Brazilian Journal
of Chemical
Engineering

ISSN 0104-6632
Printed in Brazil

Vol. 20, No. 04, pp. 343 - 355, October - December 2003

IMMOBILIZATION AND CATALYTIC PROPERTIES OF LIPASE ON CHITOSAN FOR HYDROLYSIS AND ESTERIFICATION REACTIONS

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(Received: December 13, 2002 ; Accepted: May 7, 2003)

Abstract - The objective of this study was to evaluate the immobilization of lipase on a chitosan support by physical adsorption, aiming at its application in hydrolytic and synthetic reactions. Two types of chitosan (flakes and porous) were used for immobilizing lipase from a microbial source (*Candida rugosa*) and animal cells (porcine pancreas). The best results for recovery of total activity after immobilization were obtained for microbial lipase and porous chitosan beads. This set was selected for further immobilization studies, including full characterization of the immobilized derivative in aqueous and organic media. In aqueous medium, the operational and thermal stabilities of this preparation were quantified. In organic medium, the direct synthesis of n-butyl butyrate in organic solvent was chosen as a model reaction. The influence of several parameters, such as temperature, initial butyric acid concentration and amount of enzyme in the reaction system, was analyzed. Production of n-butyl butyrate was optimized and an ester yield response equation was obtained, making it possible to predict ester yields from known values of the three main factors. Use of this immobilized preparation was extended to the direct esterification of a large range of carboxylic acids (from C_2 to C_{12}) with a variety of alcohols (from C_2 to C_{10}).
Keywords: lipase, aqueous and nonaqueous environment, hydrolytic and esterification reactions.

INTRODUCTION

Existing limitations on the synthesis of chemical intermediaries/products of commercial interest can be overcome to advantage with biotransformations. Enzymes have become a powerful catalytic tool in a wide variety of chemical processes (Dordick, 1989; Faber, 1997). Group of industrially useful enzymes is the lipases, which are hydrophobic proteins that act on carboxylic acid esters, such as glyceride lipids, at the interface between an aqueous and an oil phase. This represents approximately 20% of the biotranformations used today (Faber, 1997).

For many applications enzymes are preferably used in an immobilized state in order to easily separate the catalyst from the product steam. With immobilized lipases, improved stability, reuse, continuous operation, the possibility of better control of reactions and hence more favorable economical factors can be expected (Frense et al, 1996; Tischer and Wedekind, 1999). Many different methods of enzyme immobilization are available, each involving

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a different degree of complexity and efficiency (Malcata et al, 1990).

The high cost of popular supports (silica-based carriers and synthetic polymers) causes many to search for a cheaper substitute such as $CaCO_3$ (Rosu et al, 1998), rice husk and rice straw (Tantrakulsiri et al, 1997) or chitin and chitosan (Krajewska, 1991; Felse and Panda, 1999). Of these alternatives, the derivative of chitin, chitosan, appears to be the most attractive since chitin is the second the most abundant biopolymer in nature next to cellulose (Krajewska, 1991). In addition, this support offers several advantages as an enzyme immobilization carrier, among which the following stand out: versatility of available physical forms (flakes, porous beads, gel, fiber and membrane); low biodegradability; low cost; ease of handling; high affinity for proteins and, above all, nontoxicity (Felse and Panda, 1999). Moreover, good results were obtained in a number of previous studies in which chitosan was used to immobilize lipase (Itoyama et al, 1994; Carneiro da Cunha et al, 1999) and other hydrolases such as amyloglucosidase, papain, β-glycosidase and α-L arabinofuranosidase (Krajewska, 1991; Felse and Panda, 1999). To pursue our interest in the immobilization and subsequent use of lipases (Castro et al, 1999; Soares et al, 1999), we studied the feasibility of using chitosan as matrix for immobilizing microbial lipases. The immobilization criteria were based on the use of a low-cost method of loading enzyme onto the support. The chosen method of immobilization was simple adsorption, whereby the enzyme adheres to the surface of the support particles by van der Waals forces of attraction. Two lipase sources were tested and the efficiency of immobilization was assessed for recovery of both protein and hydrolytic activity. The best lipase source was selected for further studies, including full characterization of the immobilized derivative in aqueous and nonaqueous media. This paper reports the research developed as a Master´s thesis at the State University of Maringa and the School of Chemical Engineering of Lorena (Pereira, 1999), which won the OPP-ABEQ award in 2002. Part of this work has already been published (Pereira et al, 2001; Pereira et al, 2002).

