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ORIGINAL PAPER

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Optimization of enantioselective resolution of racemic ibuprofen by native lipase from *Aspergillus niger*

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Abstract Resolution of (*R,S*)-ibuprofen (2-(4-isobutylphenyl)propionic acid) enantiomers by esterification reaction with 1-propanol in different organic solvents was studied using native *Aspergillus niger* lipase. The main variables controlling the process (enzyme concentration and 1-propanol:ibuprofen molar ratio) have been optimized using response surface methodology based on a five-level, two-variable central composite rotatable design, in which the selected objective function was enantioselectivity. This enzyme preparation showed preferentially catalyzes the esterification of *R*(−)-ibuprofen, and under optimum conditions (7% w/v of enzyme and molar ratio of 2.41:1) the enantiomeric excess of active *S*(+)-ibuprofen and total conversion values were 79.1 and 48.0%, respectively, and the *E*-value was 32, after 168 h of reaction in isooctane.

Keywords (*R,S*)-ibuprofen · Lipases · Enantioselectivity · Resolution · *Aspergillus niger*

Introduction

Ibuprofen is an important member of the class non-steroidal anti-inflammatory drugs (NSAIDS) belonging to 2-arylpropionic acids (profens family). The anti-inflammatory activity of this class of compounds is mainly due to the active *S*(+)-enantiomer [14]. It has been reported that *S*(+)-ibuprofen is 160-fold more active than its antipode in the in vitro synthesis of prostaglandin [1]. *S*(+)-ibuprofen has been available since 1994, but until recently, all except naproxen and flunoxaprofen have been marketed and consumed as racemic mixtures. Today, there is an increasing interest in obtaining the pure enantiomeric form of biologically active drugs to prevent any side effects from using racemic mixture.

In recent years, lipases have been used for the chiral resolution of (*R,S*)-ibuprofen through mainly direct enantioselective esterification in organic media [11, 13, 24, 29] or enantioselective hydrolysis of its chemically synthesized racemic ester [18, 23, 25, 28]. Microbial lipases have a great potential for commercial applications due to their stability, enantioselectivity and broad substrate specificity [15]. Among the high number of lipases described in the literature, only the enzymes belonging to a few species have been demonstrated to have adequate stability and biosynthetic capabilities to allow routine use in organic reactions, and hence their applications as industrially relevant enzymes [3, 21]. The most widely used lipases are from *Candida rugosa*, which preferentially catalyses the esterification of *S*(+)-ibuprofen, and from *Candida antarctica*, which preferentially catalyses the *R*(−)-enantiomer. Ideally, a lipase that selectively catalyses the transformation of *R*(−)-ibuprofen should be used, leaving the required *S*(+)-ibuprofen unreacted (Scheme 1). A few works have been done exploiting the enantioselectivity from the lipases of *Aspergillus* species. Thirteen lipases were studied kinetically with the aim to exploit their enantioselectivity in the esterification of (*R,S*)-ibuprofen with primary alcohols. One of the tested lipases was from *Aspergillus niger* (Biocatalysts, UK and Amano Pharmaceutical, Japan) which did not show stereopreference towards the *R*(−)- or *S*(+)-enantiomer. Of the different lipase preparations testes, only *Candida cylindracea* and immobilized *Rhizomucor miehei* lipases were able to catalyze the esterification reaction [22]. On the other hand, in other study the comparison among three lipases from different sources in the resolution of (+−) (4 *RS*, 5 *RS*)-trans-5 (butyl rylonymethyl)-4-(4-fluorophenyl)1-methyl-piperidin-2-one precursor of

