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Kinetic Resolution of 1,3,6-Tri-*O*-benzyl-*myo*-Inositol by Novozym 435: Optimization and Enzyme Reuse

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ABSTRACT: *myo*-Inositol derivatives bearing selectively protected hydroxyl groups are relevant precursors of high-value *myo*-inositols. In the present study, we applied the response surface method to the optimization of kinetic resolution of (±)-1,3,6-tri-*O*-benzyl-*myo*-inositol by Novozym 435 (immobilized lipase B from *Candida antarctica*) with vinyl acetate in hexane. Reaction temperature, substrate, acyl donor and enzyme concentrations were set as variables. Through the constructed mathematical model, optimum condition for this enzymatic condition was established. The feasibility of enzyme recycle was demonstrated.

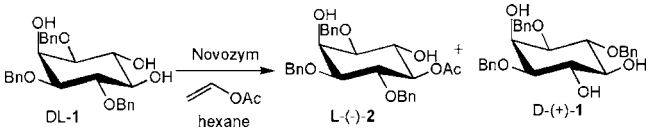
INTRODUCTION

Chiral *myo*-inositol derivatives (e.g., inositol polyphosphates) are important probes in cell biology studies. Commonly, the synthetic routes to these compounds involve optical resolution of racemic mixtures via derivatizations, which are neither practical nor efficient.[1,2] This explains in part the high prices of commercially available inositols. Chemoenzymatic routes may remove such process hurdles and increase the availability of these important compounds.

In fact, the use of enzymes, especially lipases, in organic solvents, has proved to be an excellent methodology for the preparation of chiral drugs.[3] Lipases enable high stereoselectivities and yields in kinetic resolutions or desymmetrizations. However, notwithstanding the high efficiency and economy that they bestow, few works have reported on their application to syntheses of inositols.[4−6]

We have recently shown that an immobilized lipase B from *Candida antarctica* (Novozym 435) is an outstanding biocatalyst for the kinetic resolution of a extensively protected myo-inositol.[7,8] More recently,[9,10] we determined that racemic 1,3,6-tri-*O*-benzyl-*myo*-inositol (DL-**1**) undergoes kinetic resolution by Novozym 435 to afford the acylated product, L-(−)-5-acetyl-1,3,6-tri-*O*-benzyl-*myo*-inositol (L-(−)-**2**) with a 48.30% conversion and enantiomeric excess of 97% in 112 h (Scheme 1). Substance DL-**1** is a known precursor of bioactive *myo*-inositol phosphates and analogs and a potential precursor of other inositol bisphosphates and trisphosphates.[11]

Scheme 1. Kinetic resolution of DL-1 by Novozym 435 (CaLB) using vinyl acetate in hexane



Lipase B from *Candida antarctica* (CaL-B) is one of the most used lipases in biotransformations in many different applications.[12−16] 3D-Structures of the CaL-B lipase have shown the presence of a short oligopeptide helix that may act as a lid and it has been found to adopt different conformations as a function of the medium, suggesting a great mobility of the active site environment.[8,17] CaL-B exhibits a very high degree of substrate selectivity and also has the potential for use in different reactions, for instance hydrolysis and alcoholysis reactions.[7,12,13,18−21] Different studies have discussed how the immobilization technique modulate the catalytic ability of CaL-B, endowing high performance[22,23] as it is the case of Novozym 435.

In the present work, we disclose our results of optimization of the kinetic resolution of *myo*-inositol derivative DL-**1** (Scheme 1) by Novozym 435 using vinyl acetate, as activated acyl donor, in hexane. A statistical method based upon the response surface methodology (RSM) was applied in order to determine the defining variables. By this, the statistically significant variables (for the conversions) were determined by the fractional factorial design. Once such variables are selected, the CCRD was applied to define the effect of each factor, its quadratic term and the expected curvature. The studied variables in the experimental design were temperature reaction, substrate, acyl donor and enzyme concentration, whose interactions led to the mathematical model for this enzymatic transformation. Thus, through such technique, we sought to increase the efficiency and economy of this biocatalytic transformation. In addition, the optimum conditions were explored in successful reuse experiments.