MATERIALS AND METHODS

Materials

Commercial *Candida rugosa* lipase (Type VII), porcine pancreas (Type II), bovine serum albumin (BSA) and chitosan in flake form were purchased from Sigma Chemical Co. (St Louis, MO, United States). Porous chitosan beads, supplied by SP Chemical Farma Ltda (Lorena, SP, Brazil), had the following characteristics, according to the manufacturer's information: 93% purity, 6% moisture and particle size 40 mesh. Olive oil (low acidity) was purchased at a local market. Solvents were standard laboratory grade and other reagents were purchased either from Aldrich Chemical Co. (Milwaukee, WI, USA) or Sigma Chemical Co. (St Louis, MO, USA).

Immobilization of Lipase on Chitosan

Lipase was immobilized by physical adsorption on chitosan following a previously developed methodology (Carneiro da Cunha et al, 1999) with slight modifications. Chitosan *in natura* (2 g) had been previously soaked in hexane under agitation conditions (100 rpm) for 1 hour. Then, excess hexane was removed followed by the addition of 0.5 grams of powder lipase dissolved in 10 mL of distilled water. The lipase was on the support under agitation for 3 hours at room temperature followed by an additional period of 18 hours under static conditions at 4°C. The derivative was filtered (Whatman filter paper 41) and thoroughly rinsed with hexane. Analyses of hydrolytic activities carried out with the enzyme offered for immobilization, and immobilized preparations were used to determine the activity yield by dividing catalytic activity by lipase loading (units.g^{-1}support). Further experiments were carried out to determine appropriate lipase loading by using a fixed amount of support (3 grams, dry weight) for different amounts of enzyme (0.3 to 3 grams lipase).

Protein Assay

Protein was determined according to Bradford´s method (Bradford, 1976) using bovine serum albumin (BSA) as the standard. The amount of bound protein was determined indirectly from the difference between the amount of protein introduced into the coupling reaction mixture and the amount of protein in the filtrate and the washing solutions.

Hydrolysis Assay

Hydrolytic activities of free and immobilized lipase were assayed by the olive oil emulsion method according to the modification proposed by Soares et al. (1999). One unit (U) of enzyme activity was

defined as the amount of enzyme necessary to produce 1 μmol of free fatty acid per min under the assay conditions (37°C, pH 7.0, 150 rpm).

Esterification Assay

Reaction systems consisted of heptane (20 mL), n-butanol (250 mM), butyric acid (300 mM) and immobilized lipase (1.0 gram, dry weight). The mixture was incubated at 37°C for 24 hours with continuous shaking at 150 rpm. The amount of product formed was determined by gas chromatography. Activity was expressed as μmol of butyl butyrate formed per minute per gram of dry support.

Catalytic Properties of Lipase Preparation in an Aqueous Medium

Free and immobilized hydrolytic activities were estimated with reaction mixtures containing 100 mM of the sodium phosphate buffer at different pH values in the range of 3.0 to 9.0 at 37°C. The effect of temperature on both lipase activities was determined at temperatures from 30 to 65°C under assay conditions. For determination of thermal stability, either free or chitosan lipase preparations were incubated in 2 mL sodium phosphate buffer (pH 7.0) at different temperatures (40-60°C) for 1 hour. Samples were removed and assayed for residual activity as previously described, taking an unheated control to be 100% active.

Catalytic Properties of Lipase Preparation in a Nonaqueous Medium

The butanol and butyric acid system was first studied by using a full 2^3 factorial design at two levels. The influence of temperature (37 and 50ºC), amount of lipase (0.25 and 0.5 grams) and molar ratio of butanol to butyric acid (1 and 2) on the variable response (yield of ester %) was determined. Three runs were carried out at the center point level for estimation of experimental error. The results were analyzed by Statistic (version 6.0) software.

The selectivity of the immobilized lipase was also tested by running two sets of esterification reactions: (a) butanol and different carboxylic acids (C_2 to C_{12}) and (b) butyric acid and different aliphatic alcohols (C_2 to C_{10}). Reaction took place in 100 mL closed flasks, containing an appropriate amount of reactants and immobilized lipase, for 24h under shaking (150 rpm) at 37°C.

