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# Development of a mixed solvent system for the efficient resolution of (*R*, *S*)-2-octanol catalyzed by magnetite-immobilized lipase

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ABSTRACT

In order to find a suitable reaction system for the enzymatic resolution of (*R*, *S*)-2-octanol, the effects of the molecular structure of the solvent on the enantioselectivity (*E*) and enzymatic activity of *Yarrowia lipolytica* lipase (YLL) immobilized onto magnetic nanoparticles were systematically analyzed. Both the *E* and enzymatic activity of the reaction in an acyclic, structurally linear solvent were higher than those in the corresponding branched chain solvent or cyclic solvent. In a mixed solvent system with acetone and carbon tetrachloride (v/v = 3:7), the immobilized YLL exhibited high enantioselectivity, activity, and reusability. The thermodynamic analysis showed that the enantiomer discrimination was enthalpy-driven at all temperatures tested. These results present new opportunities and challenges for understanding and intensifying the enzymatic resolution process of (*R*, *S*)-2-octanol by designing suitable solvent system.



## 1. Introduction

Biocatalysts used for industrial synthetic chemistry are on the verge of significant growth, especially for the synthesis of chiral compounds [1]. Biocatalysts are receiving increasing attention as a promising alternative to conventional catalysts due to several advantages including high catalytic efficiency, high enantioselectivity, low environmental impact, and mild reaction conditions [2,3].

Lipases are of significant interest because of their high stability in organic solvents, and the selection of the reaction medium for a lipase-catalyzed reaction is important because the selectivity of a lipase is strongly affected by the reaction medium [4]. The solvent-dependent activity and selectivity of a lipase varies greatly from one solvent to another due to their intrinsic physical properties [5], and the prediction of these effects is difficult. Therefore, it is necessary to systematically screen solvents for their utility for lipase-catalyzed reactions. Changes in the solvent have been reported to result in a reversal of enantiopreference [6,7] and in changes in substrate specificity [8,9]. Solvent engineering to improve enzyme enantioselectivity for targeted reactions is efficient because it reduces the time and resource-consuming process of screening and/or protein engineering of the biocatalyst [10–12].

In order to improve operational stability and product recovery without lipase contamination, lipase was immobilized onto magnetic $Fe_3O_4$ nanoparticles, and the immobilized lipase was easily recycled using a magnet [13–15]. Thus far, there have been few reports related to the applications of magnetically immobilized lipases for the resolution of chiral compounds, especially of chiral secondary alcohols. The objective of the current work was to understand and screen suitable solvent systems in order to improve the resolution of (*R*, *S*)-2-octanol catalyzed by *Yarrowia lipolytica* lipase immobilized onto magnetic nanoparticles.

## 2. Materials and methods

### 2.1. Materials

*Yarrowia lipolytica* lipase (YLL) was purchased from Beijing CTA New Century Biotechnology Co., Ltd. (Beijing, China). (*R*)-2-octanol, (*S*)-2-octanol, (*R*, *S*)-2-octanol and vinyl acetate were purchased from Sigma–Aldrich (Sigma–Aldrich, Shanghai, China). (*R*)-2-octanol acetate and (*S*)-2-octanol acetate were synthesized according to a previously reported method [16]. Other analytical grade organic solvents were purchased from Beijing Chemical Reagents Company (Beijing, China).

### 2.2. Preparation of magnetite-immobilized YLL

Magnetic $Fe_3O_4$ nanoparticles were synthesized using the chemical precipitation method [17]. 1.98 g of $FeCl_2 \cdot 4H_2O$ and 5.4 g

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$FeCl_3{\cdot}6H_2O$ were dissolved in 200 mL of distilled water with vigorous stirring under a nitrogen atmosphere. The solution was heated to 80 °C, 25 mL $NH_4OH$ (25 wt.%) was quickly added, and the reaction was incubated with stirring at 80 °C for 30 min. The synthesized $Fe_3O_4$ particles were collected, washed five times with distilled water, dispersed in distilled water, and stored for further use.

