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Microwave-assisted resolution of (*R*,*S*)-2-octanol by enzymatic transesterification

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Abstract

Novozym 435 (*Candida antarctica* lipase B immobilized on polyacrylic resin) was used for the resolution of (*R*,*S*)-2-octanol with vinyl acetate as the acyl donor through transesterification. Comparison was studied to illustrate the advantage of microwave irradiation superior to the conventional heating on the enzymatic resolution reaction. It was found that the activity and enantioselectivity of Novozym 435 were enhanced dramatically under microwave irradiation. Increased thermal stability and reusability of Novozym 435 under microwave irradiation were also observed. Effects of reaction conditions, such as temperature, organic solvents, water activity, substrate ratio and enzyme load on the activity as well as enantioselectivity of Novozym 435 were also investigated. Under the optimum conditions, (*S*)-2-octanol was obtained at 50.5% conversion with 99% enantiomeric excess in 2 h under microwave irradiation.

Keywords: Lipase; Resolution; Microwave irradiation; Novozym 435; Transesterification; (*R*,*S*)-2-Octanol

1. Introduction

Reactions catalyzed by enzymes have received much attention in non-aqueous media, where enzymes display higher thermal stability [1–3]. However, it is necessary to find a proper method to increase the reaction rate to promote the application of enzyme catalytic reaction, while one of the major limitations of enzymatic synthesis in non-aqueous media is its low reaction rate [4]. It is considered a simple and theoretically reliable method that the elevated temperature may help the substrate molecules to obtain adequate energy to pass over the energy barrier, increasing the reaction rate. It is reported that the reaction rate increases about 22-folds when Celite-immobilized lipase is used as the catalyst to resolve azirine-2-methanol in the range of 30–40 °C [5]. However, enzymes are believed to be temperature-depended and easily deactivated at high temperature and it is reported that the free and immobilized lipases from *Candida rugosa* lose 89 and 33% of their activities during a 120 min incubation period at 60 °C [6].

Microwave irradiation is widely used in organic chemistry [7,8] while it is proved to be clean, fast, and convenient energy source [9,10]. Traditionally, organic synthesis is carried out by conductive heating with an external heat source; this is comparatively slow and inefficient to transfer energy into the system, because it depends on the thermal conductivity that must be penetrated, resulting in the temperature of the reaction vessel being higher than that of the reaction mixture. In contrast, microwave irradiation produces efficient internal heating by direct coupling of microwave energy with the molecules (solvents, reagents, catalysts) in the reaction mixture [11], and it usually shortens the reaction time, but with a higher yield [12]. Since lipase-catalyzed reactions are rather sluggish in non-aqueous media, the synergism with microwave could be expected to enhance the reaction rate. Microwave-assisted lipase-catalyzed reaction is developed fast. Parker et al. [13] have reported recently that microwave irradiation could increase the rate of lipase-catalyzed hydrolysis reactions by two- to three-folds over conventional heating. However, most of the enzymes are readily deactivated by the fast

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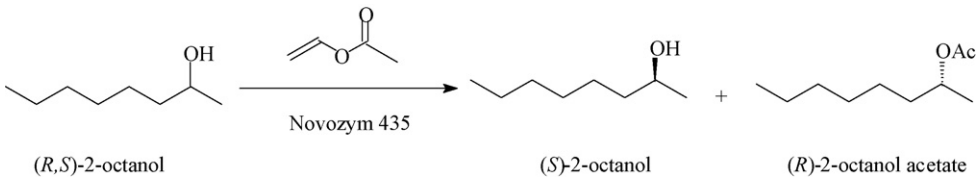

Scheme 1. Resolution of (*R*,*S*)-2-octanol catalyzed by Novozym 435.

enhancement of the temperature under microwave irradiation [14]. The problem may be solved by using immobilized enzyme which exhibits high thermal stability than the free one [15,16]. More recently, Rejasse et al. [17] have reported that the stability of Novozym 435 (*Candida antarctica* lipase B immobilized on polyacrylic resin) in organic media could be enhanced by using microwave irradiation. Yadav et al. [18] have also reported that the initial activity for transesterification of methyl acetoacetate with various alcohols in the presence of immobilized lipases is increased in the range of 2.2–4.6-folds for microwave irradiated reactions over conventional reactions. However, there still remains relatively little research so far on the applications of microwave irradiation in lipase catalytic resolution reactions, and demonstration of their utility remains a pressing concern.