Operational Stability of the Immobilized Lipase

The operational stability of the immobilized system was assayed by using 0.5 gram of immobilized lipase (dry weight) in either olive oil hydrolysis or butyl butyrate synthesis in successive batches. At the end of each batch, the immobilized lipase was removed from the reaction medium and washed with hexane to remove any substrate or product retained in the matrix. One hour later (length of time required for evaporation of the solvent), the immobilized lipase was introduced into a fresh medium. Activities were estimated at the end of each cycle and expressed as $\mu mol.min^{-1}.mg$ of catalyst. The biocatalyst half-life ($t_{1/2}$) was determined by applying the inverted linear decay model (Fonseca et al, 1993).

Analytical Methods

Reactions were monitored by measuring reactants and product concentrations by gas chromatography using a 6ft 5% DEGS on a Chromosorb WHP 80/10 mesh column (Hewlett Packard, USA) and hexanol as the internal standard. Water concentrations in liquid and solid phases were measured by the Karl Fischer method using the Karl Fischer tritrator (Mettler DL 18). Fatty acid concentrations were titrated with 0.02 M potassium hydroxide solution with phenolphthalein as an indicator.

RESULTS

Influence of Lipase Source and Chitosan Type on Efficiency of Immobilization

Table 1 summarizes the protein and activity recovery results for immobilization of both lipase sources on two types of chitosan using a fixed ratio between support (2.0 grams) and enzyme (0.5 gram). Of the two chitosan forms (flakes and porous beads), porous chitosan beads (PCB) showed the most favorable morphological properties for immobilization of both lipase preparations. It is likely that internal mass transfer occurred when the chitosan flake type was used, as lipase reaction rates tend to decrease with increasing particle size. The highest percentages of protein recovery (78.4%) and catalytic activity (15%) were obtained by microbial lipase when immobilized on porous chitosan beads at a lipase loading of 360 $units.gram^{-1}$ dry support.

Further experiments were carried out to

determine the effects of carrier loading on immobilization of *Candida rugosa* lipase on PCB. We studied the influence of the amount of lipase in aqueous solution in the range of 48 to 480 units.g^{-1}support. Results are displayed in Figure 1.

As expected, the hydrolytic activity of the immobilized enzyme increased as more lipase was loaded onto the support, but when converted to an efficiency plot (activity/loading), higher efficiencies were obtained at lower loadings (120 units.g^{-1}support). The results also suggest that for loadings over 240 units.g^{-1}support, instead of obtaining the desired crowded upright adsorption of enzyme onto the support surface, multilayer adsorption may occur, possibly blocking or inhibiting access to enzyme active sites. Therefore, most of the other experiments were carried out with immobilized preparation at lipase loadings of 120 units.g^{-1} of dry chitosan.

Table 1: Adsorption recovery, coupling yield and catalytic activities of lipase immobilized on chitosan

Chitosan Form	Lipase	Loading units.g^{-1}support	Bound Protein (%)	Coupling Yield (%)	Hydrolytic Activity (μmol.mg^{-1}.min^{-1})	Esterification Activity (μmol .mg^{-1}.min^{-1})
Flakes	PPL	245	53.7	4.3	8.6	33.3
	CRL	360	73.1	7.1	22.9	27.7
Porous beads (PCB)	PPL	245	72.3	18.2	42.6	31.9
	CRL	360	78.4	14.7	42.7	83.3

PPL - Porcine pancreas lipase
CRL - *Candida rugosa* lipase
Esterification activities are expressed as μmol butyl butyrate per minute per miligram dry support by following the rate of product formation from the reaction mixture containing butanol and butyric acid.