To the best of our knowledge, the optimization of chemoenzymatic syntheses of *myo*-inositols, as disclosed in this study, has not been previously reported in the literature.

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Table 1. Fractional factorial design matrix with real values and coded for the variables and responses for conversion (X%)

trials	substrate (mg/mL), S_1	acyl donor (mg/mL), S_2	enzyme (U), E	temperature (°C), T	conversion (%), X
1	2 (−1)	186.8 (−1)	54.56 (−1)	30 (−1)	21.46
2	14 (1)	186.8 (−1)	54.56 (−1)	50 (1)	6.15
3	2 (−1)	560.4 (1)	54.56 (−1)	50 (1)	31.21
4	14 (1)	560.4 (1)	54.56 (−1)	30 (−1)	4.10
5	2 (−1)	186.8 (−1)	136.4 (1)	50 (1)	42.00
6	14 (1)	186.8 (−1)	136.4 (1)	30 (−1)	11.86
7	2 (−1)	560.4 (1)	136.4 (1)	30 (−1)	41.00
8	14 (1)	560.4 (1)	136.4 (1)	50 (1)	42.65
9	8 (0)	373.6 (0)	81.84 (0)	40 (0)	37.00
10	8 (0)	373.6 (0)	81.84 (0)	40 (0)	35.50
11	8 (0)	373.6 (0)	81.84 (0)	40 (0)	36.00

RESULTS AND DISCUSSION

Optimization of Kinetic Resolution of DL-1 in Hexane. *Fractional Factorial Design.* The first statistical treatment of combinations of the test variables along with the measured response values (conversion, X%) are shown in Table 1. For all cases, 98% (±0.8) ee_p were obtained.

The screening by fractional factorial design revealed that the concentrations of enzyme and substrate are the most relevant variables concerning the conversion response. Figure 1 shows how significant the variables are to the conversion (X%).

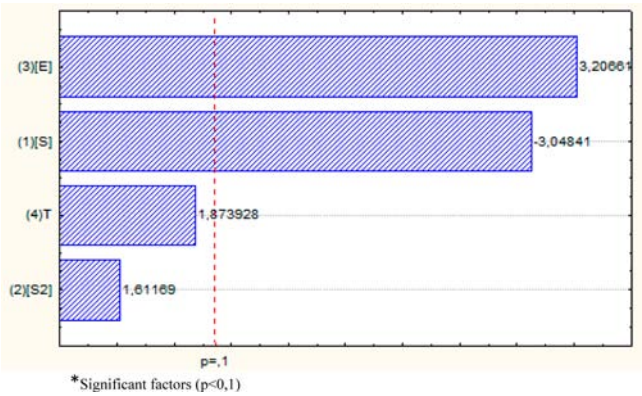

Figure 1. Pareto diagram after the fractional design showing the variables significant to the conversion (X%). [* = significant factors (p < 0.1).]

Although the temperature was not a significant factor in the fractional factorial design, this variable was included in CCRD. Previously in our studies, it proved to be the determining factor (Table 2). As acyl donor (vinyl acetate) concentration (S_2) was the least significant variable, such parameter was then fixed (0.3 mL in 1.7 mL of hexane) in the CCRD trials for conversion (X%) optimization. By means of the fractional design, we could select a minimum concentration of acylating agent in the experiments.

Table 2. Variation of conversion with the temperature in 48-h reactions[a]

temperature (°C)	conversion (X%)	ee
30	31	>98
40	41	>98
50	45.5	>98

[a]Assays with 136.4 U of Novozym 435 and 5 mg of DL-**1**.

The fractional design showed the coefficient of determination (R^2 = 96.90%) was highly significant, meaning that the model was unable to explain only 3.1% of the total variations and also indicated the significance of the model.

