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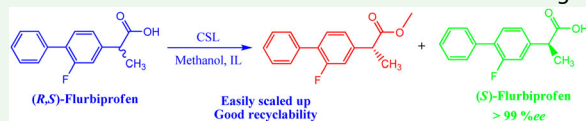
Enantioselective esterification of (*R,S*)-flurbiprofen catalyzed by lipase in ionic liquid

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ABSTRACT

The enantioselective esterification of (*R,S*)-flurbiprofen catalyzed by *Candida sp.* lipase (CSL) (a cheap commercial lipase) was successfully conducted in ionic liquid (IL). The effects of the type of IL, alcohol, temperature, substrate molar ratio and enzyme concentration were investigated. Under optimal conditions (flurbiprofen (0.2 mmol), methanol (2 mmol), CSL (4 mg/mL), [BMIM][PF₆] (5 mL), 50°C), CSL exhibited a satisfying enzyme performance (average enzyme activity, 261.5 μmol/g/h; *E* value, 20.3). The enzymatic esterification can be scaled up easily. Furthermore, CSL exhibited only a slight decrease in catalytic performance after six repetitions. This result demonstrated that this mild method has high potential for industrial production.



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KEYWORDS

Flurbiprofen; lipase; ionic liquid; enantioselective esterification

Introduction

In recent years, enzymatic resolution has become more attractive than organic synthesis due to its environmental friendliness, mild reaction conditions and high enantioselectivity (1). Many optically active pharmaceutical, agricultural and special chemicals can be prepared *via* enzymatic resolution (2). (*R,S*)-Flurbiprofen is one of the most prevalent nonsteroidal antiinflammatory drugs (3). The therapeutic action of (*R,S*)-flurbiprofen resides mainly in its *S*-enantiomer while its *R*-enantiomer enhances the gastrointestinal toxicity (4). Consequently, the preparation of (*S*)-flurbiprofen is strongly recommended. Several reports have demonstrated that (*S*)-flurbiprofen could be obtained *via* lipase-catalyzed enantioselective esterification (5–8). However, up to now, relatively low activity and enantioselectivity were generally found in those studies. Another drawback is the use of organic solvents. Conventional organic solvents are volatile and toxic to the environment. Furthermore, organic solvents also deactivate the enzyme, particularly at high temperatures.

Due to the negligible vapor pressure, high thermal stability, reuse and the excellent biocompatibility, room-temperature ionic liquid (IL) has been successfully used as an alternative medium for enzymatic reactions

(9–13). Tian et al. has reported that the enantioselectivity of lipase for the resolution of 2-methyl-1-butanol was increased about 2.3-fold in an IL than that in organic solvent (14). Pan et al. has also reported that choosing proper combination of cations and anions could boost the activity of lipase by changing the conformation of protein in ILs (15). Another advantage of ILs is that they can be easily recovered and reutilized as the enzymatic reaction media (16–17).

In this study, we report the enantioselective esterification of (*R,S*)-flurbiprofen with alcohols catalyzed by *Candida sp.* lipase (CSL, a relatively cheap commercial lipase) in IL (Scheme 1). To determine the optimal conditions for the resolution of (*R,S*)-flurbiprofen, we focused our attention on the influence of parameters such as the type of IL, alcohol substrate, temperature, substrate molar ratio and enzyme concentration. Furthermore, the reusability of the enzyme-IL system has also been studied.

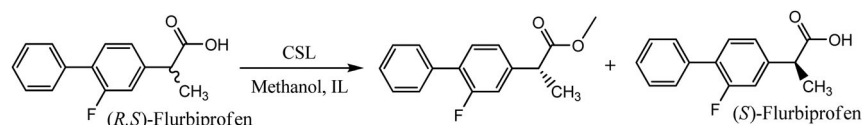
Results and discussion

In this study, different ILs were examined to select the most suitable medium for the enantioselective esterification of (*R,S*)-flurbiprofen catalyzed by CSL. As displayed in Table 1, the average enzyme activity and

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Scheme 1. Enantioselective esterification of flurbiprofen catalyzed by CSL in IL.