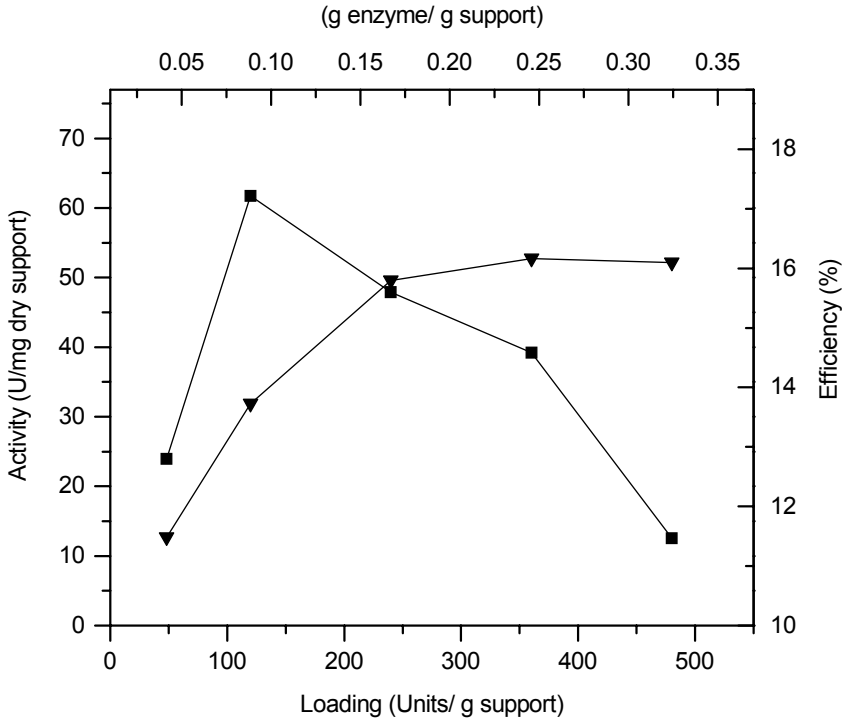


Figure 1: Effect of lipase loading on hydrolytic activity (◆) and on activity yield (■) for *Candida rugosa* immobilized on porous chitosan beads (PCB). Efficiency was calculated by dividing catalytic activity by lipase loading (see Materials and Methods).

Characterization of Immobilized Lipase for Utilization in Aqueous Medium

Immobilization of enzymes on charged supports often causes displacements in the pH activity profile, ascribed to an imbalance in the partitioning of H^+ and OH^- concentrations between the microenvironment of the immobilized enzyme and the bulk phase due to electrostatic interactions with the support. To verify the effect of immobilization upon the intrinsic activity of *Candida rugosa*, a set of experiments was carried out in which a new optimum value was established, as shown in Figures 2 to 5.

A slightly lower value for optimum pH (6.0) was found for the immobilized form than that attained for the free lipase (7.0) (Figure 2). The optimal reaction temperature shifted from 40°C to 45°C for the chitosan lipase, as observed in Figure 3. The patterns of heat stability indicate that the immobilization process tends to stabilize the enzyme (Figure 4).

The values of energy of activation, calculated by the Arrhenius equation, are presented in Table 2 and indicate that the immobilized form of the enzyme has an energy of activation lower than that of the free enzyme, with the PCB lipase having the lowest energy of activation. Activation energy lower than that of the free enzyme can be considered to be indicative of diffusion resistance of product and substrate in the case of the immobilized enzyme.

The influence of substrate concentration on hydrolytic activities was also analyzed for free and immobilized lipase in oil emulsions containing total fatty acids varying from 372 to 2604 mM (Figure 5 a-b). Plotting activity versus substrate concentration indicated that free lipase obeys the Michaelis-Menten equation, indicating that in the range studied, no inhibition by reaction products was detected (Figure 5a). For the case of the PCB lipase, different behavior was observed and a slight reduction in activity was detected for fatty acid concentrations higher than 1860 mM (Figure 5b). This can be indicative of substrate inhibition or diffusion resistance. The parameters of the Michaelis-Menten kinetic equation were determined from the double reciprocal plot of lipase activities (Table 3). Values of V_{max} were 3800 $\mu mol.mg^{-1}.min^{-1}$ and 54.5 $\mu mol.mg^{-1}.min^{-1}$ for free and immobilized lipase, respectively, while the K_m value for free lipase was 499.5 mM and for immobilized lipase was 288.5 mM.