Herein, we describe a temperature-controllable microwave-assisted resolution of (*R*,*S*)-2-octanol with Novozym 435 as the catalyst (Scheme 1). And a comparison between conventional heating and microwave irradiation on the resolution of (*R*,*S*)-2-octanol is studied. Effects of reaction conditions on the activity and enantioselectivity have also been investigated.

2. Experimental

2.1. Materials

Novozym 435 (*C. antarctica* lipase B immobilized on polyacrylic resin) and Lipozyme RM IM (*Mucor miehei* immobilized on an anionic resin) were purchased from Novo (Bagsvaerd, Denmark). Lipase from *Pseudomonas* sp. (PSL), Lipase PS-C (*Pseudomonas cepacia* lipase immobilized on ceramic particles), and *Candida cylindracea* A.Y. lipase (AYL) was purchased from Amano Pharmaceutical Co., Ltd. (Japan). *C. rugosa* lipase (CRL) was purchased from Sigma (St. Louis, MO). *Porcine pancreatic* lipase (PPL) was purchased from Shanghai Dongfeng Biochemical Reagent Co., Ltd. (China). (*R*)- and (*S*)-2-octanol were purchased from Sigma (USA). (*R*,*S*)-2-Octanol, vinyl acetate and other organic solvents of analytical grade were purchased from Shanghai Chemical Reagents Company (China).

2.2. Microwave equipment

The studies were carried out in a commercial microwave reactor (Discover, CEM-SP1245 model, CEM Corporation, USA). The reactor was 120 ml capacity fully baffled 4.5 cm i.d. cylindrical quartz vessels with provision for mechanical stirring. The temperature of the reaction mixture was kept constant (±1 °C) by using a non-contact infra-red continuous feed-back temperature system.

2.3. Resolution of (R,S)-2-octanol

Organic solvent (50 ml), (*R*,*S*)-2-octanol (50 mmol) and vinyl acetate (100 mmol) were placed in a quartz vessel (120 ml) and heated to 60 °C over a water bath (conventional heating) or in the microwave oven (microwave irradiation), respectively. Water activity of the reaction mixture was kept at 0.56. Novozym 435 (60 mg) was then rapidly added to the above reaction mixture with stirring at 200 rpm. Periodically, the reaction medium (100 μl) were withdrawn and analyzed by gas chromatography (GC). One unit (U) of enzyme activity was defined as the amount of enzyme that was necessary to produce 1 μmol 2-octanol acetate per minute in the first 0.5 h. All experiments were repeated over three times and the errors did not exceed 5%. All graphs were based on the average value.

2.4. Thermal stability study of Novozym 435

Novozym 435 (10 mg) was preincubated in *n*-heptane (10 ml) at 100 °C in a range of 0–60 min under conventional heating or microwave irradiation, respectively. Aliquots of Novozym 435 suspension were withdrawn at 10 min intervals and the residual activities were measured as described above.

2.5. Reusability of enzyme

After each batch, Novozym 435 was filtered and washed with *n*-heptane over three times and dried in air at 20 °C. Then the recycled enzyme was repeatedly used in the next reaction. The residue activity of the recycled enzyme was compared with the enzyme activity of first cycle (100%).

2.6. Determination of enantiomeric excess values and enantioselectivity

The recovered 2-octanol was analyzed directly by gas chromatography on an Agilent 6890 instrument equipped with a flame ionization detector (FID) and a chiral column (HP-Chiral Cap. 30 m × 0.25 mm × 0.25 μm). The temperatures of the injector and the detector were 200 and 280 °C, respectively. Nitrogen was used as the carrier gas at a flow rate of 60 ml/min. Temperature programming was performed between 60 and 240 °C with the increment of 4 °C/min. The retention times of (*R*)- and (*S*)-2-octanol were 45.5 and 45.7 min, respectively. The enantioselectivity was expressed as an *E*-value

calculated from the enantiomeric excess (ee_s) of the remained 2-octanol and the conversion degree (C) according to the formula previously reported by Chen et al. [19].

$$\text{enantiomeric excesses, } ee_s(\%) = \frac{[S-R]}{[S+R]} \times 100$$
$$\text{enantioselectivity, } E = \frac{\ln[(1-C)(1-ee_s)]}{\ln[(1-C)(1+ee_s)]} \quad (1)$$

where S and R represent the concentrations of the (S)-2-octanol and (R)-2-octanol, respectively.