Table 1. Effect of ILs on the esterification of flurbiprofen catalyzed by CSL^a.

Entry	Solvent	Conversion (%)	Average enzyme activity ^b (μmol/g/h)	ee _s	Enantioselectivity <i>E</i> value
1	[BMIM][OH]	12.5 ± 2.9	51.9 ± 3.1	10.4 ± 1.4	7.2 ± 2.2
2	[BMIM][NO ₃]	15.6 ± 3.0	64.8 ± 4.4	11.8 ± 2.8	5.1 ± 3.2
3	[BMIM][Ac]	15.9 ± 3.3	33.1 ± 3.9	11.7 ± 2.5	4.8 ± 3.5
4	[BMIM][OTf]	19.7 ± 2.5	197.2 ± 3.3	21.2 ± 1.7	16.9 ± 2.1
5	[BMIM][BF ₄]	20.5 ± 3.1	205.3 ± 4.6	22.0 ± 1.1	15.3 ± 1.8
6	[BMIM][PF ₆]	26.2 ± 1.9	261.5 ± 3.4	31.0 ± 1.7	20.3 ± 2.4
7	[HMIM][PF ₆]	15.8 ± 2.1	158.4 ± 2.9	16.8 ± 1.9	21.1 ± 1.6
8	[OMIM][PF ₆]	9.7 ± 2.5	96.6 ± 3.7	9.7 ± 1.5	22.8 ± 1.3
9	<i>n</i> -Hexane	13.1 ± 2.1	131.2 ± 2.5	12.8 ± 2.2	13.8 ± 2.7

^aReaction conditions: flurbiprofen (0.2 mmol), methanol (2 mmol), CSL (4 mg/mL), solvent (5 mL), 50°C.

^bReaction time: 24 h (Entry 1–2), 48 h (Entry 3), 10 h (Entry 4–9).

enantioselectivity of CSL were dramatically influenced by the anion in [BMIM][X]. The lipase was active in [BMIM][OTf], [BMIM][BF₄] and [BMIM][PF₆], but was inactive in [BMIM][OH], [BMIM][Ac] and [BMIM][NO₃]. It appears that the anion nucleophilicity is a controlling factor (18, 19). The low nucleophilicity of anion (PF₆, BF₄ or OTf) may help the ILs avoid the interference with the hydrogen bonds of enzyme, and then improve the enzyme performance. ILs with anions containing [OH], [NO₃] and [Ac] have more nucleophilic properties, which might disrupt the secondary structure of protein and then decrease the enzyme activity (20). Moreover, [BMIM][OTf] or [BMIM][BF₄] exhibited a lower catalytic performance for the CSL-catalyzed resolution of (*R,S*)-flurbiprofen compared to [BMIM][PF₆]. It may be attributed that [BMIM][OTf] or [BMIM][BF₄] is prone to desorb water from the enzyme surface and thus decrease the enzyme performance (21). The effect of the alkyl chain length on imidazolium cation based on the [PF₆] anion has also been investigated. The average enzyme activity of CSL was decreased with the increase in alkyl chain length on cation because of the increasing viscosity of ILs. In addition, the enantioselectivity of CSL was not affected by the IL cation obviously. Compared with the organic solvent (*n*-hexane), CSL exhibited apparently better enzyme performance in [BMIM][PF₆]. Thus, we selected [BMIM][PF₆] as the suitable IL for further study.

It is well known that primary alcohols are more active than secondary and tertiary alcohols in a lipase-catalyzed esterification (22). So, a series of primary alcohols was screened for this study. As shown in Figure 1, the enzyme performance of CSL was strongly influenced by the carbon chain length of primary alcohol. With the increase in the chain length, the average enzyme activity

decreased. This phenomenon is attributed that a longer alcohol may be difficult to enter the active site of enzyme due to the steric resistance (23). Besides, the enantioselectivity of CSL increased slightly when the primary alcohol with longer carbon chain length was used as the acyl acceptor (24). In the present study, methanol represents the best compromise between the average enzyme activity and enantioselectivity.