Central Composite Rotatable Design. By building on the results of the fractional design, the following variables were selected for the CCRD: substrate concentration, enzyme activity, and temperature. The results of the statistical treatment are presented in Table 3. For all cases, ee_p remained >98%.

The highest conversion that was obtained was 51.66% in trial 2. For all runs, the results were similar to the predicted values with a maximum deviation of −4.70% (trial 10).

The screening by means of CCRD revealed that the interaction between the linear terms of substrate and temperature (**S. T**) and the interaction between the linear terms of enzyme and temperature (**E. T**) showed no significance to conversion response (Table 4 shows only significant variables).

The linear and quadratic terms of substrate concentration and the quadratic terms of enzyme activity and temperature, showed a negative effect in the studied range. On the other hand, the linear terms of enzyme activity and temperature showed a positive effect in the range studied. As expected, increase of conversion was observed. In fact, a strong dependence of conversion on temperature and enzyme activity was established.

The following second-order model eq 1, which considers the statistically significant parameters (p < 0.05) in the kinetic resolution of DL-**1**, was obtained:

$$X\% = 48.37 - 7.60S - 3.29S^2 + 6.14E - 4.70E^2 + 7.80T - 5.94T^2 + 3.04S{\cdot}E \quad (1)$$

where S, E, and T were the coded values for the substrate concentration, enzyme activity, and temperature, respectively. E^2 and T^2 were the coded values for the quadratic terms of the enzyme activity and temperature, respectively. The interaction between the linear terms substrate concentration and enzyme activity are represented by SE.

In addition, the fit of the statistical model to the experimental data was confirmed by the ANOVA table (Table 5).

The F test value found for the regression (27.52) was higher than the F tabulated value (3.50). Once the F test value was 14.1 times higher than F tabulated value, with high significance (p = 0.000044), the model may be considered predictive. Furthermore, the coefficient of determination (R^2 = 96.87%) was highly significant, meaning that the model was unable to explain only 3.13% of the total variations. The adjusted R^2 value (93.35%) also indicated the significance of the model. Such

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Table 3. CCRD matrix with real values and coded for the variables and conversion (*X*%) responses

trials	substrate (mg/mL), *S*, S_1	enzyme (U), *E*	temperature (°C), *T*	conversion (%), *X*	predicted values (%)	error (%)
1	5 (−1)	136.4 (−1)	30 (−1)	27.89	27.61	−0.28
2	5 (−1)	136.4 (−1)	50 (1)	51.66	50.24	−1.42
3	5 (−1)	300.1 (1)	30 (−1)	39.00	36.98	−2.02
4	5 (−1)	300.1 (1)	50 (1)	51.00	53.28	2.28
5	11.5 (1)	136.4 (−1)	30 (−1)	10.46	10.20	−0.26
6	11.5 (1)	136.4 (−1)	50 (1)	21.04	25.09	4.05
7	11.5 (1)	300.1 (1)	30 (−1)	28.30	31.74	3.44
8	11.5 (1)	300.1 (1)	50 (1)	38.00	40.31	2.31
9	2 (−1.68)	218.2 (0)	40 (0)	50.03	51.85	1.82
10	15 (1.68)	218.2 (0)	40 (0)	31.00	26.30	−4.70
11	8.5 (0)	80.5 (−1.68)	40 (0)	25.00	24.72	−0.28
12	8.5 (0)	355.8 (1.68)	40 (0)	48.00	45.40	−2.6
13	8.5 (0)	218.2 (0)	23 (−1.68)	18.00	18.44	0.44
14	8.5 (0)	218.2 (0)	57 (1.68)	48.00	44.67	−3.33
15	8.5 (0)	218.2 (0)	40 (0)	50.00	48.37	−1.63
16	8.5 (0)	218.2 (0)	40 (0)	48.00	48.37	0.37
17	8.5 (0)	218.2 (0)	40 (0)	48.00	48.37	0.37
18	8.5 (0)	218.2 (0)	40 (0)	47.00	48.37	1.37