YLL powder (1 g) was suspended in 20 mL of 50 mM phosphate buffer (pH 8.0) at 25 °C by stirring for 30 min, and the insoluble impurities were removed by centrifugation (Sigma, 4–16 K, $1.07 \times 10^4$ g, 10 min). The magnetic nanoparticles (1 g) were added to the supernatant, and the mixture was incubated at 20 °C with shaking at 150 rpm. After 4 h stirring, 1 mL of ice-cold acetone was slowly added. The final mixture was stirred for another 20 min, and the magnetite-immobilized YYL were separated using a magnet. The immobilized YLL was washed three times with phosphate buffer (pH 8.0, 50 mM) and lyophilized for 48 h. The immobilized YLL was stored at 4 °C before use. The amount of protein was determined using the Bradford assay with BSA as the standard [18], and the amount of protein bound to the magnetic nanoparticles was calculated [19].

The immobilization efficiency and activity recovery were estimated using Eqs. (1) and (2), respectively. The specific activity of the lipase was assayed using the *p*-nitrophenol method [20].

$$\eta\ (\%) = \frac{\text{Protein content of immobized lipase}}{\text{Total protein content of loading lipase}} \times 100\% \quad (1)$$

where, $\eta$ is the immobilization efficiency.

$$\Upsilon\ (\%) = \frac{\text{Specific activity of immobilized lipase}}{\text{Specific activity of the same amount of free lipase as that immobilized on magnetite}} \times 100\% \quad (2)$$

where, $\Upsilon$ is the recovery of enzyme activity.

The immobilization efficiency and activity recovery of YYL were 80% and 90%, respectively.

### 2.3. Enzymatic resolution of (R, S)-2-octanol

#### 2.3.1. Solvent selection

The enzymatic reaction was carried out in a 25 mL conical flask capped with a stopper and stirred at 150 rpm, 30 °C, with a fixed water activity. The reaction mixture contained 10 mL solvent, 1 mmol (*R*, *S*)-2-octanol, 2 mmol vinyl acetate, and 10 mg enzyme (protein). Prior to initiating the reaction, the solvent, vinyl acetate, and the immobilized lipase were equilibrated separately with a salt hydrate pair ($Na_2SO_4{\cdot}10H_2O/Na_2SO_4$) at the reaction temperature. The water activity of the reaction system was maintained at the value of 0.83. The initial activity was used as the enzyme activity.

#### 2.3.2. Reusability

After each batch reaction, the enzyme was recovered by centrifugation (for the free lipase) or magnetic separation (for the immobilized lipase) and washed three times with fresh solvent. The collected enzyme was then re-used for the next batch under the same conditions. The residual activity and enantioselectivity of the recycled enzyme were compared with those of the first batch.

All experiments were carried out in triplicate.

### 2.4. Analysis

The (*R*, *S*)-2-octanol and product were analyzed by gas chromatography (GC) using the external standard method. The linear concentration range is $1 \times 10^{-3}$ to $1 \times 10^{-1}$ mol/L. The GC7890 (Agilent, USA) was equipped with an FID detector and a column (40 m × 0.25 mm × 0.12 μm, G-TA Capillary Column, Astech, USA). Nitrogen was used as the carrier gas at a flow rate of 40 mL/min. The split ratio was 20:1, and the injection volume was 1 μL. The injector, detector, and oven temperatures were set at 200 °C, 250 °C, and 110 °C, respectively. The enantiomeric excess (*ee*) of the (*R*, *S*)-2-octanol acetate was determined by the peak areas of the two isomers using Eq. (3). The extent of conversion (*c*) of (*R*, *S*)-2-octanol was determined by the decrease of (*R*, *S*)-2-octanol. The enantioselectivity (*E*) was calculated using Eq. (4) according to the reported method [21].

$$ee_p\ (\%) = \frac{R-S}{R+S} \times 100\% \quad (3)$$

where $ee_p$ is the *ee* of the product, *R* is the concentration of the (*R*)-enantiomer, and *S* is the concentration of the (*S*)-enantiomer.

$$E = \frac{\ln[1 - c(1 + ee_p)]}{\ln[1 - c(1 - ee_p)]} \quad (4)$$

where *c* is the extent of the conversion of (*R*, *S*)-2-octanol.