Figure 2 demonstrated the effect of the temperature on the esterification of (*R,S*)-flurbiprofen catalyzed by CSL. It could be found that low temperature can enhance the enantioselectivity of CSL and the highest *E* value could be obtained at 30°C. The result was in accordance with Hult's report (25) that enzyme exhibited their high enantioselectivity at low temperature. It could

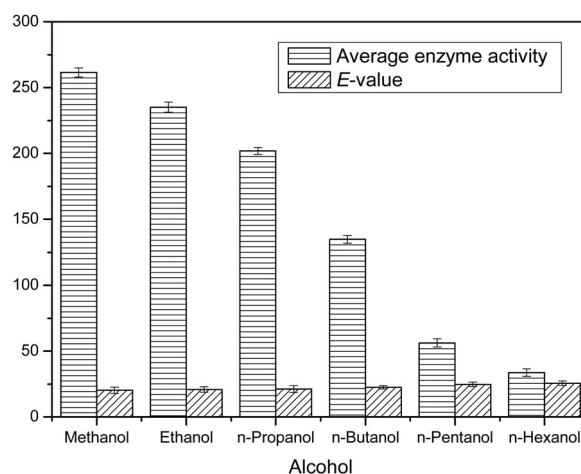


Figure 1. The effect of alcohol on the esterification of flurbiprofen catalyzed by CSL in IL. Reaction conditions: flurbiprofen (0.2 mmol), alcohol (2 mmol), CSL (4 mg/mL), [BMIM][PF₆] (5 mL), 50°C.

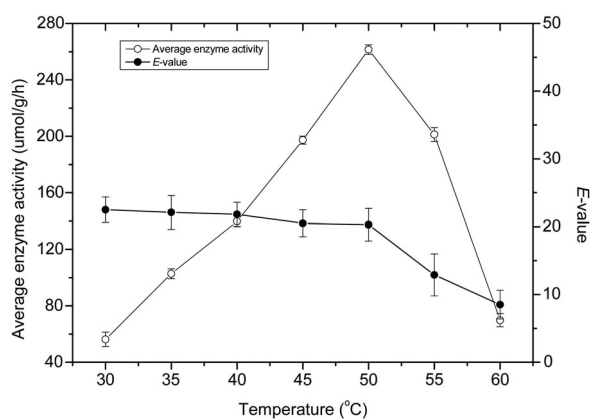


Figure 2. The effect of temperature on the esterification of flurbiprofen catalyzed by CSL in IL. Reaction conditions: flurbiprofen (0.2 mmol), methanol (2 mmol), CSL (4 mg/mL), [BMIM][PF₆] (5 mL).

also be observed that the average enzyme activity increased with increasing the temperature from 30°C to 50°C, and then declined at higher temperature (>50°C). It is known that increasing the temperature within certain limits can increase the collision chance between enzyme and substrate molecule, reduce the IL viscosity and increase the mass transfer speed, which can enhance the average enzyme activity. However, high temperatures may cause the thermal inactivation of lipase and then result in poorer enzyme performance. Therefore, 50°C was selected for this enzymatic esterification.

The effect of the substrate molar ratio (methanol/(*R,S*)-flurbiprofen) was investigated when the (*R,S*)-flurbiprofen concentration was fixed at 0.04 mmol/mL and the enzyme concentration was fixed at 4 mg/mL. It could be observed from Figure 3 that the average enzyme

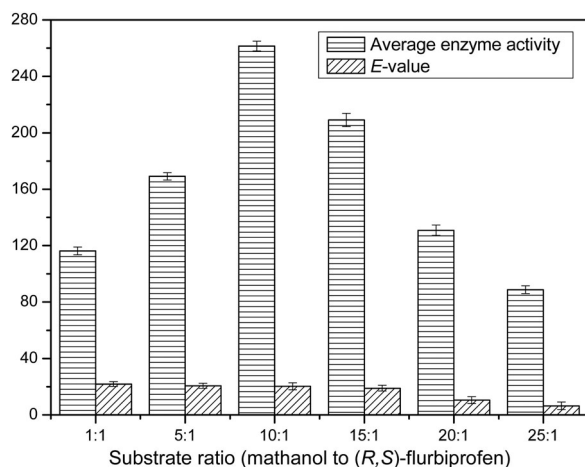


Figure 3. The effect of substrate ratio on the esterification of flurbiprofen catalyzed by CSL in IL. Reaction conditions: flurbiprofen (0.2 mmol), methanol (0.2-5 mmol), CSL (4 mg/mL), [BMIM][PF₆] (5 mL), 50°C.