Table 4. Effect estimates for the conversion (*X*%) in the kinetic resolution

factor	effect	standard error	*t* (8)	*p* value[a]
mean	48.3734	1.706094	28.35329	0.000000
S	−15.1940	1.849379	−8.21573	0.000036
$(S)^2$	−6.5745	1.921633	−3.42132	0.009070
E	12.2914	1.849379	6.64625	0.000161
E^2	−9.4129	1.921633	−4.89836	0.001196
T	15.5971	1.849379	8.43370	0.000030
T^2	−11.8877	1.921633	−6.18626	0.000263
SE	6.0875	2.416330	2.51932	0.035847

[a]Significant factors $p < 0.05$.

Table 5. ANOVA of the quadratic model for conversion in the kinetic resolution (*X*%)[a]

sources of variations sources of variation	sum of squares	degrees of freedom	mean squares	*F*-test[b]	*p* value
regression	*2892.52*	*9*	*321.39*	*27.52*	0.000044
residual	*93.42*	*8*	*11.67*		
total	2985.93	17			

[a]Coefficient of determination (R^2) = 0.9687; adj. R^2 = 0.9335. [b]$F_{0.05;\ 9;\ 8}(F_{tabulated})$ = 3.50

tests sufficed to confirm a high degree of adequacy of the model. Then, the model was used to generate contour plots and response surfaces (Figures 2 and 3).

As can be seen in Figure 2, the interaction of temperature and enzyme activity leads to the increase of conversion, indicating the beneficial effect of both variables.

The surface indicated that the best conversion was obtained at midpoint of enzyme activity axis and of temperature.

Conversely, the response surface and contour diagram (Figure 3) for the interaction of substrate concentration and enzyme activity indicated contrary effects between such variables.

Although the best value of substrate concentration in the kinetic resolution of DL-**1** was 4 mg/mL (conversion as response variable), it was desirable to increase it for the sake of economy. Fortunately, the model shows that concentrations of 8.5 mg/mL still retain a high conversion (Figure 4).

Our data showed that, within that concentration range (Figure 4 a and b), and with the use of 220 U of lipase, conversions remained high. A further increase of concentration up to 11 mg/mL is possible without significant conversion loss. Raising the substrate concentration to 14 mg/mL (Figure 4c) would require higher enzyme load (280 U) to obtain the same conversion level, which would render the process less

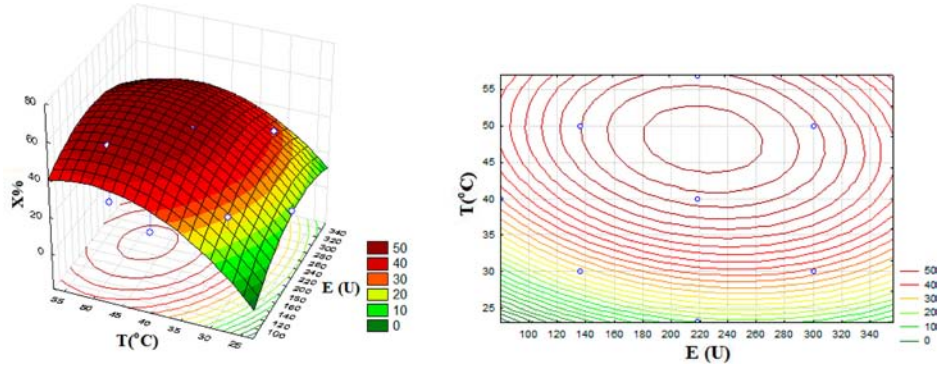

Figure 2. Response surface (left) and contour diagram (right) for conversion response (*X*%) to temperature (*T*) and enzyme activity (*E*). The value of substrate concentration was fixed at 4 mg/mL.

