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# Improved activity of lipase immobilized in microemulsion-based organogels for (*R*,*S*)-ketoprofen ester resolution: Long-term stability and reusability

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ABSTRACT

Microemulsion-based organogels (MBGs) were effectively employed for the immobilization of four commonly used lipases. During the asymmetric hydrolysis of ketoprofen vinyl ester at 30 °C for 24 h, lipase from *Rhizomucor miehe*i and *Mucor javanicus* immobilized in microemulsion-based organogels (RML MBGs and MJL MBGs) maintained good enantioselectivities ($ee_p$ were 86.2% and 99.2%, respectively), and their activities increased 12.8-fold and 7.8-fold, respectively, compared with their free forms. They gave higher yields compared with other lipase MBGs and exhibited better enantioselectivity than commercial immobilized lipases. Immobilization considerably increased the tolerance to organic solvents and high temperature. Both MJL MBGs and RML MBGs showed excellent reusability during 30 cycles of repeated 24 h reactions at 30 °C (over 40 days). The system maintained yields of greater than 50%, while the $ee_s$ values of RML MBGs and MJL MBGs remained nearly constant at 95% and 88%, respectively.



## 1. Introduction

Immobilization is often the key to optimizing the operational performance of an enzyme in an industrial process, especially for use in non-aqueous media. For lipases in particular, immobilization often provides the extra advantage of increasing the catalytic activity of the lipase compared with free enzymes [1,2]. Microemulsion-based organogels (MBGs) have become an attractive approach for enzyme immobilization to facilitate enzymatic catalysis in non-conventional media, because of their long-term stabilities [3–5]. Moreover, compared with other methods, the use of MBGs is a relatively inexpensive and facile method of enzyme immobilization. Although successful biotransformations employing enzymes immobilized in MBGs have been well documented [6,7] and were highlighted in our previous study [8], few reports have focused on the application of this method to kinetic resolution reactions.

Ketoprofen (2-(3-benzoylphenyl) propionic acid) is an important nonsteroidal antiinflammatory drug (NSAID) and is widely used as a racemic mixture to reduce inflammation and relieve pain [9]. However, these healing properties are mainly exerted by the (*S*)-enantiomer, whereas the (*R*)-enantiomer can be used as a toothpaste additive to prevent periodontal disease. Thus, efforts to obtain single enantiomer of ketoprofen are important and are expected to receive increased attention because of the pharmacological benefits of both enantiomers.

In recent years, enzymatic kinetic resolution has gained increasing importance as a versatile method for accessing optically active fine chemicals, particularly pharmaceuticals [10]. Indeed, hydrolase-catalyzed kinetic resolution remains an attractive option, because of the simplicity of the process and the high enantioselectivity of biocatalysis under mild conditions [11].

To obtain optically pure ketoprofen, commercial lipases such as Novozyme 435 (from *Candida antarctica*) and Lipozyme immobilized from *Mucor miehei* [12,13] have been used to resolve enantiomers of ketoprofen via biocatalytic resolution. However, in many cases, it has been difficult to find a commercial enzyme that exhibits both satisfactory enantioselectivity and activity for the kinetic resolution of unnatural substrates, including ketoprofen. Moreover, the high cost of nonrenewable catalysis further

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restricts its industrial application. Therefore, different strategies have been proposed to enhance the activity and enantioselectivity of existing commercial enzymes, including optimization of reaction conditions [14], modification of the substrate and/or enzyme [15], and improvement of mass transfer in the reaction [16,17].

Enzyme immobilization may facilitate the sustainability of this process and present numerous advantages for industrial applications, such as allowing repeated usage of an enzyme, simplification of product separation and improvement of enzyme stability, among other benefits [18,19].

Although aqueous solutions are frequently used in the resolution of ketoprofen, they can cause difficulties in substrate diffusion, product separation and enzyme recovery. In an attempt to ameliorate these issues, approaches involving surfactants and non-aqueous systems have been explored [17]. The practical application of surfactants has been hindered by the need to separate the surfactant from the reaction products. The use of non-aqueous solutions has been less studied because many enzymes are readily denatured in organic solvents, and enzyme-catalyzed hydrolysis generally requires the use of aqueous solutions.

In this study, several lipases were immobilized in MBGs, and their activity and enantioselectivity in the hydrolysis of ketoprofen vinyl ester in a non-aqueous solvent were examined. Lipase from *Rhizomucor miehei* immobilized in microemulsion-based organogels (RML MBGs) and lipase from *Mucor javanicus* immobilized in microemulsion-based organogels (MJL MBGs) were selected to further study the activity, selectivity and stability under various experiment conditions. The tolerance of the immobilized lipases to various organic solvents, temperatures and repeated long use was evaluated. Furthermore, the gram-scale resolutions of ketoprofen vinyl esters were tested to determine the possibility of applying the lipase MBGs in industrial chemical synthesis and other bulk applications.

## 2. Materials and methods

### 2.1. Materials

Sodium bis-(2-ethylhexyl) sulfosuccinate (AOT) was obtained from Acros Organics (New Jersey, USA). Lipase B acrylic resin from *C. antarctica* (CAL-B, ≥5000 U/g, Shanghai), lipase from *Candida rugosa* (CRL, Type VII, white powder, 739 U/mg, Japan), lipase from *M. javanicus* (MJL, Amano, white powder, ≥10,000 U/g, Japan), Lipozyme immobilized from *M. miehei* (MML, 102.5 U/g, Switzerland), lipase from *R. miehei* (RML, slightly yellow transparent liquid, ≥20,000 U/g, Denmark), lipase from *Pseudomonas cepacia* (PCL, Amano, white powder, ≥50,000 U/g, Japan) and gelatin (from porcine skin, Type A, Shanghai) were purchased from Sigma–Aldrich. Ketoprofen was obtained from Wuhan Gang Zheng Biology Technology (China). Profen vinyl esters were synthesized and purified as described by Wang et al. [20]. All of the organic solvents and other chemicals used were of analytical reagent grade from local manufacturers in China and used without further dehydration. Double distilled water was employed throughout the experiments.