### 2.5. Thermodynamic analysis

In a specific enantioselective enzyme-catalyzed reaction, the enantioselectivity, *E*, was defined as the ratio between the specificity constants for the enantiomers [21], related to the difference in activation free energy between the enantiomers as $\Delta_{R-S}\Delta G^{\#} = -RT\ln E = \Delta_{R-S}\Delta H^{\#} - T\Delta_{R-S}\Delta S^{\#}$. The enthalpic and entropic component of the *E* was calculated using Eq. (5) [22].

$$\ln E = -\frac{\Delta_{R-S}\Delta H^{\#}}{R} \cdot \frac{1}{T} + \frac{\Delta_{R-S}\Delta S^{\#}}{R} \quad (5)$$

where $\Delta_{R-S}\Delta G^{\#}$, $\Delta_{R-S}\Delta H^{\#}$, and $\Delta_{R-S}\Delta S^{\#}$ were the differences in activation free energy, activation enthalpy, and activation entropy between the reactions converting the two enantiomers, respectively. *R* was the molar gas constant, 8.31 J/(mol K), and *T* was the Kelvin temperature, K. Therefore, a thermodynamic analysis of *E* was conducted by studying the temperature dependence of *E*.

## 3. Results and discussion

### 3.1. Enzymatic resolution of (R, S)-2-octanol in solvents

The solvents were divided into two groups, cyclic and acyclic, and each group demonstrated different effects on the *E* and enzymatic activity of the magnetite-immobilized YLL. In contrast with lipase PSL which exhibited higher *E* values in the transesterification of 1-nitro-2-propanol [23] and sulcatol [24] with vinyl acetate in the cyclic solvents, YLL exhibited lower *E* values and moderate enzymatic activities in these solvents (Table 1). In the cyclic solvents, the log *P* and molecular size of the solvents showed no obvious effects on the *E* and enzymatic activity of the immobilized YLL, which were mainly dependent on the structure of the cyclic solvent.

In the acyclic solvents (Table 2), the *E* and enzymatic activity of the immobilized YLL were related to the log *P* and the molecular size of the acyclic solvent. The enzyme activity and the *E* were completely absent in dimethyl sulfoxide with a very low log *P* value of −1.49. When the log *P* of the acyclic solvent was in the range of −0.33 to 1.9, the *E* was relatively high, and the enzyme activity increased with the increasing molecular size of the acyclic solvent. When the log *P* of the acyclic solvent was in the range of 2.6–4.5, the *E* was moderate, and the enzymatic activity decreased with the increasing molecular size ($V_m$, molar volume used to characterize the molecular size) of the acyclic solvent. The solvent molecule can interact with enzyme and penetrate into the hydrophobic core of

**Table 1**
Enzymatic activity and enantioselectivity of the magnetite-immobilized YLL for the resolution of (*R*, *S*)-2-octanol in various cyclic solvents.

| Solvent | Log *P*[a] | $V_m$[b] (L/mol) | Enzymatic activity (μmol/g/min) | *E* |
|---|---|---|---|---|
| 1,4-Dioxane | −1.10 | 0.085 | 16.9 ± 0.1 | 38 ± 1 |
| Tetrahydrofuran | 0.49 | 0.081 | 16.9 ± 0.1 | 38 ± 2 |
| 2-Methyltetrahydrofuran | 0.99 | 0.101 | 17.7 ± 0.1 | 39 ± 1 |
| Nitrobenzene | 1.85 | 0.102 | 17.8 ± 0.1 | 41 ± 1 |
| Benzene | 2.0 | 0.089 | 17.1 ± 0.1 | 39 ± 1 |
| Anisole | 2.11 | 0.109 | 17.7 ± 0.1 | 42 ± 2 |
| Toluene | 2.50 | 0.107 | 17.9 ± 0.1 | 39 ± 1 |
| Chlorobenzene | 2.84 | 0.102 | 17.8 ± 0.1 | 40 ± 0.5 |
| Cyclohexane | 3.20 | 0.108 | 17.2 ± 0.1 | 41 ± 0.5 |
| Cycloheptane | 4.00 | 0.121 | 17.5 ± 0.1 | 40 ± 1 |
| Diphenyl ether | 4.20 | 0.158 | 16.9 ± 0.1 | 39 ± 1 |
| Cyclooctane | 4.45 | 0.134 | 17.5 ± 0.1 | 42 ± 1 |

[a] The values were taken from Ref. [4] or calculated using the Kirkwood theory (J. G. Kirkwood, J Chem. Phys. 2 (1934) 351–361).
[b] The molar volume of the solvent, $V_m$ (L/mol), was calculated according to the following equation: $V_m$ = MW/1000 d, where *d* and MW are the density and molecular weight of the solvent, respectively.

the enzyme to result in a site-specific interaction [4,24,25]. The interaction between the cyclic solvent and the YLL were weaker than those between the acyclic solvent and the YLL because the molecular structure of the cyclic solvents resulted in greater steric hindrance in comparison with that of the acyclic solvents.

