substrate for resolution.

2.4. Preparation of Immobilized Lipase Bioreactor

2 mL water was added to a 50 mL conical flask with 0.1g PCL, and then the flask with mouth open was settled in an incubator shaker at 37°C and 170 rpm for more than 10 h. Then a simple bioreactor with a layer of PCL on the inner wall was prepared.

2.5. Kinetic Resolution of (R,S)-1-Chloro-3-(1-Naphthyloxy) -2-Propanol 3

A mixture of **3** (1.18 g, 5 mmol) and vinyl acetate (10 mL) was added to the bioreactor, and then it with mouth close was kept in an incubator shaker at 37°C and 170 rpm. Simultaneity in another 50 mL conical flask, a mixture of 3 (1.18g, 5mmol), vinyl acetate (10mL) and 0.1g PCL powder was added, and then the flask with mouth close was also settle in the above incubator shaker. The reaction conversions were detected by GC analysis. When the conversion achieved 50.4% after 74 h, the reaction was stopped by pouring reaction compound out of bioreactor. And the bioreactor was washed by petroleum ether. Removal of vinyl acetate and petroleum from Combined organic mixture under vacuum yielded an oil followed by separation on a silica gel column (petroleum ether-ethyl acetate, 6:1, v/v) gave 0.52 g (R)-**3** and 0.62 g (S)-**4** (oils, 88% yield). The optically corresponding parameters on intermediate (R)-3 for synthesis of (S)-propranolol were $[\alpha]_D^{25}$ (2.56, EtOH) -8.5, 95% ee, respectively. The attempt to

determine ee of 3 using chiral HPLC (Chiral park AD-H) was not successful. Hence, the ee and absolute configuration were determined by the conversion of chiral **3** to chiral propranolol, and comparing the observed rotation with that of literatures reported values [5, 6, 11, 22, 23].

2.6. Reusability and Reactivation of Bioreactor

In a used bioreactor in section *2.5*, **3** (1.18g, 5 mmol) and vinyl acetate (10 mL) were added. And then the bioreactor with mouth closed was keep in a rotary incubator shaker at 37°C and 170 rpm. The reaction conversion was measured by GC analysis. After 3 d, reaction compound was poured out of bioreactor, which was washed with petroleum ether and dry in air. Afterwards the empty bioreactor was added 2 mL water and settled again in an incubator shaker at 37°C and 170 rpm for more than 10 h. Then a reactivated bioreactor was obtained.

3. Results and Discussion

3.1. Modification of Chemical Synthesis Process

The synthesis of 3 was improved relative to the previous way [11]. In the course of transformation from **2** to **3**, ethanol and hydrochloric acid were used instead of chloroform and hydrochloric acid. By this means, not only the dosage of hydrochloric acid and organic solvent was only 1/5 of that report before [1, 5, 11, 22], but also reaction mixture became a homogeneous phase and reaction proceeded more quickly. In the end, the obtained **3** could be directly used as substrate for resolution subsequently without further purification by column chromatogram separation like that before [11].

3.2. Usage, Reusage and Reactivation of Bioreactor

To refer results reported on lipases in some literatures [1, 11, 23], it is found that the enantioselectivity of PCL (E>100 [11]) is the best among those lipases used for resolution of 3 in nonaqueous medium. But its activity in organic medium is very low like that of common lipases. So it is selected as a bioactivator to be immobilized on the glass wall of a conical flask to prepare a bioreactor to improve its catalytic activity in organic media. Although the weight and water content of immobilized PCL was same to free lipase, the activity of the former was 10 times more than that of the latter at the beginning of reaction (seen in Figure 3. ■ and ◆).

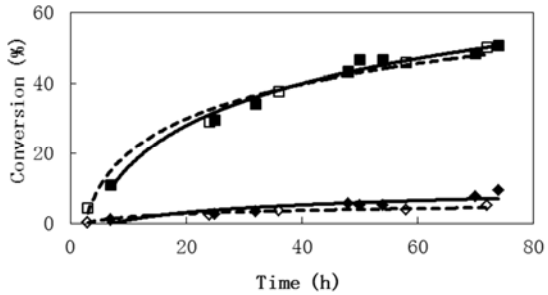

Figure 3. *Kinetics of (R, S)-1-chloro-3-(1-naphthyloxy)-2-propanol* ***3*** *reacting with vinyl acetate catalyzed by free lipase (◆), immobilized lipase (■), used immobilized lipase (◇) and reactivated immobilized lipase (□).*