activity increased gradually when the substrate molar ratio increased from 1:1 to 10:1. A further increase in substrate molar ratio resulted in a moderate decrease in average enzyme activity. Besides, the enantioselectivity remained stable with the increasing of the substrate ratio, followed by a steady decrease in a higher substrate ratio (> 15:1). These results were in accordance with those studies that reported that lipase could be inhibited by higher concentration of methanol in the lipase-catalyzed esterification (26–28). In this study, 10:1 was chosen to be the preferred substrate ratio for further investigation. Generally, the shortcoming of esterification catalyzed by lipase is the possibility for giving a reversible reaction, and greatly decrease the yield and optical purity of remained (*S*)-flurbiprofen (29). In this work, the concentration of (*R,S*)-flurbiprofen is relatively low (0.04 mmol/mL); so the possible reversibility in the present kinetic resolution could be greatly decreased.

The enzyme concentration can influence the reaction rate during the enzymatic resolution of (*R,S*)-flurbiprofen. In this study, the effect of enzyme concentration has been investigated and the results were shown in Figure 4. It was found that the catalytic activity of CSL increased with increasing lipase concentration and decreased slightly when the concentration was above 4 mg/mL. When the enzyme concentration was increased, more enzyme molecules would take part in the reaction and then increased the efficiency of the reaction. However, high enzyme concentration may lead to the aggregation of enzyme, which might prevent the active sites of the enzyme molecules from exposure to the substrates, and then decrease the average enzyme activity slightly (30). The enantioselectivity of CSL was not affected by the variety of enzyme concentration.

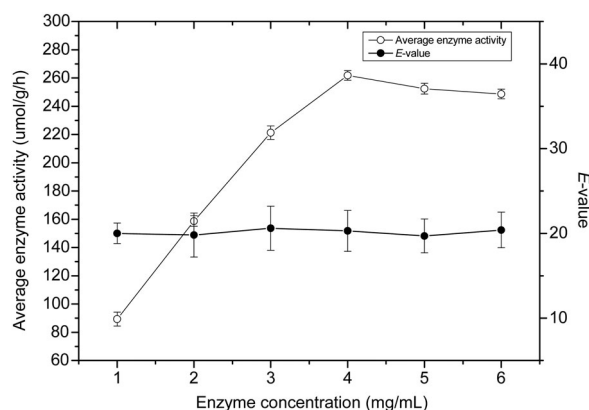


Figure 4. The effect of enzyme concentration on the esterification of flurbiprofen catalyzed by CSL in IL. Reaction conditions: flurbiprofen (0.2 mmol), methanol (2 mmol), CSL (1-6 mg/mL), [BMIM][PF₆] (5 mL), 50°C.

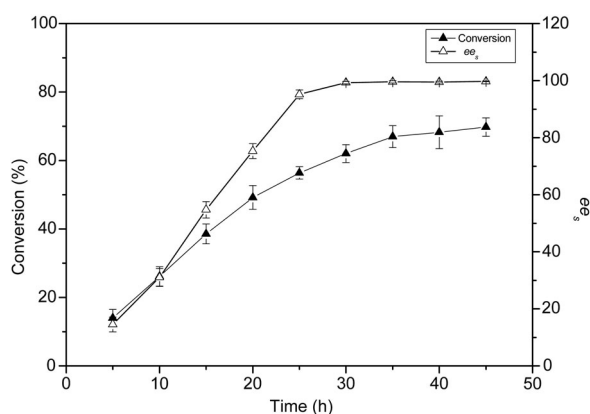


Figure 5. The time course of the esterification of flurbiprofen catalyzed by CSL in IL. Reaction conditions: flurbiprofen (10 mmol), methanol (100 mmol), CSL (4 mg/mL), [BMIM][PF₆] (250 mL), 50°C.