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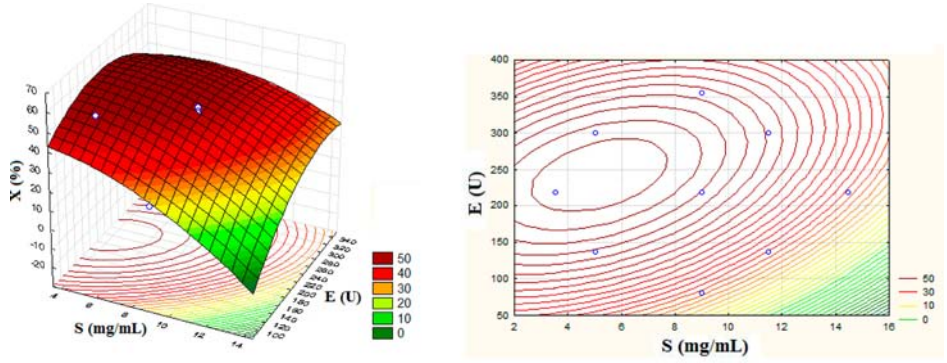

Figure 3. Response surface (left) and contour diagram (right) for conversion response (X%) to substrate concentration (S) and enzyme activity (E). The temperature was fixed at 40 °C.

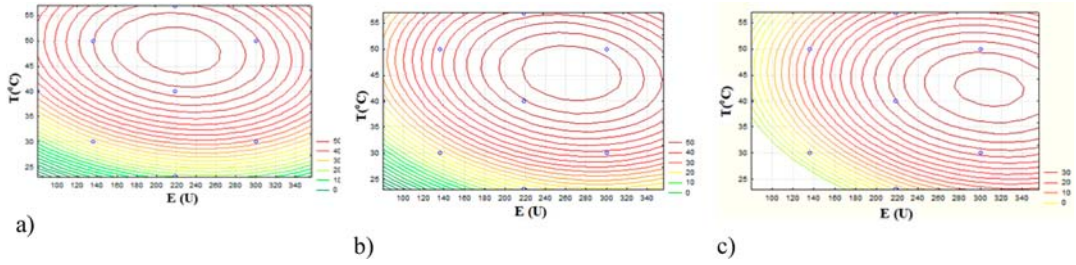

Figure 4. Contour diagram for variable conversion response (X%) using 220 U of lipase. Values of substrate concentration were fixed at (a) 4 mg/mL, (b) 8.5 mg/mL, and (c) 14 mg/mL.

economical. Although the productivity study pointed out to 57 °C as the temperature, we continued to employ 45 °C (the optimal temperature by CCRD) in order to avoid enzyme destabilization.

Thus, our model for kinetic resolution of DL-**1** by Novozym 435 with vinyl acetate in hexane set as the optimum condition (in the range) 45 °C, 4 mg/mL of substrate, and 222.8 U of enzyme. The accuracy of the model was validated with three replicates under the aforementioned optimum conditions. The experimental conversion was 49.7 ± 0.2% after 24 h of reaction against a 52% predicted conversion. The ee_p was >98% (E > 200) in all samples collected during the 24-h reaction (Figure 5).

The time course of this enzymatic transformation, under the optimum conditions, was determined (Figure 5). Clearly, the experimental design reduced the reaction time (48.3% after 112 h in the original condition using hexane) in the kinetic resolution of inositol DL-**1**.[10]

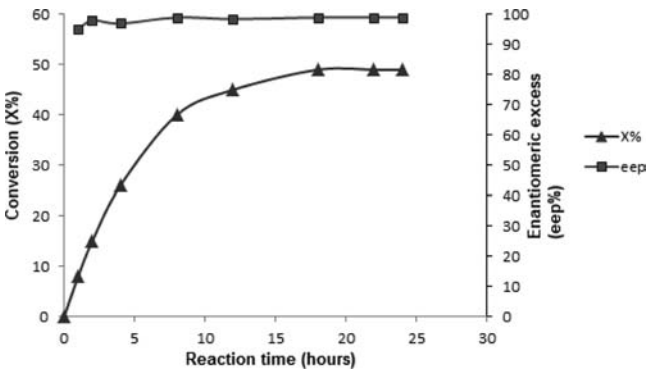

Figure 5. Time course of the kinetic resolution of DL-**1** in hexane under optimum conditions: 45 °C, 4 mg/mL of substrate and 222.8 U of enzyme activity.