3. Results and discussion

3.1. Screening of enzymes

Lipase catalytic resolutions depended mainly on the type and origin of the enzyme. In this study, the desirable enzyme was screened and selected for the resolution of (*R*,*S*)-2-octanol under microwaves irradiation (Table 1). It was supposed that the enzyme activity and enantioselectivity varied markedly with the type of enzyme, and the immobilized enzymes exhibited higher activity than that of the free ones. Novozym 435 (a thermostable lipase) was selected as the catalyst for further study because of its highest activity and enantioselectivity.

3.2. Microwave irradiation versus conventional heating

The comparison of enzymatic resolution of (*R*,*S*)-2-octanol heated by microwave irradiation and conventional heating was studied. The microwave assistance was found to enhance both initial rates and enantioselectivity, compared with the results obtained from the conventional heating. As shown in Fig. 1, 50% conversion was obtained only after 3 h under microwave irradiation while it required about 12 h to obtain the same conversion under the conventional heating. It meant that the reaction rate by microwave heating was much higher than that of the conventional heating. Control experiment in the absence of enzyme did not show any conversion which might indicate that there must have a definite synergism between enzyme catalysis and microwave irradiation. It was known that microwave heating involved directed absorption of energy by functional groups that bear ionic conductivity or a dipole rotational effect, and this energy was then released into the surrounding solution. In our systems, the adsorption of microwave irradiation caused the functional groups of the substrates to be of much higher reactivity than that incubated at the same temperature [20], and the reaction rate was improved.

Table 1
Comparison between the activity and enantioselective of different enzymes in resolution of (*R*,*S*)-2-octanol under microwave irradiation

Enzyme	Enzyme activity (μmol/min mg)	*E*-Value
Lipozyme RM IM	24	—
Lipase PS-C	59	105
Novozym 435	165	328
AYL	1	—
CRL	4	7
PSL	33	76
PPL	5	11

Conditions: Reactions were carried out in *n*-heptane (50 ml), (*R*,*S*)-2-octanol (50 mmol), vinyl acetate (100 mmol), enzyme (60 mg) and water activity of 0.56 at 40 °C and 200 rpm under microwave irradiation.

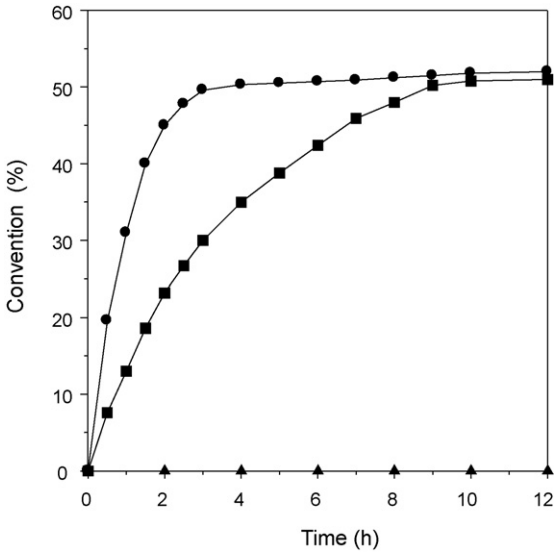

Fig. 1. Comparison of reaction process without enzyme under microwave irradiation (▲), with enzyme under microwave irradiation (●), and with enzyme under conventional heating (■). *Conditions*: Reactions were carried out in *n*-heptane (50 ml), (*R*,*S*)-2-octanol (50 mmol), vinyl acetate (100 mmol), Novozym 435 (60 mg) and water activity of 0.56 at 40 °C and 200 rpm under microwave irradiation and conventional heating, respectively.