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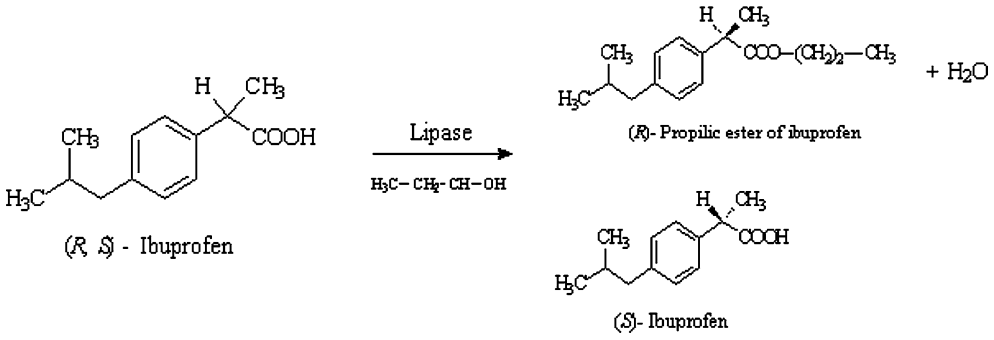
paroxetine was shown. Using *Aspergillus oryzae* lipase, the *E*-value could be improved from 3.5 to 16 in the presence of 20% of dioxane [19].

Scheme 1 Enantioselective esterification of (*R,S*)-ibuprofen with 1-propanol using lipase with (*R*)-stereochemical preference



Recently, we described a lipase, from *A. niger* AC-54 which was able to esterify preferentially *R*(−)-ibuprofen and that provided the best results in terms of enantioselectivity and thermostability compared with others natives lipases [6, 7].

This study refers a research to enhance knowledge about the reaction parameters which affect the enantioselective resolution of (*R,S*)-ibuprofen by this lipase and optimize the process of obtaining an economical enzymic esterification. The influence of organic solvents and the other variables that control the resolution of (*R,S*)-ibuprofen by lipase *A. niger* (such as enzyme concentration and ratio molar propanol:ibuprofen) were studied. A statistical method is presented to indicate the parameter for an optimized synthesis process, which uses the response surface methodology (RSM) [4, 16].

Materials and methods

Chemicals

Isoctane, 1-propanol, (*R,S*)-ibuprofen and pures enantiomers were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Yeast extract and Bacto peptone were purchased from Difco Laboratories (Detroit, MI, USA). The components from culture media, chemical reagents and the other solvents were obtained from Merck (Darmstadt, Germany) and from Sigma-Aldrich Chemical Co. in the highest purity available. Low acidity olive oil (carbonel) was purchased at a local market.

Lipase

This study was carried out using *A. niger* AC-54 lipase, which achieved a better performance in the previous experiment [6, 7]. The lipase was produced in a basal medium with an initial pH value of 6.0 and consisted of 2% (w/v) peptone, 0.5% (w/v) yeast extract, 0.1% (w/v) $NaNO_3$, 0.1% (w/v) KH_2PO_4, 0.05% (w/v) $MgSO_4 \cdot 7H_2O$ and 2% (w/v) of olive oil. Cultures were grown in Erlenmeyer flasks (500 mL) containing 120 mL of the growth medium. The cultures were inoculated with 1 mL of spores suspension (10^5–10^6 spores/mL) and the flasks were stirred on a rotary shaker (130 rpm) at 35°C for 72 h. After this period, the cultures were filtered and the supernatants were treated with ammonium sulphate (80% saturation). The precipitates were dialyzed in water and lyophilized for the use as an extracellular crude lipase preparation in powder form. The residual water in the lyophilized lipase was 0.2% w/w. Lipase activity was quantified by triolein using olive oil as a substrate [26]. One unit (U) is defined as 1 μmol of oleic acid released per minute.

Esterification reaction

The standard reaction mixture was composed of (*R,S*)-ibuprofen (66 mM), 1-propanol (66 mM) and isooctane (10 mL) without addition of water. The reaction started by the addition of 0.4 g crude lipase to the solution and carried out in sealed up Erlenmeyer's on a shaker with orbital magnetic stirring at 180 rpm, at 35°C. Hexane (log *P* 3.50), toluene (log *P* 2.50), chloroform (log *P* 2.00), dichloromethane (log *P* 0.93), diethyl ether (log *P* 0.85) and acetone (log *P* −0.25) were tested as solvents and the final reaction volume was 10 mL. Experiments without addition of the enzyme were carried out to evaluate the spontaneous esterification percentage of the system. Samples of 100 μL of the solution were withdrawn at different times and diluted in 1.4 mL of isooctane. The amount of ester (conversion degree) formed during the reaction and the enantiomeric excess of (*S*)-ibuprofen were determined by gas chromatography and high performance liquid chromatography, respectively, as described below.