Under the optimal reaction conditions, we scaled up the lipase-catalyzed esterification to 50-fold ((*R,S*)-flurbiprofen (10 mmol), methanol (100 mmol), CSL (1 g), [BMIM][PF₆] (250 mL)) and illustrated the time course of the esterification of (*R,S*)-flurbiprofen catalyzed by CSL. As shown in Figure 5, 99% ee of the un-reacted (*S*)-flurbiprofen could be obtained at the conversion of 62% (reaction time, 30 h).

To ensure that the lipase-catalyzed esterification in ILs is economical at a large scale, the recyclability of the enzyme-IL system must be taken into account. It could be observed from Figure 6 that the catalytic activity was decreased significantly after six repetitions, which could be attributed to the enzyme inactivation in the process of repetition. Novozym 435 is reported to be the best enzyme for the resolution of (*R,S*)-flurbiprofen (31–32). Nevertheless, the catalytic performance of CSL was much higher than Novozym 435 ($E < 15$) when IL was used as the reaction media. Besides, the price of

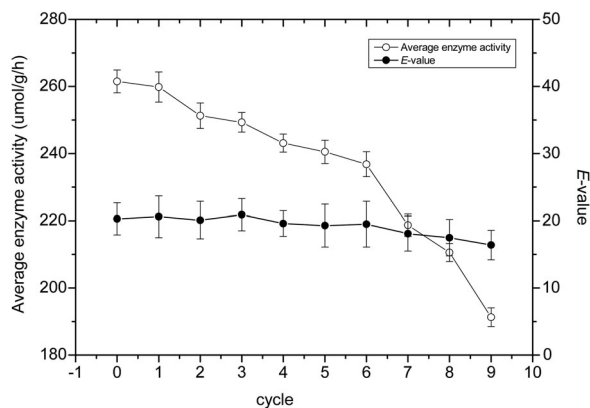


Figure 6. The reusability of the enzyme-IL system. Reaction conditions: flurbiprofen (10 mmol), methanol (100 mmol), CSL (4 mg/mL), [BMIM][PF₆] (250 mL), 50°C.

CSL was much lower than that of Novozym 435. Compared with the previous works (5–8), CSL exhibited a high average catalytic activity (261.5 μmol/g/h), good enantioselectivity ($E = 20.3$) and satisfying stability in [BMIM][PF₆]. However, the stability and reusability of CSL could be further increased by the enzyme immobilization. It is well known that immobilization is a powerful tool to improve enzyme features (activity, reusability, stability, etc.) in modern biotechnology (33–34). In order to improve the reusability of the enzyme-IL system and cut the costs, a study adopting the technique of immobilization is currently in progress and will be reported in due course.

Experimental

Materials

CSL was obtained from Beijing CTA New Century Biotechnology Co., Ltd. (Beijing, China) and was used after lyophilization for enzymatic reaction without further purification. (*R,S*)-Flurbiprofen was kindly donated by Dr Yazhuo Li (College of Chemistry of Jilin University). ILs were purchased from Shanghai Chengjie Chemical Co. Ltd. (Shanghai, China). Other reagents were purchased from Shanghai Chemical Reagent Company (Shanghai, China). Commercially available reagents and solvents were used without further purification. High performance liquid chromatography (HPLC) analysis was performed on an Agilent 1200 HPLC with a UV detector.

Enantioselective esterification of (*R,S*)-flurbiprofen catalyzed by lipase

The reaction was performed with (*R,S*)-flurbiprofen (0.2 mmol), methanol (2 mmol), IL (5 mL) and CSL (4 mg/mL), which was incubated at 50°C. The conversion (C) and the enantiomeric excess (ee_s) of the remained (*S*)-flurbiprofen were determined by HPLC. The average enzyme activity (μmol/g/h) was defined as the amount of flurbiprofen decreased (determined by HPLC) per hour per gram of enzyme, and calculated by using Equation (1).

$$\text{Average enzyme activity} = \frac{[C] \cdot M_{\text{flurbiprofen}}}{W_{\text{CSL}} \cdot T}, \quad (1)$$

$[C]$ is the conversion of flurbiprofen (ranged from 15% to 30%); $M_{\text{flurbiprofen}}$ is the initial dosage of (*R,S*)-flurbiprofen (μmol); W_{CSL} is the dosage of CSL (g); T is the reaction time (hours).