When the enantioselectivity was considered, the detected *E*-value was significantly enhanced from 104 under conventional heating to 328 under microwave irradiation (Table 2). In order to clarify the possible mechanism of enantioselectivity improvement, the transesterification was performed under microwave irradiation and conventional heating by using (*R*)-2-octanol and (*S*)-2-octanol as the substrate, respectively. As shown in Table 2, both of the enzyme activities for transesterification of (*R*)-2-octanol and (*S*)-2-octanol under microwave irradiation were higher than that under conventional heating. The enzyme activity for transesterification of (*R*)-2-octanol increased about 3.1-folds whereas it increased only 1.3-folds for (*S*)-2-octanol. The results showed that microwave irradiation acted as activator to both of (*R*)- and (*S*)-2-octanol during the reaction except for the variation of the activation degree. And the increasing enantioselectivity under microwave irradiation were due to the higher activation effect on (*R*)-2-octanol than on (*S*)-2-octanol.

3.3. Effect of reaction conditions on transesterification under microwave irradiation

3.3.1. Effect of temperature

The effect of temperature on the activity and enantioselectivity of Novozym 435 was examined in the range of 40–100 °C. The results in Fig. 2 shows that the enzyme activity increased as temperature increased from 40 to 80 °C, followed by a decrease at higher temperature. When the reaction temperature elevated,

Table 2
The activity and enantioselectivity of Novozym 435 in transesterification under microwave irradiation and conventional heating

Substrate	Microwave irradiation		Conventional heating	
	Enzyme activity (μmol/min mg)	*E*-Value	Enzyme activity (μmol/min mg)	*E*-Value
(*R*)-2-Octanol	202.80	332	65.50	109
(*S*)-2-Octanol	0.64		0.47	
(*R*,*S*)-2-Octanol	203.25	328	66.18	104

Conditions: Reactions were carried out in *n*-heptane (50 ml), enantiomeric purity or (*R*,*S*)-2-octanol (50 mmol), vinyl acetate (100 mmol), Novozym 435 (60 mg) and water activity of 0.56 at 40 °C and 200 rpm for 2 h under microwave irradiation and conventional heating, respectively.

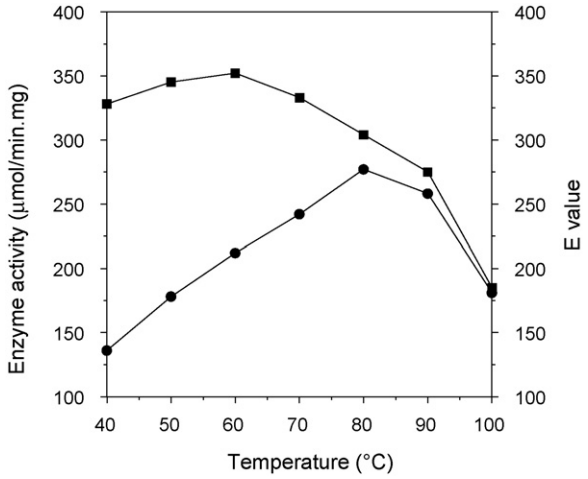

Fig. 2. Effect of temperature on enzyme activity (●) and enantioselectivity (■) in the resolution of (*R*,*S*)-2-octanol. *Conditions*: Reactions were carried out in *n*-heptane (50 ml), (*R*,*S*)-2-octanol (50 mmol), vinyl acetate (100 mmol), Novozym 435 (60 mg) and water activity of 0.56 at 200 rpm for 2 h under microwave irradiation. Temperature varied from 40 to 100 °C.