Experimental design

A five-level, two-variable central composite rotatable design (CCRD) was adopted for optimizing the

esterification reaction. The variables studied in the process of esterification of (*R,S*)-ibuprofen were concentration of enzyme (1.0–7.0% w/v of the total system) and 1-propanol:ibuprofen molar ratio (MR) (3:1–1:3). The independent variables, their levels and real values are presented in Table 1 and the experimental design is shown in Tables 3–5. This study required 11 experiments, which included four factorial points, and three central points to provide information about the interior of the experiment region, allowing to check for curvature. Significance of data was tested using the analysis of variance (ANOVA) statistical test. The time of reaction was not considered a significant variable in this experimental design, since the experiments were accomplished at five different time frames in order to study the reaction kinetics and the temperature was kept constant at 35°C for a more economical process and, under these conditions, no denaturation was observed for this lipase [6].

Chromatography analysis

Gas chromatography was performed using a CHROMPACK CP 9001 gas chromatography equipped with a flame ionization detector (FID) and a CP-Sil 5 CB column (10 m × 0.25 mm × 0.12 μm). Injector temperature was 300°C and the detector was 350°C; oven temperature was maintained at 180°C. Carrier gas was hydrogen with a flow of 12 mL/min. An external standard method was employed to quantify the formed ester and the remaining acid. The enantiomers of the unreacted ibuprofen were separated by HPLC using a chiral column (Chiralcel OD, Daicel Chemical Industries, Ltd., Japan). The mobile phase was a mixture of *n*-hexane/isopropanol/trifluoracetic acid (HPLC grade) (100/1/0.1 v/v/v) at a flow rate of 1.0 mL/min and detection was by UV at 254 nm.

Table 1 Variable and levels for a central composite design in the enantioselective resolution of racemic ibuprofen

Variables	Coded variable levels				
	−1.41	−1	0	+1	+1.41
Molar ratio (1-propanol:ibuprofen)	3:1	2.41:1	1:1	1:2.41	1:3
Lipase concentration (% w/v)	1	1.88	4	6.12	7

Enantioselectivity-value measurements

The value of enantioselectivity (*E*) was calculated from the enantiomeric excess of remaining S-ibuprofen (ee_s) and the conversion (*c*) according to the method described by Chen et al. [9] (Eq. 1):

$$E = \frac{\ln[(1 - c)(1 - ee\ s)]}{\ln[(1 - c)(1 + ee\ s)]}. \quad (1)$$

Results and discussion

Selection of solvent of the esterification reaction

The solvent hydrophobicity has an important effect on the esterification reactions catalyzed by microbial lipases. This parameter is often correlated with log *P* values, logarithm of the partition coefficient (*P*) of the solvent between 1-octanol and water. As shown in Table 2, solvents of different hydrophobicities had marked influence in the activity and enantioselectivity of *A. niger* lipase. Increasing the solvent hydrophobicity resulted in the enhancement of both the enzyme activity and in the active enantiomeric excess of (*S*)-ibuprofen and also in the improvement of the enantioselectivity (*E*) of this lipase. The highest enantiomeric excess of (*S*)-ibuprofen (ee = 18.9%), enantioselectivity (*E* = 4.5) and activity (240 U/mL) was obtained using isooctane. These values are considerably higher than those previously found for the *A. niger* lipase in the resolution of (*R,S*)-ibuprofen

Table 2 Esterification of (*R,S*)-ibuprofen (66 mM) with 1-propanol (66 mM) with different organic solvents by *Aspergillus niger* lipase (0.4 g) at 35°C after 144 h of reaction

Solvent	log *P*[a]	Hidrolytic activity[b] (U/mL)	*c*[c] (%)	ee[d]	*E*[e]	Stereopreference
Isoctane	4.50	240	25	18.9	4.5	*R*
n-Hexane	3.50	180	25	12.3	2.4	*R*
Toluene	2.50	138	19	5.8	1.8	*R*
Chloroform	2.00	86	16	4.2	1.7	*R*
Dichloromethane	0.93	25	8	1.9	ND	*R*
Diethyl ether	0.85	NA	9	2.1	ND	*R*
Acetone	−0.25	NA	ND	< 1.0	ND	*R*