In fact, the effect of the optimization on the productivity and reaction kinetics was dramatic (Figure 6). Despite the sharp decay over time, the productivity of the optimized reaction overcomes that of the nonoptimized one throughout the reaction course (>20 h). Little variation of productivity is shown in the latter condition. This explains the much higher conversion in the optimized reaction.

Thus, through the experimental design strategy by CCRD, a 15-fold increase in productivity (0.006 $mg_{product}/mg_{enzyme}$/h for an 8-h reaction) over that from the original protocol[9] (0.0004 $mg_{product}/mg_{enzyme}$/h for a 112 h reaction). Besides boosting the catalytic performance (higher conversion and shorter reaction time) in the kinetic resolution of DL-**1** by Novozym 435, the present optimization fosters higher economy, as lesser quantities of solvent and catalyst are required in the process. Moreover, such high conversion (49.7 ± 0.2%) enables the synthesis of both the acylated product (ee > 99%) and the remaining (unreacted) substrate (ee = 95%) in optically pure form.

This condition could be reproduced in a 1-g scale, which afforded high conversion (50% ± 1) and selectivities (ee_p = 99% and ee_s = 99%, E > 100).

■ REUSABILITY

Naturally, the reuse of an immobilized enzyme without appreciable loss of enzyme activity is important for the economic viability of enzymatic processes. We inquired whether the designed conditions for the enzymatic reaction under study would enable such reusability. The results are in Figure 7.

The immobilized lipase was operated over 14 d (with 24-h cycles), nearly without loss of conversion values up to the

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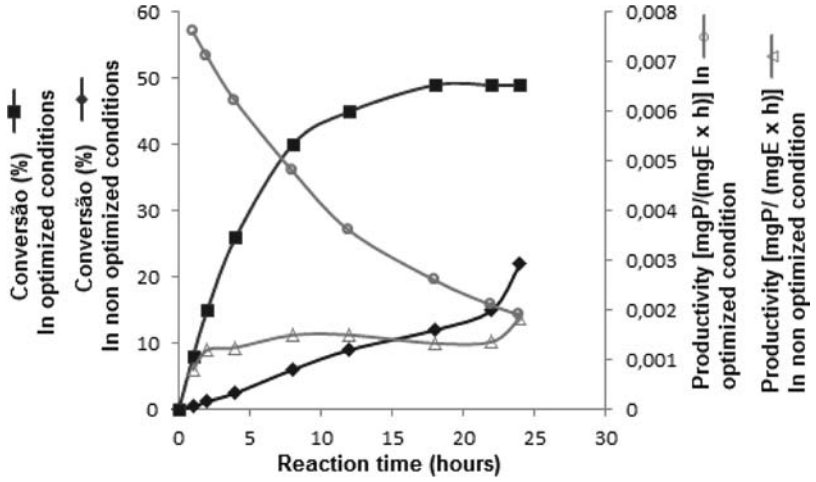

Figure 6. Productivity and conversion evolution in the kinetic resolution of DL-**1** in hexane under the optimum (45 °C, 4 mg/mL of substrate and 222.8 U of enzyme activity) and nonoptimized conditions.[9].