### 2.2. Preparation of lipase MBGs

The method used for the preparation of lipase MBGs was similar to that described in a previous paper by our group [8]. Lipase (15 mg/mL) was incubated overnight at 4 °C in phosphate buffer (pH 7.0, 0.1 M) in the presence of 1 mM AOT. After 24 h, the lipase solution was added to reverse micellar solutions of AOT/buffer/isooctane (Wo = 60). A thermodynamically stable reverse micellar solution was prepared by mixing each component in a suitable ratio. Lipase-containing microemulsions were obtained by adding the previously prepared AOT-coated lipase solution to the AOT reverse micellar solution with the appropriate water content in isooctane. This solution was shaken briefly, then immediately added to a second solution of 14% (w/v) gelatin in phosphate buffer (pH 7.0, 0.1 M) at 55 °C, stirred vigorously until reaching homogeneity, and cooled to 25 °C. The gelatin solution was prepared by dissolving gelatin obtained from porcine skin in phosphate buffer, followed by autoclaving and cooling to 55 °C before use. The obtained gel was then poured into plastic plates and left overnight to air-dry. The following day, the dried gel was cut into small pieces (approximately 1–2 $mm^2$ in size) and refrigerated at −18 °C for later use.

### 2.3. Enzymatic reaction conditions

In addition to the solvent experiments, additional experiments were performed in isopropyl ether. Lipase MBGs were weighed and added to 2 mL of isopropyl ether containing 10 mg ketoprofen vinyl ester, unless otherwise noted. Each reaction mixture was incubated at 200 rpm in a temperature–controlled shaker at the desired temperature. The reaction was initiated by the addition of 10 mg of lipase or lipase MBGs containing 10 mg lipase. Samples were withdrawn from the reaction medium at regular intervals and analyzed via high-performance liquid chromatography (HPLC). The hydrolysis reaction was conducted at a larger scale using 5 g of ketoprofen vinyl ester, as described in detail in Section 3.5. All of the experiments were repeated at least three times.

### 2.4. Tolerance of lipase MBGs to solvents and temperatures

RML MBGs and MJL MBGs were used for the hydrolysis of ketoprofen vinyl ester in various organic solvents. Free lipase served as the control. Hexane (water content ≤ 0.05%), isopropyl ether (water content 0.1%), *tert*-butyl methyl ether (water content ≤ 0.05%), toluene (water content 0.03%), isopropanol (water content ≤ 0.2%), tetrahydrofuran (THF, water content ≤ 0.01%), 2-methyl tetrahydrofuran (2-MeTHF, water content ≤ 0.05%), 1, 4-dioxane (water content ≤ 0.1%), acetone (water content ≤ 0.3%) and acetonitrile (water content ≤ 0.01%) were selected as solvents.

Tolerance of temperature changes was examined by performing the above reaction at three different temperatures: 20, 30 and 50 °C. A control setup was included at each temperature, with free lipase as the catalyst.

### 2.5. Reusability of RML MBGs and MJL MBGs

To analyze the reusability of lipase MBGs, the hydrolysis reaction was performed as described above for ketoprofen vinyl ester. Upon the completion of one cycle, the immobilized enzyme was recovered via filtration. The recovered lipase MBGs were washed three times with isopropyl ether (3 mL × 3) to ensure complete removal of the product and substrates. The residual solvent was subsequently removed using $N_2$, and fresh solvent was reintroduced into the system. This procedure was repeated for several cycles.

### 2.6. Analytical procedures

Quantitative analysis of the samples was performed via HPLC through a CHIRALPAK column (Chiral AD-H, 5 μm, 4.6 mm × 250 mm; Daicel Chemical) using a Shimadzu LC-2010A HT apparatus equipped with a 254 nm UV detector. Hexane with 10% (v/v) isopropanol was employed as the mobile phase with a split flow

rate of 0.5 mL/min. Retention times were as follows: (*R*)-ketoprofen vinyl ester, 12.24 min; (*S*)-ketoprofen vinyl ester, 13.00 min; (*R*)-ketoprofen, 25.18 min; and (*S*)-ketoprofen, 29.14 min.

### 2.7. Computational methods

All molecular simulations were performed using Discovery Studio 3.1 (Accelrys, San Diego, CA, USA). Accelrys Discovery Studio 3.1 is available from Accelrys Inc.-San Diego, CA 92,121, USA. Protein structures were obtained from the Protein Data Bank (PDB). All protein structures were prepared before molecular docking. Active sites were defined using "From Current Selection" tools based on active residues including catalytic triad residues and oxyanion hole residues. All chemical compounds were constructed manually using Discovery Studio Visualizer and were subjected to the "Minimization" module for full structural refinement with 5000 steps of the steepest descent algorithm, followed by 2000 steps of the conjugate gradient algorithm energy minimization, utilizing the generalized born implicit solvent model and the CHARMM forcefield. Molecular docking was then performed using the "Flexible Docking" module [21] implemented in Discovery Studio 3.1 [22]. Finally, conformations with highest -CDocker interaction energy in each docking process were analyzed and visualized in Discovery Studio (detailed in Supporting information). For the substrate binding step, -CDocker Interaction Energy (-CDIE) and -CDocker Energy (-CDE) were used to evaluate the interaction energy and enzyme-substrate complex stability, respectively.