the collision chance between enzyme and substrate molecules increased which might help to form enzyme-substrate complexes and then the reaction rate was improved. On the other hand, the molecules of the proteins can fluctuate to relieve the steric repulsion by elevating the temperature, and such a fluctuation could be contributed to the rate acceleration [21]. However, the decreasing reaction rate with further increasing the temperature higher than 80 °C might be due to the denaturation (alteration) of protein structure resulting from the breakdown of the weak ionic and hydrogen bonding that stabilized the three dimensional structure of the enzyme. As far as the enantioselectivity was considered, it was worthy of noting that unlike most of the enzymatic resolution reaction under conventional heating where *E*-value decreased as the temperature increased [22], the *E*-value increased as temperature increased from 40 to 60 °C, followed by a decrease at higher temperature. It is interesting that both enantioselectivity and activity could be increased by simply elevating the reaction temperature. Since *E*-value was found to be greatest at 60 °C, maintaining a higher enzyme activity, 60 °C was selected as the optimal reaction temperature. Further increasing of the temperature may destroy the conformation of enzyme by heat-induced destruction of non-covalent interactions [23], resulting in decreasing the enantioselectivity.

3.3.2. *Effect of reaction media*

It was well described in the literature that enzyme activity and enantioselectivity were strongly affected by the organic solvent [24]. On the other hand, the effect of microwave irradiation on the solvent varied. In the present study, the effects of several organic solvents with various log *P* (the partition coefficient of the solvent for the standard octanol/water two-phase system) [25] were investigated for the resolution of (*R*,*S*)-2-octanol. As shown in Table 3, there was an increase in both enzyme activity and enantioselectivity as the solvent hydrophobicity increased. The highest enzyme activity and enantioselectivity were obtained when *n*-heptane was used as the reaction media. Klibanov pointed out that a minimum of water was required to preserve the conformation of the enzyme [26]. In hydrophilic solvent, such as acetone, acetonitrile, DMF and 1,4-dioxane,

Table 3
Effect of solvents on the enzyme activity and enantioselectivity in resolution of (*R*,*S*)-2-octanol under microwave irradiation and conventional heating

Solvent	log *P*[a]	Microwave irradiation		Conventional heating	
		Enzyme activity (μmol/min mg)	*E*-Value	Enzyme activity (μmol/min mg)	*E*-Value
n-Heptane	4.6	203	352	55	99
n-Hexane	3.5	196	344	53	96
Toluene	2.5	182	313	48	87
Cyclohexane	1.2	155	265	36	71
Acetone	−0.23	36	58	27	46
Acetonitrile	−0.33	29	45	22	38
DMF	−1.0	9	10	5	7
1,4-Dioxane	−1.1	6	7	4	5

Conditions: Reactions were carried out in organic solvents (50 ml), (*R*,*S*)-2-octanol (50 mmol), vinyl acetate (100 mmol), Novozym 435 (60 mg) and water activity of 0.56 at 60 °C and 200 rpm for 2 h under microwave irradiation and conventional heating, respectively.

[a] Source of date: references [28,29].

water had higher affinity in hydrophilic solvent rather than bound to the enzyme. As a consequence, the enzyme lost its flexibility conformation due to the lack of bound water and then losing its activity [27].

The influence of solvent also seemed to be of great importance with regard to the microwave irradiation. It had been shown that the effect of microwave on the reaction was decreased as the polarity of solvent increased [21]. If high polar solvents were involved, the absorption may occur mainly between microwaves and the polar solvent. In this case, energy was transferred from the solvent to reaction mixture and reactants. Consequently, the results should be nearly the same under conventional heating [30]. As shown in Table 3, when high polar solvents, such as DMF and 1,4-dioxane were used as the reaction media, both of enzyme activity and enantioselectivity were probably the same under microwave irradiation and conventional heating. It was interesting to use non-polar solvents as they were almost transparent to microwave. During the reaction, energy was transferred from the reactants to the solvent which might mean that when the temperature heated by microwave was the same as that by conventional heating, the reactants might possess higher activity and the reaction group on the reactants was more active. So the reaction rate was improved.