In addition, the magnetite-immobilized YLL exhibits a low enzymatic activity and enantioselectivity in acyclic solvents containing branched chain(s), such as 2,2-dimethylbutane, 2,4-dimethylpentane, and 2,2,4-trimethypetane which are the isomers of hexane, heptane, and octane, respectively (Table 2). These results further confirmed that the enzymatic activity and enantioselectivity of the immobilized YLL were strongly affected by the solvent structure.

The interaction sites and force between the acyclic hydrophilic solvent ($\log P \leq 1.9$) and the immobilized YLL were different from those between the acyclic hydrophobic solvent ($\log P \geq 2.6$) and the immobilized YLL, and this difference led to the decrease of enzymatic activity when the molecular size of the acyclic hydrophilic solvent increased. Among the acyclic hydrophilic solvents tested, dimethyl sulfoxide interacted more strongly with the immobilized YLL compared to other acyclic solvents. It was considered that the dimethyl sulfoxide induced a drastic change in the conformation of the YLL that was completely inactivated. A previous report [26] indicated that a hydrophilic solvent can inactivate the enzyme by stripping the essential water from the enzyme. In our study, however, the enzymatic reactions were conducted at a fixed water activity (the most convenient parameter for correlating enzymatic activity in a non-aqueous media [27,28]). This largely eliminated solvent effects due to differences in water partitioning [29] and indicated that the effect of the solvent on the lipase arose from the solvent itself. The enzymatic activity of the immobilized YLL increased as the $V_m$ increased because the number of solvent molecules effectively interacting with the YLL decreased. As a result, the immobilized YLL was released from the adverse effects of the acyclic hydrophilic organic solvent. Acetone and acetonitrile can interact with lipase via intramolecular hydrogen bonds, which play an important role in the native structure of the lipase [30]. The structure of YLL is disrupted by the formation of intermolecular hydrogen bonds, such as (YLL)—N—H. . .O=C($CH_3$)$_2$ (acetone) and (YLL)—N—H. . .N≡C—$CH_3$ (acetonitrile), which lead to severe distortions in its conformation. *R*-2-octanol and *S*-2-octanol were not able to correctly bind to the deformed active site, which resulted in the significant decrease in the reactivity of *S*-2-octanol. Therefore, the *E* of YLL in acetone and acetonitrile was high.

Enzymatic activity and *E* of an enzyme are crucial factors in the enzymatic process and influence its potential applications. In a typical enzymatic process, the reaction time is less than 24 h, and the *ee* of the product or substrate obtained is not less than 99% [31]. This requires that the enzyme exhibits both a high activity and a high *E* during the catalytic process. To identify a reaction medium in which YLL exhibited a relatively high activity and *E* for the resolution of (*R*, *S*)-2-octanol, the effect of a mixture of acetone and carbon tetrachloride on the immobilized YLL performance was investigated (Table 3). As the percentage of carbon tetrachloride in the mixed reaction increased, the *E* value decreased significantly while the reaction rate was significantly enhanced. This suggested that each component of the mixed solvent interacted with the YLL and thereby influenced the conformation, activity, and *E* of the enzyme [32]. Based on these results, a mixed solvent system composed of 30% acetone and 70% carbon tetrachloride was recommended for the resolution of (*R*, *S*)-2-octanol by the magnetite-immobilized YLL.

**Table 2**
Enzymatic activity and enantioselectivity of the magnetite-immobilized YLL for the resolution of (*R*, *S*)-2-octanol in various acyclic solvents.

| | Solvent | Log *P* | $V_m$ (L/mol) | Enzymatic activity (μmol/g/min) | *E* |
|---|---|---|---|---|---|
| Without branched chain(s) | Dimethyl sulfoxide | −1.49 | 0.071 | – | – |
| | Acetonitrile | −0.33 | 0.052 | 3.90 ± 0.1 | 659 ± 8 |
| | Acetone | −0.23 | 0.074 | 6.20 ± 0.2 | $2.10 \times 10^3$ ± 11 |
| | Butanone | 0.29 | 0.09 | 7.60 ± 0.2 | 216 ± 5 |
| | Ethyl acetate[a] | 0.68 | 0.098 | 8.20 ± 0.2 | 185 ± 6 |
| | Isopropyl ether | 1.90 | 0.142 | 37.5 ± 0.1 | 89 ± 5 |
| | Carbon tetrachloride | 2.60 | 0.0967 | 50.3 ± 0.2 | 78 ± 2 |
| | Hexane | 3.50 | 0.131 | 38.2 ± 0.1 | 87 ± 2 |
| | Heptane | 4.00 | 0.147 | 35.8 ± 0.1 | 95 ± 3 |
| | Octane | 4.50 | 0.163 | 32.5 ± 0.2 | 97 ± 3 |
| With branched chain(s) | 2,2-Dimethylbutane | 3.00 | 0.133 | 29.5 ± 0.1 | 67 ± 1 |
| | 2,4-Dimethylpentane | 3.40 | 0.150 | 25.4 ± 0.1 | 76 ± 1 |
| | 2,2,4-Trimethypetane | 3.80 | 0.165 | 22.2 ± 0.1 | 81 ± 2 |