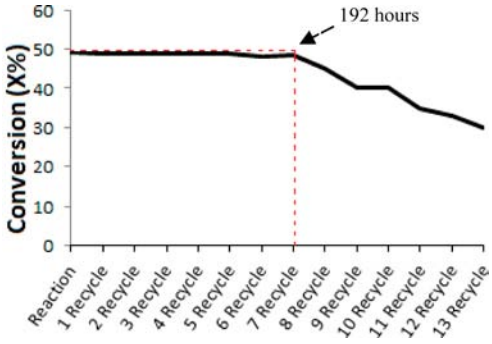

Figure 7. Operational stability in the kinetic resolution of DL-**1** by Novozym 435 under the designed conditions: 4 mg/mL of substrate, 222.8 U of enzyme, and 45 °C of temperature.

seventh recycle (49% ± 0.7). From that point up to the 10th recycle, acceptable conversions were still attained (40% ± 0.5). Throughout the process 98% ee_p (E > 100) was obtained. These results fully justify the use of immobilized lipase and qualify the optimized procedure for gram-scale chemoenzymatic syntheses of *myo*-inositols.

CONCLUSION

The successful optimization of the kinetic resolution of *myo*-inositol DL-**1** by Novozym 435 enables a practical and economical synthesis of both enantiomorphs D-(+)-**1** and (by deacylation) L-(−)-**1**, besides acetate L-(−)-**2**, in high ee. This process promises to make different high-value *myo*-inositols and other synthetic derivatives more available for cell biology investigations and medicinal applications. In fact, by means of CCRD, the optimization of this enzymatic transformation in hexane with vinyl acetate led to a 15-fold increase of productivity over the original protocol. Moreover, the constructed mathematical model informed us that higher substrate concentration (over 4 mg/mL) can be employed without any significant decrease of conversion. Finally, these results were explored in assays that demonstrated the reusability of Novozym 435 in the kinetic resolution of *myo*-inositol DL-**1**.

EXPERIMENTAL SECTION

Substrate. The substrate (±)-1,3,6-tri-*O*-benzyl-*myo*-inositol, DL-**1**, was synthesized by literature procedures.[24,25]

Enzyme. Novozym 435 (Lipase B of *Candida antarctica* immobilized on a macroporous acrilic resin, 2728 U/g) was purchased from Novozymes Brasil (Araucária-PR). The enzyme activity was determined as the initial rate in esterification reactions between oleic acid and ethanol at a molar ratio of 1:1, temperature of 40 °C and enzyme concentration of 5 wt % in relation to the substrates.[26] One lipase activity unit (U) was defined as the amount of enzyme necessary to consume 1 μmol of oleic acid per minute at the established experimental conditions previously presented. All enzymatic activity determinations were replicated at least three times.

Experimental Conditions. The enzymatic reactions were realized under magnetic stirring in closed thermostatized flasks (water bath) containing DL-**1**, Novozym 435 and vinyl acetate in hexane (Final volume 2.0 mL) (*vide* discussion for details). After 24 h, the reactions were stopped by catalyst removal by filtration. The assays were run in triplicate. The samples containing product L-(−)-**2** and D-(+)-**1** had the volatiles evaporated and the resulting material was subjected to HPLC analysis of conversion and separation for ee determination (*vide infra*).

For the 1-g scale (DL-**1**) reaction under the optimized protocol (45 °C), 10.2 g of Novozym 435 and a 250-mL vinyl acetate/hexane mixture (containing 37.5 mL of vinyl acetate) were employed (see text).

HPLC Analysis of Conversion of DL-1 to L-(−)-2. Conversion analyses were done via HPLC on a Shimadzu-C18 column (40 °C in a CTO-20A oven), eluted with an acetonitrile/H_2O (60:40) mixture (0.5 mL/min) by a Shimadzu LC-20AT pump. A Shimadzu SPD-M20A variable-wavelength UV/vis detector was employed, with the detection set at 215 nm, and the Shimadzu LC solution software was used for chromatogram integration. The samples to be analyzed were filtered through a 0.22 μm PTFE filter. The retention times of the substrate DL-**1** and the acetate L-(−)-**2** were 8 min and 13 min, respectively.