## 3. Results and discussion

### 3.1. Different lipases immobilized in MBGs

In a preliminary study, we demonstrated that entrapment within microemulsion-based organogels was an efficient and facile method for immobilizing lipase from *C. rugosa*, as immobilized *C. rugosa* lipase showed remarkable stability in organic solvents and at high temperatures [8]. To extend this work, experiments were performed to investigate the differences among different lipases immobilized in MBGs. Changes in the activity and selectivity of the enzymes following immobilization were detected through the hydrolysis of ketoprofen vinyl ester. Initially, to investigate the role of the immobilization system in the hydrolysis reaction, the control experiments were performed using single gelatin, surfactant, reverse micelle solution and MBGs without lipase as catalyst. The reaction without lipase was investigated as well. There was no product observed in all these reactions even after 48 h, suggesting that the catalytic effect of the polymeric support was excluded. In the present work, four lipases that are differ in species, taxa and structural characteristics were selected. Lipases from *M. javanicus* (MJL) and *R. miehei* (RML) were selected as representatives of the fungal taxon. Lipases from *C. rugosa* (CRL) and *P. cepacia* (PCL) were chosen to represent the yeast and prokaryotic bacterial taxa, respectively. The results are shown in Table 1. After 72 h, most of the free lipases showed very low activity in isopropyl ether, with a maximum yield of 11.8%. Additionally, only MJL and RML exhibited good enantioselectivity. Following immobilization, MJL MBGs and RML MBGs demonstrated maximum increases in activities and gave higher yields compared with other lipases. After 24 h, the

**Table 1**
Kinetic resolutions of ketoprofen vinyl ester catalyzed by free or immobilized lipases from different sources[a].

| Source | Name | Abbreviation | Time | Yield[b] (%) | $ee_p$[b] (%) | $ee_s$[b] (%) | $E$[c] |
|---|---|---|---|---|---|---|---|
| Fungus | *Mucor javanicus* lipase | MJL | 24 h | 3.7 ± 0.5 | 99 ± 0.2 | 6 ± 0.5 | >200 |
| | | | 48 h | 10.9 ± 1.3 | 99 ± 0.2 | 14 ± 0.7 | >200 |
| | | | 72 h | 11.8 ± 0.7 | 83 ± 1.2 | 20 ± 1.3 | 13.4 |
| | | MJL MBGs | 24 h | 29.0 ± 1.1 | 99 ± 0.5 | 54 ± 0.9 | >200 |
| | | | 48 h | 36.7 ± 0.8 | 95 ± 1.1 | 87 ± 1.3 | 104.7 |
| | | | 72 h | 43.1 ± 0.6 | 88 ± 2.1 | 98 ± 1.2 | 71.6 |
| | *Rhizomucor miehei* lipase | RML | 24 h | 3.2 ± 0.3 | 99 ± 1.7 | 6 ± 1.4 | >200 |
| | | | 48 h | 7.3 ± 0.6 | 99 ± 1.3 | 9 ± 0.5 | >200 |
| | | | 72 h | 7.3 ± 0.7 | 98 ± 1.1 | 11 ± 1.6 | 116.5 |
| | | RML MBGs | 24 h | 41.1 ± 2.4 | 86 ± 0.6 | 88 ± 2.1 | 35.2 |
| | | | 48 h | 44.9 ± 1.3 | 77 ± 1.8 | 95 ± 1.7 | 27.9 |
| | | | 72 h | 48.9 ± 0.8 | 67 ± 1.3 | 97 ± 2.6 | 17.8 |
| | *Mucor miehei* lipase | MML | 24 h | 25.0 ± 1.5 | 68 ± 2.3 | 86 ± 1.6 | 14.5 |
| | | | 48 h | 52.0 ± 2.3 | 45 ± 1.9 | 91 ± 3.1 | 7.7 |
| | | | 72 h | 66.1 ± 1.4 | 28 ± 1.2 | 89 ± 1.9 | 4.7 |
| Yeast | *Candida rugosa* lipase | CRL | 24 h | 2.0 ± 0.4 | 36 ± 2.4 | 1 ± 0.3 | 2.1 |
| | | | 48 h | 3.8 ± 0.7 | 35 ± 2.2 | 1 ± 0.5 | 2.1 |
| | | | 72 h | 4.0 ± 1.1 | 26 ± 1.9 | 3 ± 1.4 | 1.8 |
| | | CRL MBGs | 24 h | 3.0 ± 1.1 | 57 ± 0.7 | 2 ± 0.9 | 3.8 |
| | | | 48 h | 4.9 ± 1.4 | 64 ± 1.6 | 5 ± 1.3 | 4.8 |
| | | | 72 h | 6.0 ± 1.7 | 70 ± 1.5 | 6 ± 1.1 | 6.1 |
| | *Candida antarctica* lipase B | CAL-B | 24 h | 75.7 ± 1.7 | 0 ± 0.0 | 9 ± 0.5 | 1.1 |
| | | | 48 h | 80.6 ± 2.6 | 8 ± 0.4 | 5 ± 0.7 | 1.2 |
| | | | 72 h | 85.5 ± 3.5 | 5 ± 0.3 | 2 ± 0.6 | 1.1 |
| Bacteria | *Pseudomonas cepacia* lipase | PCL | 24 h | 1.1 ± 1.5 | 49 ± 1.6 | 0 ± 0.0 | 2.9 |
| | | | 48 h | 1.4 ± 1.7 | 31 ± 1.4 | 0 ± 0.0 | 1.8 |
| | | | 72 h | 2.8 ± 1.1 | 15 ± 0.7 | 0 ± 0.1 | 1.4 |
| | | PCL MBGs | 24 h | 1.2 ± 0.5 | 12 ± 0.6 | 0 ± 0.0 | 1.3 |
| | | | 48 h | 1.8 ± 0.9 | 18 ± 1.2 | 0 ± 0.1 | 1.4 |
| | | | 72 h | 2.4 ± 0.7 | 35 ± 1.5 | 1 ± 0.4 | 2.1 |