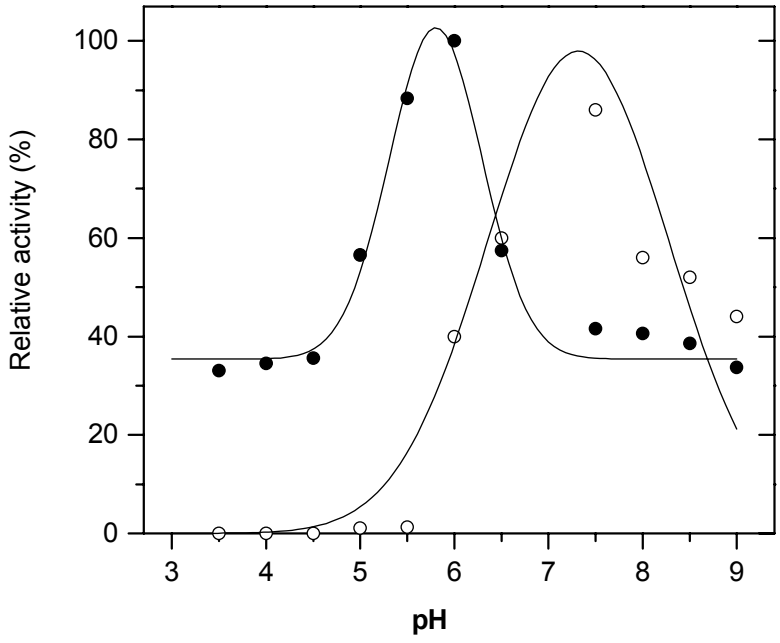


Figure 2: Effect of reaction pH on hydrolytic activities of lipase preparations. Enzymes were assayed with olive oil emulsion as substrate at 37°C, (∓) free lipase, (ℓ) PCB lipase. Initial activities (free lipase: 3400 units.mg^{-1}; PCB lipase: 51.4 units.mg^{-1}) were defined as 100%.

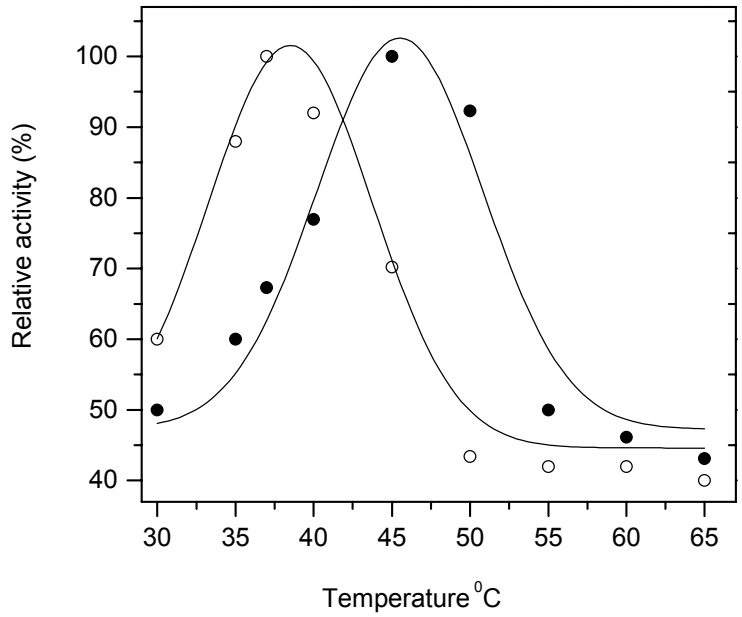


Figure 3: Effect of reaction temperature on hydrolytic activities of lipase preparations. Enzymes were assayed with olive oil emulsion as substrate at pH 7.0, (∓) free lipase, (ℓ) PCB lipase. Initial activities (free lipase: 3400 units.mg^{-1}; PCB lipase: 51.4 units.mg^{-1}) were defined as 100%.