Determination of Enantiomeric Excesses (ee). Unreacted substrate, D-(+)-**1**, and monoacetylated product, L-(−)-**2**, were separated by HPLC, under elution condition used for the conversion determination (*vide supra*). Then, the

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solvents of the obtained solutions were evaporated prior to direct analysis (D-**1**) or further treatment (methanolysis) and following analysis (L-**2**). In the case of L-(−)-**2**, the substance was subjected to methanolysis reaction ($MeOH/K_2CO_3$) to give triol L-(−)-**1** prior to the HPLC analyses.[5c]

Chromatographic determination of the ee's of both D-(+)-**1** (ee_s) and L-(−)-**1** (ee_p) were done on the same equipment mentioned above, carrying a Chiralcel OD-H column (5 μm; 4.6 mm × 250 mm), eluted with a 7:3 hexane/2-propanol mixture (0.6 mL/min). The retention times of these enantiomorphs were 24.5 and 28.5 min, respectively. The enantiomeric ratio (E) was calculated by using the equation of Chen et al.[27]

Experimental Design Strategy for Kinetic Resolution of DL-1: Optimization Experiments. Five independent test variables were chosen for the statistical experimental design: substrate concentration (S_1, mg/mL); acyl donor concentration (S_2, mg/mL), enzyme concentration (E, mg/mL) and temperature (T, °C). A previous study by our group had shown anhydrous hexane as the best solvent for this enzymatic transformation.[9,10]

First, a fractional factorial design including 2^{4-1} runs, with three central points, was carried out to evaluate which variables have a major effect on the kinetic resolution (see Table 6).

Table 6. Experimental values and levels of the independent variables of fractional factorial design for the kinetic resolution of (±)-1 by Novozym 435

	range and levels		
test variables	−1	0	1
substrate (mg/mL), S_1	2	8	14
acyl donor (mg/mL), S_2	186.8	373.6	560.4
enzyme (U), E	54.56	81.84	136.4
temperature (°C), T	30	40	50

The preliminary fractional factorial design allowed for the selection of the statistically significant variables for conversion of the reaction. After selection of the variables, a CCRD with 23 trials, including four replicates at the central point and six axial points, were employed to obtain a second-order model for the prediction of conversion (dependent variable) as function of the studied variables (independent variables). The real variable values and coded levels were varied according to the experimental design of the kinetic resolution under study are given in Table 7.

The data obtained from the CCRD were used to fit an empirical quadratic polynomial model related to the response by a multiple regression procedure.[28−30] For three factors, the model takes the form of eq 1a.

Table 7. Experimental values and levels of the independent variables of CCRD for the kinetic resolution of (±)-1 by Novozym 435

	range and levels				
test variables	−1.68	−1	0	1	1.68
substrate (mg/mL), S_1	2	5	8.5	11.5	15
enzyme (U), E	80.5	136.4	218.2	300.1	355.8
temperature (°C), T	23	30	40	50	57

$$Y = a_0 + \sum_{i=0}^{n} a_i x_i + \sum_{i=0}^{n-1} \sum_{j=i+1}^{n} a_{ij} x_i x_j \quad (1a)$$

where Y is the predicted response, i and j range from 1 to the number of variables (n), a_0 is the intercept term, a_i values are the linear coefficients, a_{ij} values are the quadratic coefficients, and x_i and x_j are the levels of the independent variables.

All the experiments of the fractional design and the CCRD were performed randomly, and the data were treated with the aid of the software STATISTICA 7.0 (Statsoft Inc., Tulsa, OK, USA).

Enzyme Reusability. In the assays of Novozym 435 reuse, after each 24-h batch run (2 mL), the reaction mixture was centrifuged. Then, the liquid phase (for chromatographic analyses) was decanted and the solid catalyst was used in the next run under the optimum condition. Such procedure was repeated 13 times. In each run, a sample of 100 μL was taken for determination of the enzyme activity. No decline in activity was observed. When washing of the catalyst (with vinyl acetate/hexane mixture) was carried out after each run, a significant loss of activity occurred.

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Notes

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