3.3.3. Effect of water activity

Since the desired balance between rigidity and flexibility of the enzyme was correlated with the water activity (a_w), effect of water activity on the activity and enantioselectivity for the resolution of (*R*,*S*)-2-octanol under microwave irradiation were investigated in *n*-heptane as the solvent at a wide range of initial a_w values (0.06–0.97). In the present study, the a_w was controlled by addition of salts or salt hydrates in the organic solvent or substrate as described by Halling [31]. The enzyme activity exhibited a bell shaped curve with the water activity changing in microwave irradiation (Fig. 3). At low a_w values (0.06–0.11), low enzyme activity were observed. Especially, no transesterification was detected with dried enzymes. In non-aqueous media, a certain amount of water was necessary for the enzyme to maintain its proper conformation so as to keep its catalytic activity. At low a_w, the conformation of Novozym 435 was excessively rigid which disturbed the "induced-fit" process of CALB and decreased the enzyme activity [32]. Novozym 435 exhibited the highest activity at $a_w = 0.56$ and a further increase in the initial a_w value to 0.97 resulted in an obvious decrease in enzyme activity. The decrease in enzyme activity at higher initial a_w values can be attributed to the excessively flexible conformation, and the water in the reaction mixture may act as competing nucleophile for acyl-enzyme thus suppressing the expected acyl transfer and cause unfavourable equilibrium position in reversed hydrolysis. Overall, these results suggested that water activity strongly influenced the hydration level of the enzyme that in turn affected the transesterification activity. When concerning the effect of a_w on the enantioselectivity, the results in this study indicated that the *E*-value remained almost the same with the variation of a_w values under microwave irradiation. The possible explanation may be that water would only participate in the enantioselective step of reaction when the acyl part of the substrate was chiral [33,34]. Since the acyl part was not chiral in this reaction, the enantioselectivity was not affected by the water activity.

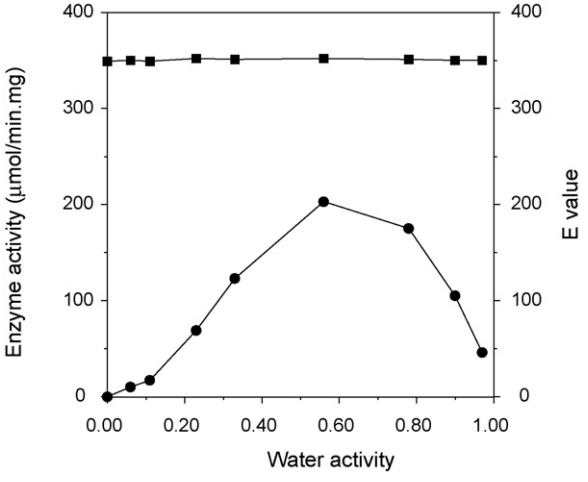

Fig. 3. Effect of water activity on the activity (●) and enantioselectivity (■) of Novozym 435 in the resolution of (*R*,*S*)-2-octanol under microwave irradiation. *Conditions*: Reactions were carried out in *n*-heptane (50 ml), (*R*,*S*)-2-octanol (50 mmol), vinyl acetate (100 mmol), Novozym 435 (60 mg) and water activity (0.06–0.97) at 60 °C for 2 h.

3.3.4. Effect of substrate ratio

The rate of an enzyme catalytic reaction depended on the concentrations of enzyme and substrate. In this reaction, the effect of microwave was mainly on substrate as described above, so the variation of substrate ratio may have great effect on the reaction. In the present experiments, the substrate ratio (vinyl acetate to (*R*,*S*)-2-octanol) was changed by keeping the moles of (*R*,*S*)-2-octanol constant at 50 mmol, whereas the moles of vinyl acetate varied from 50 to 400 mmol. It had been shown experimentally that the enzyme activity increased until the substrate concentration was gradually increased to the maximum, keeping the amount of the enzyme constant. After this point, increased in substrate concentration could not increase the enzyme activity. Indeed, when using higher ratio of vinyl acetate over (*R*,*S*)-2-octanol, the enzyme activity increased from 130 to 203 μmol/min mg and a ratio of 2:1 turned out to be sufficient (Fig. 4). Further increasing of substrate ratio could not increase the enzyme activity. It might be that enzyme/substrate complex had to be dissociated before the active sites were free to accommodate more substrate. In addition, although the substrate ratio greatly affected the enzyme activity, it seemed not influence the enantioselectivity of enzyme.