[a] The reaction of ethyl acetate reacting with 2-octanol was not significantly detected.

**Table 3**
Effect of the acetone and carbon tetrachloride mixture on the resolution of (*R*, *S*)-2-octanol catalyzed by the magnetite-immobilized YLL.

| Volume ratio[a] (%, v/v) | | Reaction time (h) | Conversion (%) | $ee_p$ (%) | *E* |
|---|---|---|---|---|---|
| Acetone | Carbon tetrachloride | | | | |
| 90 | 10 | 36 | 19.5 ± 0.1 | 99.8 ± 0.001 | $1.69 \times 10^3 \pm 8$ |
| 80 | 20 | 36 | 25.8 ± 0.1 | 99.7 ± 0.002 | $1.12 \times 10^3 \pm 7$ |
| 70 | 30 | 36 | 29.9 ± 0.1 | 99.7 ± 0.001 | 949 ± 1 |
| 60 | 40 | 36 | 34.5 ± 0.1 | 99.6 ± 0.001 | 867 ± 0.2 |
| 50 | 50 | 24 | 39.5 ± 0.1 | 99.5 ± 0.002 | 799 ± 6 |
| 40 | 60 | 24 | 42.6 ± 0.1 | 99.4 ± 0.001 | 777 ± 2 |
| 30 | 70 | 24 | 45.1 ± 0.1 | 99.3 ± 0.001 | 694 ± 3 |
| 20 | 80 | 24 | 46.7 ± 0.2 | 98.2 ± 0.001 | 299 ± 3 |
| 10 | 90 | 24 | 48.3 ± 0.1 | 95.2 ± 0.001 | 121 ± 1 |

[a] The total volume of the mixture is 10 mL.

**Table 4**
Thermodynamic components of the *E* for the resolution of (*R*, *S*)-2-octanol in various reaction systems by the magnetite-immobilized YLL.

| Reaction system | *E* (298 K) | $\Delta_{R-S}\Delta G$ (298 K) (kJ/mol) | $\Delta_{R-S}\Delta H$ (kJ/mol) | $\Delta_{R-S}\Delta S$ (J/mol) | $T_r$[a] (K) |
|---|---|---|---|---|---|
| Acetone | $2.37 \times 10^3$ | −19.3 | −20.8 | −4.92 | $4.22 \times 10^3$ |
| Acetone (30%) + carbon tetrachloride (70%) | 768 | −16.5 | −18.5 | −6.81 | $2.17 \times 10^3$ |
| Carbon tetrachloride | 99 | −11.4 | −17.4 | −21.6 | 808 |

$\Delta\Delta G$ was calculated at 298 K using the mean of *E*. $\Delta\Delta H$ and $\Delta\Delta S$ values were determined from the straight line of ln *E* vs. 1/*T*. *T* was in the range of 293–313 K.
[a] $T_r$ was defined as the racemic temperature, and $T_r = \Delta\Delta H/\Delta\Delta S$.

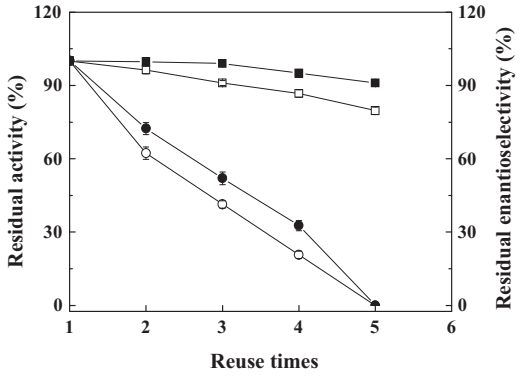


**Fig. 1.** Reusability of the free and magnetite-immobilized YLL. Reaction medium: acetone and carbon tetrachloride (v/v = 3:7), 10 mL. Residual activity: □ magnetite-immobilized, ○ free; residual enantioselectivity: ■ magnetite-immobilized, • free.