NA No activity, *ND* no detected
[a]The values of log *P* are from Laane et al. [17], *P* partition coefficient
[b]Hidrolytic activity was quantified by using olive oil as a substrate. One unit (U) is defined as 1 μmol of oleic acid released per minute
[c]Conversion is given as the percentage of initial racemic ibuprofen esterified after the reaction time
[d]Enantiomeric excess of the (*S*)-ibuprofen activity
[e]Value of enantioselectivity calculated according to the method described by Chen et al. [9]

enantiomers [22] and of (*R*,*S*)-naproxen enantiomers [8, 27]. Decreasing the hydrophobicity of solvent resulted in low product conversions which was related by a decrease in the enzyme activity. More hydrophylic organic solvents can result in the enzyme conformation changes affecting the affinity of the substrate-binding site for this ligand and the enantioselectivity [5, 10, 30]. In all cases, this lipase showed enantiopreference for the (*R*)-enantiomer. Among the organic solvents tested, *n*-hexane, cyclohexane and isooctane are the most commonly used in biocatalyzed reactions by lipases. Thus, the isooctane was selected as the solvent in furthers studies.

Characterization of the optimal conditions: effect of enzyme concentration and 1-propanol:ibuprofen molar ratio

The study of the main variables that control the process (enzyme concentration and 1-propanol:ibuprofen MR) affecting the enantioselective esterification of racemic ibuprofen was optimized using RSM. The selection of these factors combined with the chosen levels implied the need for 11 experiments, which were carried out following in each case both enantiomeric excess of (*S*)-ibuprofen and total conversion with reaction time.

Maximum enantioselectivity (*E*) was chosen as an objective function; and this maximum value was obtained at different reaction times for each experiment. The results obtained for each reaction time, 144, 168 and 192 h, are shown separately in Tables 3–5.

The maximum *E*-value observed was 26 after 168 h of reaction with an MR (1-propanol:ibuprofen) at 3:1 and 4% (w/v) of lipase (run 5). The enantiomeric excess of (*S*)-ibuprofen and total conversion values were 68.2 and 44.1%, respectively (Table 4). In general it can be observed that the condition with MR at 1:2.41 and 1.88% (w/v) of lipase (run 2) were not efficient for the enantioselective resolution of racemic ibuprofen and that the change of MR from 3:1 (run 5) to 1:3 (run 6) caused a great decrease in the *E*-value after 144 and 168 h of reaction time.

When the reaction time increased, conversion exceed 50%, thus (*R*)-ibuprofen was esterified and thus the enantiomeric excess of (*S*)-ibuprofen decreased. After 192 h of esterification, enantiomeric excess and *E*-value were 56% and 4.6, respectively (run 5) (Table 5). In principle, since *A. niger* lipase has *R* stereopreference, it seems possible to obtain (*S*)-ibuprofen with high enantiomeric excess, carrying the reaction to conversion levels beyond 50%. However, the reversibility of esterification reaction causes a worsening of the enantiomeric purity with increased incubation time. In fact, the gradual accumulation of water produced in the forward reaction will facilitate the reverse reaction and the enantiomer esterified faster will be the one that suffers hydrolysis more easily, thus resulting in a decrease in the enantiomeric excess of (*S*)-ibuprofen. Anyway, (*S*)-ibuprofen can be obtained with good enantiomeric excess (68.2%) and good *E*-value (26) quenching the reaction near 44% conversion after 168 h reaction (Table 4). As a rule of thumb, enantiomeric ratios below 15 are unacceptable for practical purposes. They can be

Table 3 Central composite design and responses in the enantioselective resolution of racemic ibuprofen after 144 h of reaction

Run	Coded variable levels		*c* (%)	ee	*E*
	MR	LC			
1	−1	−1	19.0	15.0	5.5
2	+1	−1	12.2	6.2	2.8
3	−1	+1	30.4	31.1	8.3
4	+1	+1	17.1	11.0	3.8
5	−1.41	0	31.9	39.0	18.0
6	+1.41	0	18.3	12.8	4.5
7	0	−1.41	14.8	12.0	5.9
8	0	+1.41	35.0	38.1	8.1
9	0	0	25.0	18.8	4.5
10	0	0	24.5	18.6	4.8
11	0	0	21.4	16.4	5.0