[a] Reactions were performed with 10 mg ketoprofen vinyl ester and 10 mg lipase or lipase immobilized in microemulsion-based organogels (MBGs) containing 10 mg lipase in 2 mL isopropyl ether at 30 °C for 24 h, 48 h and 72 h.
[b] Yield and *ee* were determined via high-performance liquid chromatography (HPLC) using a Chiral AD-H column.
[c] $E = \ln[1 - c\,(1 + ee_p)]/\ln[1 - c\,(1 - ee_p)]$, $c = ee_s/(ee_s + ee_p)$ [23].

activities of MJL MBGs and RML MBGs increased 7.8-fold and 12.8-fold, respectively, compared with their free forms. In addition, MJL MBGs exhibited a high *E*-value greater than 200 after 24 h, whereas RML MBGs maintained a moderate *E*-value. In the case of CRL, both activity and enantioselectivity improved 1.5-fold after immobilization in MBGs. Moreover, PCL MBGs showed almost constant activity and decreased enantioselectivity.

To compare the performance of the lipase MBGs with that of commercial immobilized lipases addressed in the literature, we also measured the activities and enantioselectivities of Novozyme 435 (*C. antarctica*) and Lipozyme MML. MML showed high activity and low *E*-values, whereas CAL-B exhibited almost no selectivity. Compared with other processes using commercial or laboratorial immobilized lipases (Table 2), RML MBGs and MJL MBGs showed obvious superiority in the asymmetric hydrolysis of ketoprofen vinyl ester at 30 °C. The biotransformation catalyzed by MBGs resulted in improved enzymatic conversion efficiency and good enantioselectivity. Further advantages of MBGs reaction systems without the addition of water are improved solubility of aromatic substrates and products, excellent recyclability and low enzyme loading.

These results may be attributed to the specific active sites and spatial conformations of the different lipases. The conformational behaviors of the tested lipases indicate remarkable differences among them, not only in terms of the accessibility of the active site but also regarding the modification of the geometry of the catalytic machinery.

A unique property that distinguishes lipases from esterases is the enhanced activity of lipases at or near the lipid/water interface [26], which is related to the presence of a flexible protein domain called the lid [27]. The lid opens upon contact of the lipase with an interface. This leads to the restructuring of the lipase by creating an electrophilic region (the oxyanion hole) around the serine residue by exposing hydrophobic residues and burying hydrophilic ones. This process stabilizes the transition state intermediate during catalysis [28,29].

**Table 3**
Residues forming the catalytic machinery of the tested lipases.

| Enzyme | PDB code | Catalytic triad | Oxyanion hole |
|---|---|---|---|
| RML | 4TGL | Ser144, His257, Asp203 | Ser82, Leu145 |
| CAL-B | 1TCA | Ser105, His224, Asp187 | Thr40, Gln106 |
| CRL | 1CRL | Ser209, His449, Glu341 | Gly123, Gly124, Ala210 |
| PCL | 3LIP | Ser87, His286, Asp264 | Leu17, Glu88 |

It has been reported that PCL exhibits a broad lid domain and a stable open conformation, even in aqueous environments [30]. Therefore, the MBG system likely has little effect on the conformation of this lipase. In contrast to PCL, the lids of RML and MJL are much smaller and, consequently, can be easily altered in the immobilization environment. In addition, CRL has an intermediate-sized lid domain, between the sizes of the wide lid of the *Pseudomonas* lipase and the small lid observed in the fungal RML and MJL lipases, and it displayed intermediate increases in activity and selectivity. Finally, CAL-B represents an atypical case in these experiments because this enzyme has a very small lid, which is not able to cover the active site. Accordingly, CAL-B showed the highest activity but the lowest enantioselectivity.

### 3.2. Computational simulations

In light of these clear differences in activity and selectivity among lipases from different species, we then performed computational docking simulations to consider the variations

**Table 2**
Comparison of the efficiency of ketoprofen kinetic resolution using different immobilized lipases.

| Enzyme | Reaction conditions | Product | Reaction efficiency | | | Ref. |
|---|---|---|---|---|---|---|
| | | | Yield (%) | *ee* (%) | *E* | |
| Lipozyme IM (commercial immobilized RML) | Esterification isopropyl ether, 37 °C | *R*-Ketoprofen 1-butanol ester | 20 (72 h) | 9.5 | 2.4 | LoÂpez -Belmonte et al. [13] |
| Immobilized MJL | Hydrolysis pH 7.0 buffer, 30 °C (containing 30% v/v acetone) (2 mg substrate versus 30 mg MJL) | *R*-ketoprofen (after 5 cycles remaining 50% activity) | 53 | 86 | 55 | Kato et al. [24] |
| Immobilized CRL | Hydrolysis pH 3.5 buffer with Tween 80 as additive, 30 °C | *S*-Ketoprofen | 22.3 (72 h) | 94 | 50 | Liu et al. [25] |
| Immobilized CAL-B in an enzymatic membrane reactor | Esterification dichloropropane: hexane = 20: 80, 40 °C | *R*-ketoprofen 1-butanol ester (after 5 cycles remaining 50% activity) | 73 (24 h) | $ee_p$ 87 $ee_s$ 57 | 27 | Ong et al. [15] |
| RML MBGs | Hydrolysis acetate vinyl ester, 30 °C | *R*-Ketoprofen | 41 (24 h) | $ee_p$ 86 $ee_s$ 88 | 35 | This study |
| | | | 53 (after 30 cycles) | $ee_p$ 89 $ee_s$ 91 | 67 | |
| MJL MBGs | | | 29 (24 h) | $ee_p$ 99 $ee_s$ 54 | >200 | |
| | | | 55 (after 30 cycles) | $ee_p$ 90 $ee_s$ 81 | 48 | |