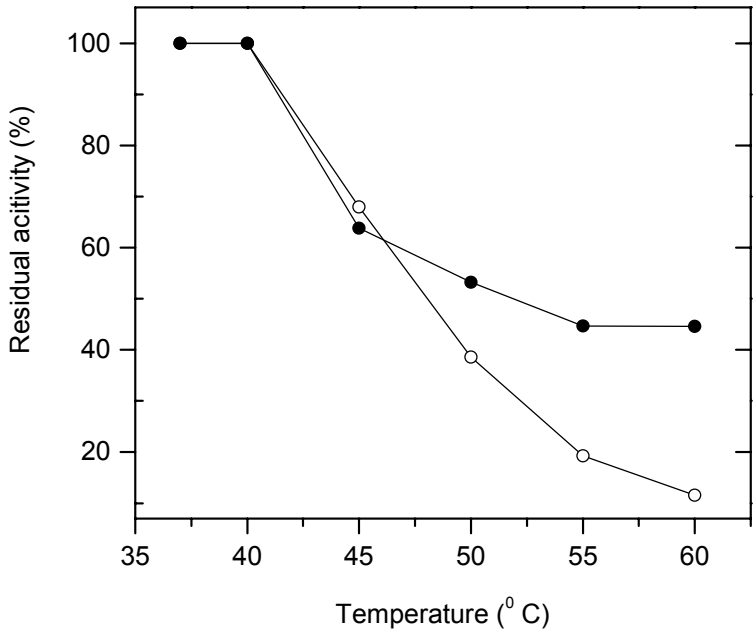
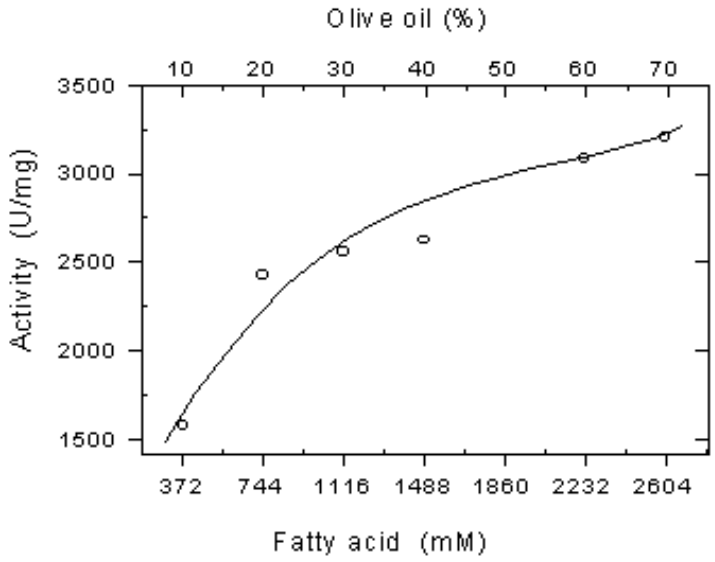


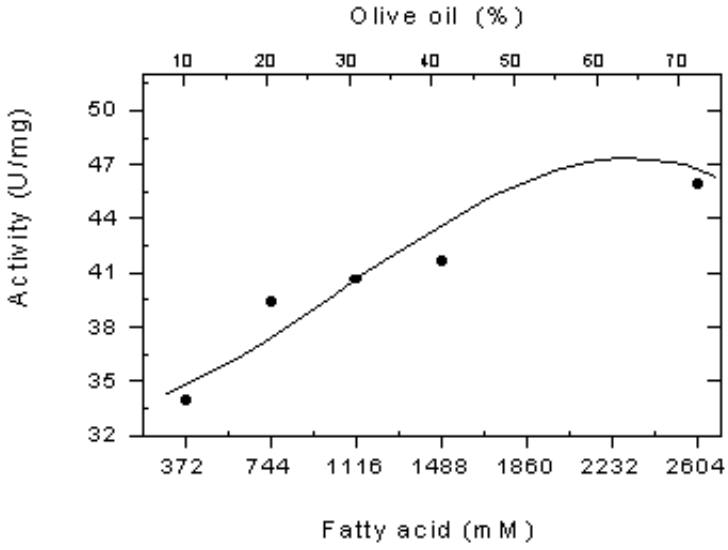
Figure 4: Temperature deactivation for free lipase (∓) and PCB lipase (ℓ) at different temperatures. Experiments were carried out in 100 mM phosphate buffer (pH 7.0). Initial activities (free lipase: 3400 units.mg^{-1}; PCB lipase: 51.4 units.mg^{-1}) were defined as 100%.

Table 2: Activation energy of free and PCB lipase preparation calculated with the Arrhenius Equation.

Enzyme preparation	Energy of activation ($kcal.mol^{-1}$)
Free lipase	9.90
PCB lipase	7.26



(5a)



(5b)

Figure 5: Influence of substrate concentration on hydrolytic activities for free lipase (a) and PCB lipase (b). Reactions were carried out with olive oil emulsion at different proportions at pH 7.0 and 37ºC.