Table 4 Central composite design and responses in the enantioselective resolution of racemic ibuprofen after 168 h of reaction

Run	Coded variable levels		*c* (%)	ee	*E*
	MR	LC			
1	−1	−1	27.9	26.3	6.2
2	+1	−1	20.2	13.2	3.4
3	−1	+1	42.8	59.8	16.0
4	+1	+1	22.3	16.0	4.0
5	−1.41	0	44.1	68.2	26.0
6	+1.41	0	21.7	17.1	4.7
7	0	−1.41	17.0	14.2	5.8
8	0	+1.41	38.0	46.5	10.4
9	0	0	32.2	35.2	9.8
10	0	0	30.1	32.9	11.0
11	0	0	32.1	38.3	12.0

Table 5 Central composite design and responses in the enantioselective resolution of racemic ibuprofen after 192 h of reaction

Run	Coded variable levels		*c* (%)	ee	*E*
	MR	LC			
1	−1	−1	40.0	27.3	3.0
2	+1	−1	28.9	16.0	2.7
3	−1	+1	60.2	52.1	3.3
4	+1	+1	39.1	18.9	2.2
5	−1.41	0	57.2	56.2	4.6
6	+1.41	0	31.3	18.4	2.8
7	0	−1.41	20.1	10.0	2.6
8	0	+1.41	56.0	54.0	4.1
9	0	0	52.3	40.4	3.1
10	0	0	52.9	43.8	3.4
11	0	0	52.2	40.2	3.1

considered as moderate to good from 15 to 30, and above this value they are excellent [12].

This tendency had not been observed in the reaction time of 72 and 96 h, in which (*R*)-ibuprofen was not esterified and low *E*-value were obtained (data not shown).

The estimated regression coefficients for *E*-value of each variable after 168 h reaction are presented in Table 6. ANOVA is present in Table 7.

The highest values of regression coefficient of MR propanol:ibuprofen (0.55) indicated that it is the most important factor effecting the resolution of (*R,S*)-ibuprofen (95% of confidence level), whereas lipase concentration (LC) had the lowest influence on the *E*-value (0.24).

This result was statistically validated by means of the *F* test. Experimental value of *F* test was 4.8 times higher than the tabulated value for a confidence of 99%, which was 2.4. This means that the significance of factor effects was significant with a confidence equal or greater than 99%, which reflected a very low variance due to lack of fit. The pure error was very low, indicating good reproducibility from the data obtained.

The adjusted correlation coefficient ($R^2 = 64.9$) and the result of the *F* test were good enough indicators for a model (Eq. 2) representative of the actual relationship between variables and *E*-value:

$$E(148) = 2.11 - 0.55\ \mathrm{MR} + 0.24\ \mathrm{LC}. \quad (2)$$

The contour diagram (Fig. 1) represents the predicted model, indicating the variable levels for an optimal process. An amount of *A. niger* lipase around 6.12–7% and MR between 2.41:1 and 3:1 increased the *E*-value. The optimum conditions were 7.0% for LC and 2.41:1 for MR.

So, these optimal conditions were chosen for the resolution of (*R,S*)-ibuprofen using *A. niger* lipase. One single experiment (three replications) under the optimal conditions was carried out. After 168 h of esterification, the enantiomeric excess of (*S*)-ibuprofen and total conversion values were 79.1 and 48.0%, respectively, and the *E*-value was 32. This value was considerably higher than those we previously reported using *A. niger* lipase [6], in which the best enantioselectivity value was 1.8. However, the reaction conditions were different, the concentration of both, ibuprofen and alcohol, was 66 mM and the amount of biocatalyst was at least 7 times smaller than that in this experiment. Mustranta et al. [22] reported that *A. niger* lipase (Biocatalyst, UK) was almost inactive in the ester formation in the resolution of racemic ibuprofen.