occurring at the level of the catalytic machinery of each enzyme in more detail. The first priority was to identify the residues forming the catalytic machinery of each considered enzyme, namely the catalytic triad and the oxyanion hole (Table 3).

It is well established that there are two steps required for enzyme catalysis: connection between substrate and enzyme and catalytic transformation from substrate to product through catalytic residues. The ability to bind and the efficiency of catalysis are of equal importance and are therefore considered in molecular docking analyses. For the substrate binding step, we used -CDocker Interaction Energy (-CDIE) and -CDocker Energy (-CDE) to evaluate the interaction energy and enzyme-substrate complex stability, respectively. The ability of an enzyme to bind substrates can be estimated using -CDIE. Higher -CDIE values indicate better binding abilities, whereas higher -CDE values indicate a greater ability of stable substrates to remain in the correct position. For the enzyme catalysis step, the distances between the substrate atoms involved in catalysis and the catalytic residues were considered, including the catalytic triad and oxyanion hole residues. In particular, the distance between the carbon atom of the carbonyl in the vinyl ester and the hydroxyl oxygen of the nucleophilic serine side chain ($D_1$) and the distance between the oxygen atom of the carbonyl and the closest hydrogen donor of the oxyanion hole ($D_2$) were analyzed (Fig. 1).

As shown in Table 4, the -CDIE and -CDE values between RML and the (*R*)-enantiomer were 34.9154 kcal/mol and 26.4078 kcal/mol, respectively, indicating that RML is apt to interact with the (*R*)-enantiomer and that the interaction is stable. Although both the -CDIE and -CDE values of RML and the (*S*)-enantiomer were greater than those of RML and the (*R*)-enantiomer, the carbonyl oxygen of the (*S*)-enantiomer is far away from the catalytic triad and oxyanion hole residues (Fig. 1). Moreover, as can be seen in Fig. 1(c), the phenyl ring in (*S*)-enantiomer can form a Pi-sigma interaction with Ser82 residue, which enhanced the stability of RML-substrate complex and hindered catalysis. This might account for the low activity and high enantioselectivity of the native RML. After activated in the immobilization environment, lipase was stabilized in an open form. Therefore, RML MBGs showed efficiently improved yield while maintained good enantioselectivities. CAL-B showed high -CDIE and -CDE values to both enantiomers, which explained the ability of CAL-B to interact efficiently. Additionally, the oxygen atom of the carbonyl in (*R*)-ketoprofen formed three hydrogen bonds with the residues in the oxyanion hole in CAL-B, which enhanced the interaction between the (*R*)-enantiomer and CAL-B and aligned the carbonyl group in the catalytic sites to facilitate subsequent catalysis. However, because of the similar $D_1$ and $D_2$ values of the (*R*)- and (*S*)-enantiomers, CAL-B showed almost no selectivity. The enantioselectivity of CRL was poor because of the similar connection ability and activity to both enantiomers. Higher -CDIE and -CDE values of the (*S*)-enantiomer indicated the binding of the (*S*)-enantiomer with PCL was more efficient. However, the incorrect orientation of the (*S*)-enantiomer towards the oxyanion hole inhibited catalysis. Therefore, PCL showed intermediate enantioselectivity and afforded low yields in the reaction. Furthermore, PCL showed little interfacial activation, thus performed limited improvement in activity after immobilization.

Although it is difficult to design a reasonable experimental scheme to verify the influence of organic solvents in the molecular simulations using the "Flexible Docking" module, because of the complexity of the lipase/MBGs/solvent system, the docking results can help explain the low catalytic efficiency of these lipases in their native forms during the hydrolysis of ketoprofen vinyl ester. As a consequence, by combining the conformational behaviors of these lipases and our experimental data, this experiment could demonstrate that MBGs can efficiently improve the activity of RML after immobilization.

### 3.3. Tolerance of organic solvents and temperatures

To meet the demands of a technical process, the possibility of adapting an enzyme to various solvents is crucial to exploring novel applications and optimization for industrial needs. Therefore, various organic solvents were examined to determine their effects on the activity and stability of MJL MBGs, RML MBGs and