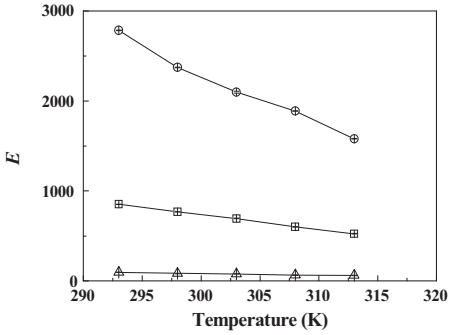


**Fig. 2.** Effect of temperature on enantioselectivity of the magnetite-immobilized YLL. □ Acetone + carbon tetrachloride, ○ acetone, △ carbon tetrachloride.

### 3.2. Reusability of the magnetite-immobilized YLL

Reusability of the immobilized enzyme is an important property for its applications [33]. Therefore, the reusability of the magnetite-immobilized YLL in the repeated batch resolution of (*R*, *S*)-2-octanol was studied. As shown in Fig. 1, the operational stability of YLL was significantly improved after being immobilized on magnetite. This was in agreement with sol–gel immobilized *Candida antartica* lipase B used to resolve 2-octanol [34]. The free *Candida antartica* lipase B lost 15% of its activity after the first reuse, while the sol–gel immobilized *Candida antartica* lipase B maintained its catalytic activity during reuse. The free YLL lost its enzymatic activity and enantioselectivity entirely after four batches, whereas the magnetite-immobilized YLL retained 90% of its initial enantioselectivity and approximately 80% of its initial activity after five cycles. Together, these results indicated that the magnetite-immobilized YLL had potent reusability in the kinetic resolution of (*R*, *S*)-2-octanol.

### 3.3. Thermodynamic analysis

Thermodynamic analysis was conducted to study the effects of substrate, reaction medium, and lipase type on the temperature dependence of *E* in lipase-catalyzed kinetic resolutions [35–37]. In order to evaluate the effects of the reaction medium on the *E* of the immobilized YLL, the resolution of (*R*, *S*)-2-octanol with vinyl acetate as the acyl donor in the selected reaction medium was thermodynamically analyzed.

As shown in Fig. 2, the *E* of YLL decreased as the temperature increased. Linear relationships were identified between ln *E* and $T^{-1}$ in various reaction systems, from which $\Delta_{R-S}\Delta H$ and $\Delta_{R-S}\Delta S$ were estimated. The difference in $\Delta_{R-S}\Delta G$ for the transient states of *R*- and *S*-reacting enantiomers can be separated into the differences in $\Delta_{R-S}\Delta H$ and $\Delta_{R-S}\Delta S$. It can then be clearly visualized whether the enantiomer discrimination is either enthalpy- or entropy-driven [37]. As shown in Table 4, $-\Delta_{R-S}\Delta H$ was greater than $-T\Delta_{R-S}\Delta S$ at 298 K. This indicated that the enantiomer discrimination was enthalpy-driven at this temperature. The increase of $-\Delta_{R-S}\Delta H$ from 17.4 kJ/mol in carbon tetrachloride to 20.8 kJ/mol in acetone indicated that acetone was favorable for enhancing the interactions between the fast-reacting (*R*)-2-octanol at the transition state and YLL residues to lower the enthalpy. In addition, the racemic temperature ($T_r$), was above 313 K, the maximum in the experimental temperature range for all the reaction systems tested, and the *E* of YLL decreased with the increase in temperature. If the experimental temperature is above the $T_r$, the enantioelectivity changes to the entropically preferred enantiomer, and the enantioselectivity will increase with the increase in temperature [5].

## 4. Conclusion

The solvent structure was found to be the key factor governing the performance of the resolution of (*R*, *S*)-2-octanol by *Yarrowia lipolytica* lipase immobilized onto magnetic nanoparticles. A suitable acyclic structurally linear solvent resulted in sufficient enantioselectivity or enzymatic activity of the immobilized YLL for this resolution. A mixed solvent system with acetone and carbon tetrachloride was developed for enhancing both the enantioselectivity and the enzymatic activity, and the thermodynamic analysis demonstrated that the enantiomer discrimination was enthalpy-driven at the temperatures tested.

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