Arroyo et al. [2] reported high ester yields (40% conversion) and 72% of enantiomeric excess of (*R*)-ibuprofen using lipase from *C. cylindracea*. The amount of enzyme added to 10 mL of isooctane was 75 mg, 1-propanol:ibuprofen MR was of 1:1 and 66 mM of ibuprofen concentration at 30°C.

An optimized synthesis using RSM of short chain citronellyl esters by lipase from *Rhizopus* sp was described by Macedo et al. [20]. The statistical method (surface response, ANOVA and contour diagram) indicated that the best results were obtained with more alcohol than acid and high yields of citronellyl valerate was obtained, reaching 75% after 48 h.

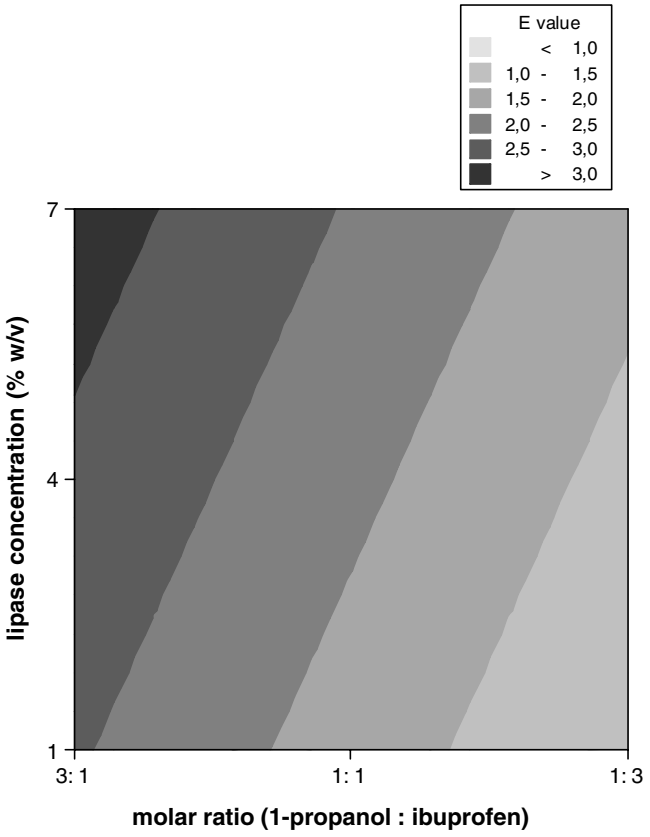


Fig. 1 Contour diagram of enantioselectivity (*E*)-value as a function of molar ratio and lipase concentration for the resolution of (*R,S*)-ibuprofen

Table 6 Estimated regression coefficients for *E*-value after 168 h reaction

Term	Coefficient	SE Coefficient	*T*	*P*
Constant	2.11	0.10	19.85	< 0.001
MR	−0.55	0.12	−4.40	0.002
LC	0.24	0.12	1.93	0.089

Table 7 Analysis of variance (ANOVA) for *E*-value after 168 h reaction

Term	Degrees of freedom	Sum of square	*F* test	*P*
Regression linear	2	2.89	11.58	0.004
Residual error	8	0.99		
Lack-of-fit	6	0.97	15.81	0.061
Pure error	2	0.02		
Total	10	3.89		

Sánchez et al. [24] optimized the conditions of the enantioselective esterification of ibuprofen by *R. miehei* lipase (Lipozyme, 10 mg/mL) in a batch system using an orthogonal full factorial experimental design. *E*-value was maximal at a butanol:ibuprofen MR of 1.9:1and 50 mM of ibuprofen concentration at 40°C. After 112 h of esterification, enantiomeric excess and total conversion values were 93.8 and 49.9%, respectively, and the *E*-value was 113.

The native lipase from *A. niger* catalyzes the esterification of racemic ibuprofen with 1-propanol and isooctane as organic solvent to furnish enantiopure (*S*)-ibuprofen of pharmaceutical interest. The experimental design procedure provided a powerful tool to optimize the esterification conditions that permit an important improvement of the enantiomeric excess of active (*S*)-ibuprofen and enantioselectivity of lipase in this process. Under optimum conditions, a good enantioselective resolution of (*R,S*)-ibuprofen has been achieved, which is considerably higher than those results previously reported using this lipase.

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