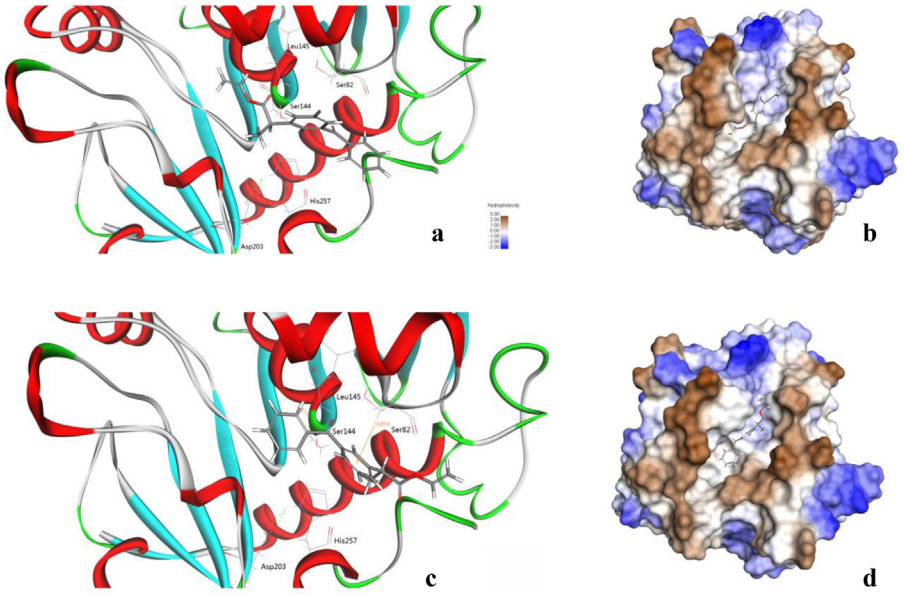
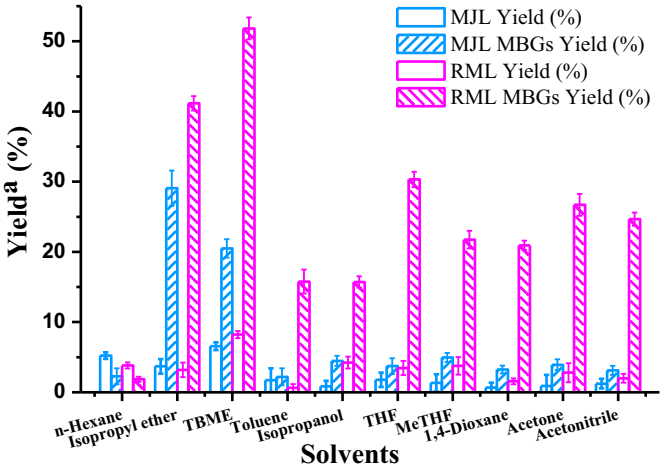



**Fig. 1.** Comparison between (*R*)- and (*S*)-ketoprofen vinyl ester binding with lipase from *Rhizomucor miehei* (RML). (a) Three-dimensional structure of the binding between (*R*)-enantiomer and RML. (b) Binding surfaces of the complex of (*R*)-enantiomer and RML. The hydrophilic areas are displayed in blue and the hydrophobic areas are visualized in brown. (c) Three-dimensional structure of the binding between (*S*)-enantiomer and RML. (d) Binding surfaces of the complex of (*S*)-enantiomer and RML. The hydrophilic areas are displayed in blue and the hydrophobic areas are visualized in brown.

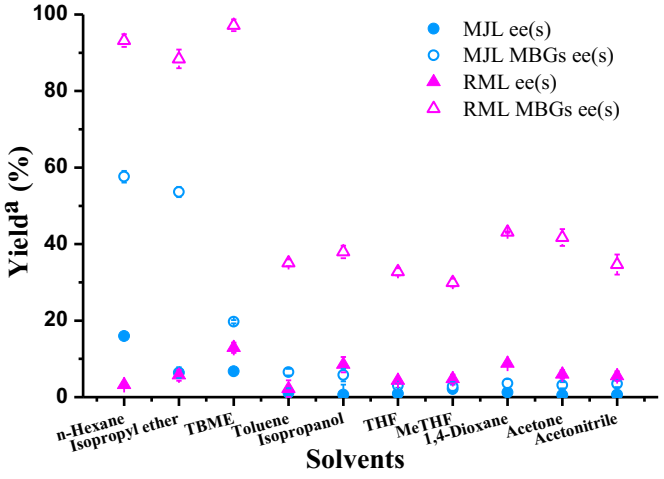
**Table 4**
The docking results of different lipases[a].

| Lipase | Chirality | Distance 1 (Å) | Distance 2 (Å) | -CDIE (kcal/mol) | -CDE (kcal/mol) |
|---|---|---|---|---|---|
| RML | R | 3.20 | 3.05 | 34.92 | 26.41 |
| | S | 6.51 | 8.71 | 39.64 | 28.91 |
| CAL-B | R | 4.05 | 2.31 (3*HB[b]) | 43.17 | 32.56 |
| | S | 4.04 | 1.97(1*HB[c]) | 40.96 | 28.81 |
| CRL | R | 3.05 | 2.01 (1*HB[d]) | 29.88 | 20.20 |
| | S | 3.63 | 3.26 | 30.56 | 21.48 |
| PCL | R | 3.52 | 2.98 | 35.89 | 24.78 |
| | S | 4.61 | 6.83 | 37.10 | 28.86 |

[a] All molecular simulations were performed using Discovery Studio 3.1 (Accelrys, San Diego, CA, USA). Protein structures were obtained from Protein Data Bank. PDB codes for lipase from *Rhizomucor miehei* (RML), lipase B from *Candida antarctica* (CAL-B), lipase from *Candida rugosa* (CRL) and lipase from *Pseudomonas cepacia* (PCL) are 4TGL, 1TCA, 1CRL and 3LIP, respectively. All protein structures were prepared before molecular docking.
[b] The oxygen atom of the carbonyl in (*R*)-ketoprofen formed three hydrogen bonds with the residues in the oxyanion hole in CAL-B.
[c] The oxygen atom of the carbonyl in (*S*)-ketoprofen formed a hydrogen bond with the residues in the oxyanion hole in CAL-B.
[d] The oxygen atom of the carbonyl in (*R*)-ketoprofen formed a hydrogen bond with the residues in the oxyanion hole in CRL.