Table 3: Kinetic parameters of *Candida rugosa* lipase

Enzyme preparation	K_{mapp} (mM)	V_{maxapp} ($\mu M.mg^{-1}.min$)
Free lipase	499.5	3800
PCB lipase	288.5	54.5

Characterization of Immobilized Lipase for Utilization in Organic Medium

To study the properties of lipase immobilized on porous chitosan beads in organic medium, a direct esterification of butanol with butyric acid was first studied. A factorial design was performed to identify the factors that influence ester synthesis and to verify whether any changes should be made in their settings to improve this reaction (Box et al, 1978).

As shown in Table 4, the ester yield (Y%) varied widely (from 26.9 to 73.1%) and independently of the other variables, increasing the lipase level from 0.25 to 0.5 grams substantially increased the ester yield from 26.9 to 57.7 (runs 1 and 3). A further increase in ester yield was dependent on the other factors. Run 7 (high level of molar ratio and low level of temperature) gave the highest ester yield (73%). Therefore, of the three factors studied, temperature (x_1), lipase level (x_2) and substrate molar ratio (x_3), all seem to have played a critical role in the butyl butyrate synthesis.

Table 5 summarizes results of the statistical analyses and shows standard errors and Student's *t*-test values for the yield factor (Y%). According to the Student's *t*-test results, the most important factor was the initial lipase concentration (x_2), which had a highly significant effect (99% - confidence level). The effects of temperature (x_1), molar ratio (x_3) and interaction (x_2x_3) were also significant ($p < 0.05$).

Table 5 also reveals that while substrate molar ratio (x_3) had a significant positive effect ($p < 0.05$), temperature exerted a negative influence on ester yield. The influence of temperature is in agreement with thermal stability data for this immobilized lipase preparation in aqueous medium (Pereira et al, 2001). The results also suggest that the PCB lipase, like other preparations, is greatly influenced by substrate molar ratio in formation of the product. For example, studying the same reaction system with Lipozyme as the catalyst, Castro et al. (1997) verified that the molar ratio between butanol and butyric acid was a critical factor for attaining a high yield of butyl butyrate, requiring an amount of butyric acid on the order of 1.5 times that of butanol.

The main effects were fitted by multiple regression analysis to a linear model, and the best fitting response function can be demonstrated by Eq. (1).

$$\hat{y} = 45.39 - 4.34\, x_1 + 15.86\, x_2 + \\ +4.33\, x_3 - 2.41 x_1.x_3 \qquad (1)$$

where $\hat{y}$ is ester yield (%) and x_1, x_2 and x_3 are temperature, lipase loading and molar ratio, respectively.

The statistical significance of this model was evaluated by the F-test (Table 6), which revealed that this regression is statistically significant at a 99% - probability level. The model did not show lack of fit, and the determination coefficient (R^2=0.99) indicates that the model can explain 99% of the variability.

According to this study, the maximum ester yield can be obtained at the lowest level of temperature (37°C), highest level of lipase concentration (0.5 grams) and middle level of initial acid/alcohol molar ratio (1.5). The run conducted to study particular conditions arising from the results of the experimental design attained a concentration of 26 grams butyl butyrate/L, which corresponds to a yield of 83%. A detailed presentation of the optimum value predicted from the results using the response surface model is given in Figure 6.

Further experimental runs were also carried out by testing a large range of carboxylic acid (from C_2 to C_{12}) with a variety of alcohols (from C_2 to C_{10}). The analysis in Table 7 shows that after 24 h, the different acids tested (C_2 to C_{12}) achieved similar conversions of butanol in the range of 40-45%. The only exception occurred for acetic acid, and in this case butanol was not consumed. This is in agreement with data reported by Oguntimein et al. (1995) and Castro et al. (1997) who used Lipozyme (immobilized lipase preparation manufactured by Novozymes). The presence of acetic acid in the reaction medium may cause modification of the original hydration state of the enzyme or of its structure, resulting in partial or total inhibition of the immobilized enzyme. Table 7 also shows that the different alcohols (C_2 to C_{10}) resulted in butyric acid conversions higher than 40%, except for ethanol in which case only 26% of the butyric acid was converted. This result can probably be explained by the high dehydration capacity of ethanol, which causes similar but weaker effects than acetic acid.