3.3.5. Effect of enzyme loading

The effect of enzyme loading on the resolution of (*R*,*S*)-2-octanol was studied under microwave irradiation. The mole ratios of the reactants were kept constant while changing the amount of enzyme from 10 to 200 mg. As the loading of Novozym 435 increased, the conversion rate was also increased (Fig. 5). Considering the high expense of Novozym 435,

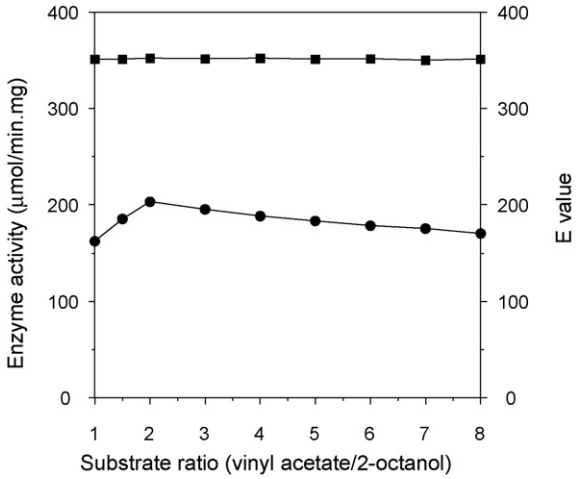

Fig. 4. Effect of vinyl acetate to (*R*,*S*)-2-octanol on enzyme activity (●) and enantioselectivity (■) in the resolution of (*R*,*S*)-2-octanol. *Conditions*: A quartz vessel containing *n*-heptane (50 ml), (*R*,*S*)-2-octanol (50 mmol) and Novozym 435 (60 mg) were performed at 60 °C at 200 rpm and water activity of 0.56 for 2 h under microwave irradiation. Vinyl acetate varied from 50 to 400 mmol.

60 mg Novozym 435 was good enough; further increasing of Novozym 435 may not increase the reaction rate obviously. Under the optimum conditions (60 °C; *n*-heptane, $a_w = 0.56$; substrate ratio 2:1, enzyme loading = 60 mg), the (*S*)-2-octanol was obtained at 50.5% conversion with 99% enantiomeric excess in 2 h.

3.4. Thermal stability of Novozym 435

Comparison between the residual enzymatic activities obtained after preincubation under microwave irradiation and under conventional heating is shown in Fig. 6. Under conventional heating, the enzyme maintained 49% of its initial activity against 83% under microwave irradiation. Results suggested that Novozym 435 was more stable under microwave irradiation. It was known that enzymatic inactivation was probably caused by the loss of the catalytically active conformation. The stability of this active conformation was closely dependent on the microenvironment of enzyme. Indeed, different non-covalent forces, such as hydrogen bonds, hydrophobic, ionic and Van der Waals interactions maintained enzymatic structure. Under conventional heating, these forces may reduce at high temperature and enzyme molecules unfold, so the enzymatic activity decreased very fast. However, microwave may enhance the interaction of chemical bond that maintained enzymatic structure, and the microwave changed the interactions between the enzyme and its microenvironment, preventing the enzyme from thermodenaturation [17].

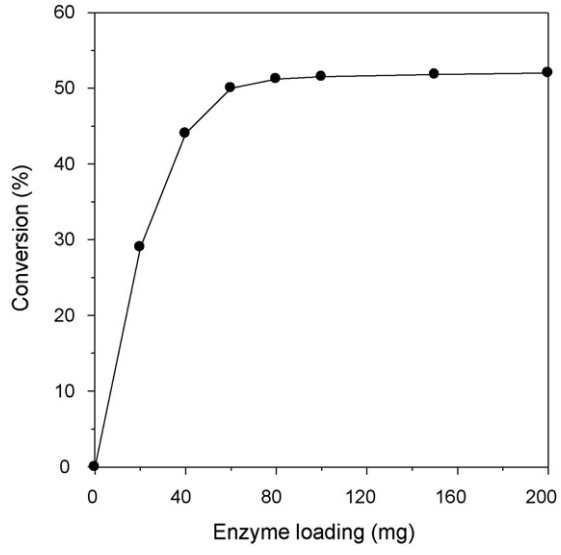

Fig. 5. Effect of enzyme loading on the resolution of (*R*,*S*)-2-octanol. *Conditions*: Reactions were carried out in *n*-heptane (50 ml), (*R*,*S*)-2-octanol (50 mmol), vinyl acetate (100 mmol), and water activity of 0.56 at 60 °C for 2 h. Novozym 435 varied from 10 to 200 mg.