**(a)**. Yields of hydrolysis of ketoprofen vinyl ester.

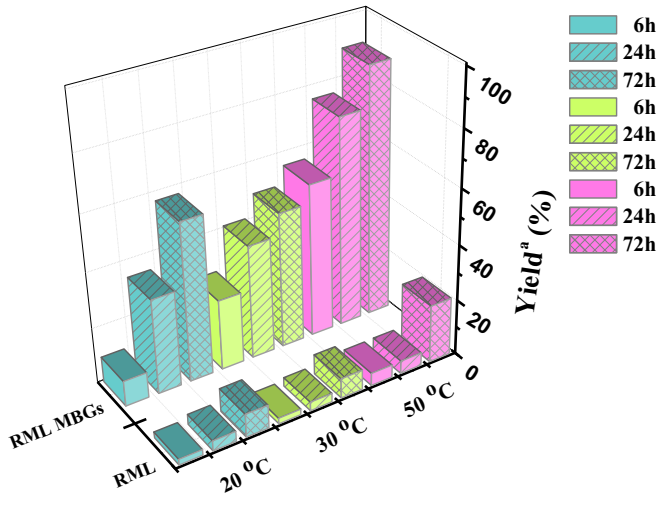



**(b)**. $ee_s$ of hydrolysis of ketoprofen vinyl ester.

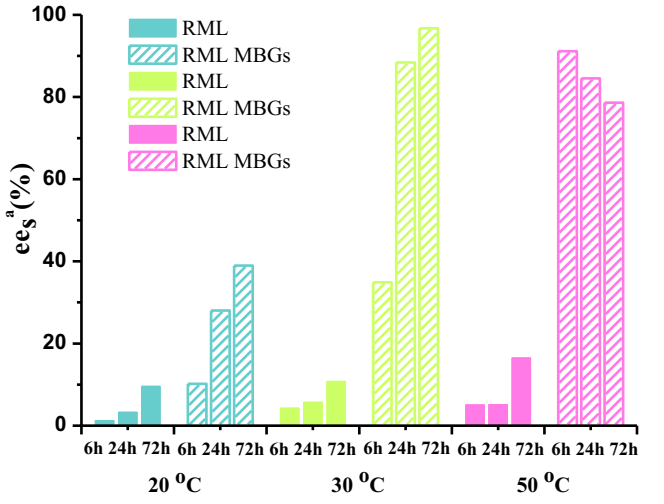
**Fig. 2.** Tolerance to solvents of lipase from *Rhizomucor miehei* immobilized in microemulsion-based organogels (RML MBGs) and lipase from *Mucor javanicus* immobilized in microemulsion-based organogels (MJL MBGs). (a) Yields of hydrolysis of ketoprofen vinyl ester. (b) $ee_s$ of hydrolysis of ketoprofen vinyl ester. a. Reactions performed with 10 mg ketoprofen vinyl ester and 10 mg lipase or lipase MBGs containing 10 mg lipase in 2 mL solvent at 30 °C and 200 rpm for 24 h.

their original forms, and these results are presented in Fig. 2. Ketoprofen vinyl ester is soluble under all experimental conditions. In all of the solvents except for hexane, the activity and selectivity of the lipases immobilized in MBGs were improved, most notably for RML MBGs. After 24 h, RML MBGs produced yields greater than 20% in all of the solvents except for toxic toluene and viscous isopropanol, whereas little product was observed when free RML was used. The relative lower yields of immobilized lipase in more polar solvents might also because of their deleterious effects to gelatin MBGs. These results suggested that the stability of these enzymes in the presence of organic solvents depends on both the enzyme and the nature of the organic solvent being used. Notably, the RML MBGs produced a 21.7% yield after 24 h in the bio-based green solvent 2-methyl-tetrahydrofuran (2-MeTHF), which is easily biodegradable. Concerning the environmental characteristics of these processes, the use of green organic solvents that combine an awareness of the ecological footprint with enzymatic efficiency may allow more environmentally friendly measures to be applied in industrial syntheses. When hexane were used as the solvent, it can be observed from the HPLC chromatograms that the peak of (*R*)-ketoprofen vinyl ester reduced obviously, whereas (*R*)-ketoprofen had a slender peak (Figs. A. 1 and A. 2 of Supplementary information). As shown in Fig. 2, the MBGs in hexane exhibited high $ee_s$ values and low yields. Therefore, we tested mixtures of different ratios of hexane and isopropanol as solvents to investigate the resultant reactions (Fig. A. 1 of Supplementary information). In hexane with 20% isopropanol, the RML MBGs afforded a 33.6% yield with a 70.7% $ee_s$, whereas a 50% isopropanol mixture gave a 17.9% yield with a 47.7% $ee_s$. These results could indicate that the diffusion of ketoprofen in the MBGs was hindered in non-polar hexane, because of the strong polarity of this compound.