The enantiomeric ratio (E value), which can dictate the enantioselectivity of a kinetic resolution, was calculated according to Chen et al. (35). The experiments were

performed triplicate, and all data were obtained based on the average values.

The reusability of the enzyme-IL system

To ensure that the lipase-catalyzed esterification in ILs is economical at a preparatory scale, batch esterification of (*R,S*)-flurbiprofen (10 mmol) and methanol (100 mmol) was catalyzed by CSL (4 mg/mL) in [BMIM][PF₆] (250 mL) at 50°C for 30 h. Then, the reaction mixture was extracted three times with diethyl ether, and almost no substrate or product could be detected in the IL phase. The enzyme-IL mixture was vacuum-dried overnight after extraction. Following that, the IL phase containing CSL was reused in the next run under the same conditions.

Analytical methods

The reaction was monitored when the conversion of flurbiprofen ranged from 15% to 30%. The reaction mixture (100 µL) was withdrawn from the vial, and was extracted with diethyl ether (200 µL), followed by chiral HPLC analysis (Chiralpak AD-H column, Diacel) employing hexane – isopropanol (90:10) as mobile phase at 0.75 mL/min and monitored by UV at 254 nm (Figure 7). The absolute configuration of the enantiomers of flurbiprofen was determined by comparison with commercial standards of the optically pure compounds. The retention time of (*R*)-flurbiprofen was 8.9 min and that of (*S*)-flurbiprofen was 11.7 min.

To quantify the concentration of flurbiprofen in diethyl ether, a linear calibration curve was constructed based on the peak area and the quantity of flurbiprofen. The degree of flurbiprofen conversion (*C*) was calculated from the reduction of flurbiprofen. The enantiomeric excess of the remained flurbiprofen (*ee_s*) was determined by calculating the peak areas of the two enantiomers with Equation (2) and the enantiomeric ratio (*E* value)

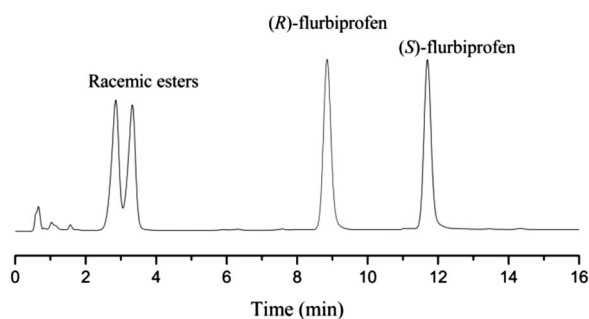


Figure 7. The chromatogram of racemic flurbiprofen and its corresponding methyl esters on HPLC.

was determined by using Equation (3):

$$\text{enantiomeric excesses } ee_s(\%) = \frac{[S - R]}{[S + R]} \times 100, \quad (2)$$

$$\text{enantioselectivity, } E = \frac{\ln[(1 - C)(1 - ee_s)]}{\ln[(1 - C)(1 + ee_s)]}. \quad (3)$$

Conclusion

In this study, we disclosed an efficient lipase-catalyzed enantioselective esterification of (*R,S*)-flurbiprofen in IL. The anion and alkyl chain of the imidazolium cation greatly influenced the catalytic performance of lipase. Under the optimal conditions, CSL exhibited a satisfying catalytic performance in [BMIM][PF₆]. The scale-up esterification of (*R,S*)-flurbiprofen was also examined, and a high enantiomeric excess for (*S*)-flurbiprofen (*ee* > 99%) was achieved when the conversion was up to 62% in 30 h. Furthermore, the enzyme-IL system exhibited good recyclability for six repetitions. Therefore, this mild method demonstrated high potential for industrial production.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

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Mei Li was born in 1972. She was graduated from Bethune nursing school in 1994. Now, she is mainly investigating endocrine and metabolic diseases.

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