The great difference in their catalytic activities should be ascribed to different conformations of enzyme molecules [14]. The low activity of native lipases was because they usually suffered precipitation and denature of organic solvent during the manufacture course of them separated from water solution [24,25], which caused their molecule conformation transform from a hydrophobic-closed form (HCF) with little diffusion limitations (LDL) to a hydrophobic-open form (HOF) with much diffusion limitation (MDL) (Figure 4. i). Namely the hydrophobic amino acid regions of enzyme molecules expose out continually and enzyme precipitated in the end. The precipitation resulted in that the active sites located in hydrophobic regions of enzyme molecules were embedded and only low active site concentration could be employed in organic media. In another way, the enhanced activity of wall-immobilized PCL in bioreactor was because the water diffused enzyme molecules and caused their hydrophilic amino regions expose out (Figure 4. ii), and which conformation with LDL was retained after air-drying. Once the wall-immobilized lipase was used for resolution in organic media, its molecule conformation transformed reversibly from HCF with LDL to HOF with MDL (Figure 4. i) in that organic solvent favored to HOF [26]. During the course of conformational transform, the active sites in hydrophobic regions expose out gradually, which endowed wall-immobilized PCL with great catalytic activity.

$$\text{HCF} + \text{LDL} \underset{\text{ii treated by water}}{\overset{\text{i in organic media}}{\rightleftharpoons}} \text{HOF} + \text{MDL}$$

Figure 4. *Conformational transform of enzyme between HCF with LCL and HOF with MDL.*

However, the used bioreactor showed very low catalytic activity in resolution of **3** (Figure 3. ◇) though the weight and water content of enzyme in it had not obviously changes. But, once it was reactivated by the way repeating immobilization performance, its activity came back (Figure 3. □). The results confirmed once again the conformational transforms discussed in Figure 4. Because of a long contact between lipase and organic media in first resolution of 3, the conformation of wall-immobilized PCL had become a HOF with MDL by and large. Actually the first usage of bioreactor gradually completed a change process exhibited by Figure 4. i. So the used bioreactor displayed low activity like native lipase. By contraries, the reactivation of bioreactor actually repeated the change of Figure 4. ii. Then the reactivated bioreactor showed an activity similar to that used firstly.

3.3. Further Discussion on Wall-Immobilized PCL Bioreactor

From above results and analysis, it is found that the bioreactor for resolution of **3** in nonaqueous medium is only endowed with excellent catalytic activity during the course of conformational transform in Figure 4. i. So enhancing enzyme

activity must firstly obtain an immobilized lipase with HCF and LDL by immobilization in existence of water and then use it in an organic media, which is actually a kind of simulation to the interfacial activation. On interfacial face, there exists a reversibly dynamic equilibrium between two enzyme conformations (Figure 4). The equilibrium cause enzyme with HOF towards organic media and with LDL for water present, then the lipases at water/oil interface always display high activity. But in nonaqueous medium, the 'dynamic' equilibrium is carried out only by stages for that there is no normal interface as biphasic media. The immobilization with water and the usage of bioreactor in organic media cause enzyme with LDL and HOF, respectively. As a result, the wall-immobilized PCL in bioreactor displayed an excellent catalytic activity (50% conversion after 3d) in resolution of **3**. While the reaction was catalyzed by same amount of native PCL, it took 13 d to achieve equal conversion in the previous report [11]. Furthermore, the immobilized lipase had same good enantioselectivity as free PCL (E≈110, calculated in terms of the relevant formula $E = \ln[(1-C)(1-ees)]/\ln[(1-C)(1+ees)]$) [27].

In addition, the protocol on preparation and use of this immobilized bioreactor was more facile. Because there are no special materials and complicated performances in its preparation and also the separation is do nothing but pouring reaction compound out of bioreactor without filtration. Certainly this bioreactor is not fit for those resolutions in water or biphasic media.

4. Conclusions

In a practical chemoenzymatic synthesis of (S)-propranolol, the key step was the resolution of intermediate **3** in nonaqueous media by PCL. For enhancing the catalytic activity of PCL, a simple immobilized bioreactor was prepared and employed on the resolution of **3**, which demonstrated greatly catalytic activity in organic media. The reason for enhancement in activity is that the immobilization and subsequent use of bioreactor actually simulated interfacial activity of lipase by stages. Based on conformational transform and diffusional limitation, the critical performance is that treating PCL with water and then drying it by air. As an example, this tells us a way for enhancement of enzyme activity in pure organic media. Namely, the commercial lipases should be pretreated with water like fore mentioned method or other methods having same effect before them used in organic phases. However, the study work on this bioreactor employed in product at industrial scale still need carried out in future.

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