Because of their high tolerance to organic solvents, the RML MBGs were then tested to determine their thermostability. The influence of temperature on the activities of native and immobilized RML was studied using three different temperatures, as shown in Fig. 3. The activity and selectivity of free RML were low at all temperatures tested and showed limited increases over time. However, it is noteworthy that after being immobilized in MBGs, RML showed a significant enhancement of activity and selectivity at all temperatures, most notably at 30 °C. The activity of the RML MBGs increased significantly as the temperature increased, and the reaction yield was greater than 50% following the incubation at 50 °C for 24 h. Thus, $ee_p$ decreased over time when the reaction was conducted at 50 °C. These results demonstrate that MBGs offer a more conducive microenvironment at high temperatures and




**(a)**. Yields of hydrolysis of ketoprofen vinyl ester.




**(b)**. $ee_s$ of hydrolysis of ketoprofen vinyl ester.

**Fig. 3.** Tolerance to temperatures of lipase from *Rhizomucor miehei* immobilized in microemulsion-based organogels (RML MBGs). (a) Yields of hydrolysis of ketoprofen vinyl ester. (b) $ee_s$ of hydrolysis of ketoprofen vinyl ester. a. Reactions performed with 10 mg ketoprofen vinyl ester and 10 mg RML or RML MBGs containing 10 mg RML in 2 mL isopropyl ether at various temperatures and 200 rpm for 24 h.

might therefore protect the correct lipase conformation from destruction at high temperatures.

### 3.4. Reusability

One of the greatest advantages of immobilization is to facilitate an enzyme's reusability, which is essential to render the process technologically and economically viable. The stability of immobilized RML and MJL was assessed in repeated 24 h long runs of ketoprofen vinyl ester hydrolysis with excessive washings between the runs. Fig. 4 shows the impressive stability of the catalyst during repeated use in our system. In the first three runs, there is an obvious increase in yield and $ee_s$ of RML MBGs and MJL MBGs, because of the imprinting effect of the residual substrate molecules in immobilized lipase [31]. One of the most successful strategies to enhance enzyme activity in organic solvents involves tuning the enzyme active site by molecular imprinting with substrates or

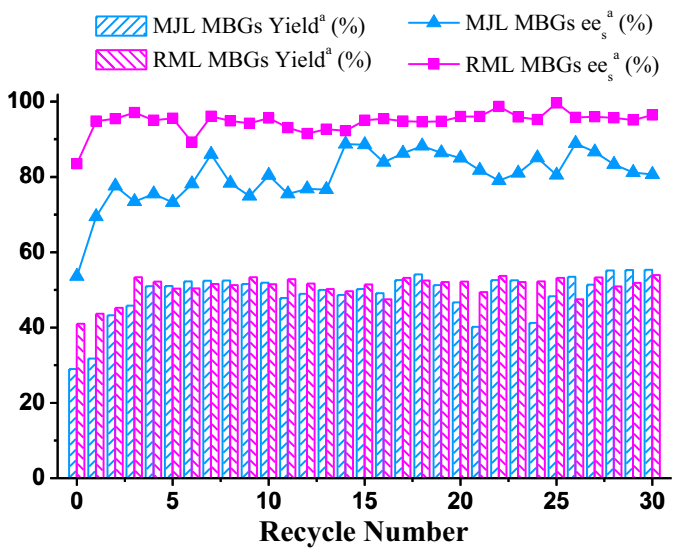



**Fig. 4.** Reusability of lipase from *Rhizomucor miehei* immobilized in microemulsion-based organogels (RML MBGs) and lipase from *Mucor javanicus* immobilized in microemulsion-based organogels (MJL MBGs). a. Reactions performed with 10 mg ketoprofen vinyl ester and 10 mg lipase or lipase MBGs containing 10 mg lipase in 2 mL isopropyl ether at 30 °C and 200 rpm for 24 h.

their analogues [32]. By combining the imprinting of the lipase surface with surfactant coating and reverse micellar activation, known as dual bioimprinting [33], the RML MBGs and MJL MBGs showed high activity and no significant reduction in activity or enantioselectivity over 30 cycles. The system maintained yields of greater than 50%, even after 30 cycles of repeated use. For the RML MBGs, the $ee_s$ values remained nearly constant at 95%, whereas the $ee_s$ values for the MJL MBGs fluctuated by approximately 88%. Together, these results suggest that immobilized lipase demonstrates good durability and reusability. The proven high stability of the enzyme during 30 cycles (over 40 days) shows promise for effective catalyst recycling.

### 3.5. Gram-scale experiment

To investigate the effect of substrate structure on this process, we synthesized several ketoprofen esters for hydrolysis, including the methyl ester, ethyl ester, 1-butyl ester, trifluoroethyl ester, 2-chloroethyl ester, benzyloxy ester and phenylethyl ester. Free RML showed trace activity for many different substrates with different leaving groups, whereas the RML MBGs were significantly more active. However, the activity of the RML MBGs was significantly affected by the properties of the leaving groups in the substrates. After 72 h, trifluoroethyl ester and 2-chloroethyl ester showed 47.8% and 35.1% yields, while maintaining 69% and 87% $ee_p$, respectively, because of their good leaving groups.

To verify the feasibility of the proposed process on a gram-scale, RML MBGs with 100 mg of RML were added to a mixture of 5 g of ketoprofen vinyl ester in 100 mL isopropyl ether and shaken at 30 °C. The isolated yield from the hydrolysis, as measured by column chromatography, reached 46.8% after 72 h with a 91% $ee_p$. The significant increase in the enantioselectivity factor ($E$) from 35.2 in analytical scale trials to 54.1 in the gram-scale experiment also underscores the possibility of using RML MBGs in industrial chemical syntheses and other bulk applications.

## 4. Conclusions

In summary, these results demonstrate that MBGs protect the correct lipase conformation from destruction by polar solvents and

high temperatures. The findings of this study clearly indicate that MBGs is an ideal candidate for RML and MJL immobilization. Moreover, this work expands the application of RML MBGs and MJL MBGs to the asymmetric synthesis of (*S*)-ketoprofen. The ease of preparation and operation, the high activity of several lipases and the ability to conduct straightforward product separation make this process promising for industrial applications.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.btre.2015.04.003.

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