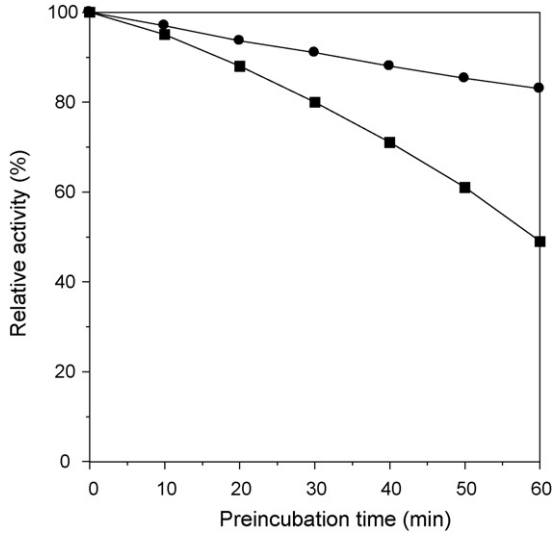

Fig. 6. Thermal stability of Novozym 435 under microwave irradiation (●) and conventional heating (■).

Control experiments were also conducted to observe how Novozym 435 may react when it was irradiated alone without organic solvent. In these conditions, the biocatalyst weakly interacted with the microwave: after 30 min radiation at 200 W, the temperature of Novozym 435 did not exceed 60 °C and only 9% of the enzymatic activity was lost.

3.5. Reusability of enzyme

The catalyst reusability studies were carried out to make a comparison on the stability of the enzyme under microwave irradiation and conventional heating. As shown in Fig. 7, only slightly decrease (8%) in enzyme activity was observed under microwave irradiation after five reuses, which might be due to loss of the enzyme during filtration and drying. However, only 70% activities was recovered under conventional heating after five reuses. It can be concluded that Novozym 435 did not get deactivated or denatured under microwave irradiation, and showing the good stability and the possibility of recycling of such systems.

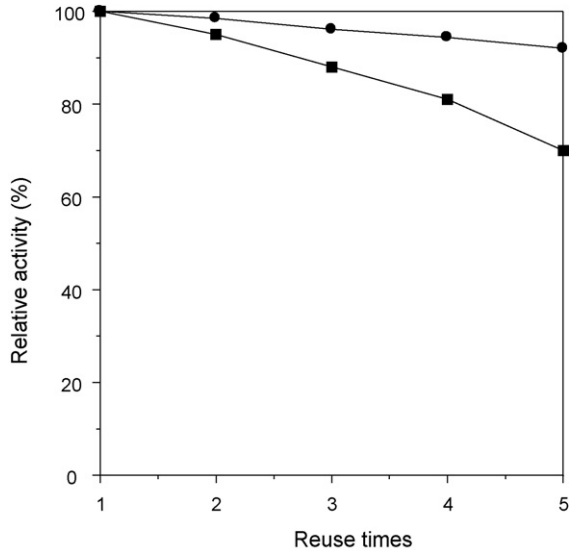

Fig. 7. Reusability of Novozym 435 under microwave irradiation (●) and conventional heating (■).

4. Conclusion

The ability to decrease the reaction time and reuse the biocatalyst is a decisive parameter for the economic viability of a biocatalytic process. In this study, we have taken advantage of the complementarily of two eco-friendly technologies, using immobilized enzymes catalysis and microwave activation to enhance both reactivity and enantioselectivity of Novozym 435 in resolution of (*R*,*S*)-2-octanol. Reaction conditions exhibit various effects on the enzyme activity and enantioselectivity under microwave irradiation compared with those under conventional heating. We have shown that thermal stability and reusability of Novozym 435 could also be enhanced when heated by microwave irradiation. However, microwave as a clean, fast, and convenient energy source has not yet been extensively studied in enzyme catalyzed resolution reaction, therefore one is likely to see more work in this area in the coming years.

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