Operational Stability

The immobilized lipase was used repeatedly in hydrolytic and synthetic batch reactions (Figures 7 a-b). In the olive oil hydrolysis (10 min/ 37°C) a half-life of 4.34 hours was observed (Figure 7a). For the esterification reaction of butanol with butyric acid (24 hours/ 37°C), a slow decrease in the esterification activity was verified with a total reduction of 83% at the end of the seventh recycle, corresponding to a half-life ($t_{1/2}$) of 86 hours (Figure 7b).

The contrasting behavior for hydrolytic and synthetic activities suggests that desorption of the enzyme from the support occurred only in aqueous medium. In organic medium it appears that reactants and product bind to the solid enzyme phase, resulting in drastic changes in the enzyme esterification activity for the next cycle. Therefore, techniques allowing the removal of these potential inhibitors from the enzyme solid phase are expected to improve the operational stability of the immobilized lipase on chitosan beads. In this work, hexane was used as a solvent based on previous studies that demonstrated its feasibility to dehydrate immobilized lipase preparations for consecutive batch runs (Castro et al, 1999). Further research should be carried out in order to study other dehydration techniques (Yahya et al, 1998) such as sparging air through the enzyme, use of molecular sieves or washing with polar solvents (e.g., acetone).

Table 4: Matrix for a 2^3 full factorial design and experiment results

Run	X_1	X_2	X_3	T(°C)	[E] (g)	MR	Y(%)
1	-	-	-	37	0.25	1	26.92
2	+	-	-	50	0.25	1	23.10
3	-	+	-	37	0.50	1	57.70
4	+	+	-	50	0.50	1	53.80
5	-	-	+	37	0.25	2	38.50
6	+	-	+	50	0.25	2	26.90
7	-	+	+	37	0.50	2	73.10
8	+	+	+	50	0.50	2	57.70
9	0	0	0	43	0.37	1.5	48.15
10	0	0	0	43	0.37	1.5	46.70
11	0	0	0	43	0.37	1.5	46.70

MR: initial acid/alcohol molar ratio

Table 5: Estimated effects, standard errors and Student's *t*-test for ester yield (Y%) using the 2^3 factorial design

Factors	Effects	Standard errors	*t*-values	p-values
Average	45.38	± 0.61	74.28	
Temperature (x_1)	-8.68	± 1.43	-6.06	0.037[b]
Enzyme (x_2)	31.72	± 1.43	22.13	0.000[a]
Molar ratio (x_3)	8.67	± 1.43	6.05	0.037[b]
x_1x_2	-0.97	± 1.43	-0.67	0.53
x_1x_3	-4.68	± 1.43	-3.36	0.028[b]
x_2x_3	0.98	± 1.43	0.68	0.33

[a] $p < 0.01$ [b] $p < 0.05$

Table 6: Analysis of variance of the model regression

Source	SS	DF	MS	F-value	P
Model	2360.00	4	589.99	175.21	0.000
Residue	20.20	6	3.36		
Lack of fit	15.02	2	7.5	10.72	0.08
Pure error	1.42	2	0.7		
Total	2380.20				

R^2= 0.991
SS = Sum of Squares; DF = Degree of Freedom;
MS = Mean Square

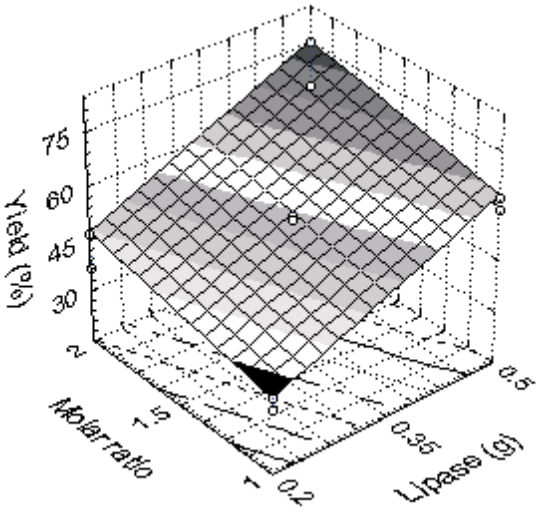
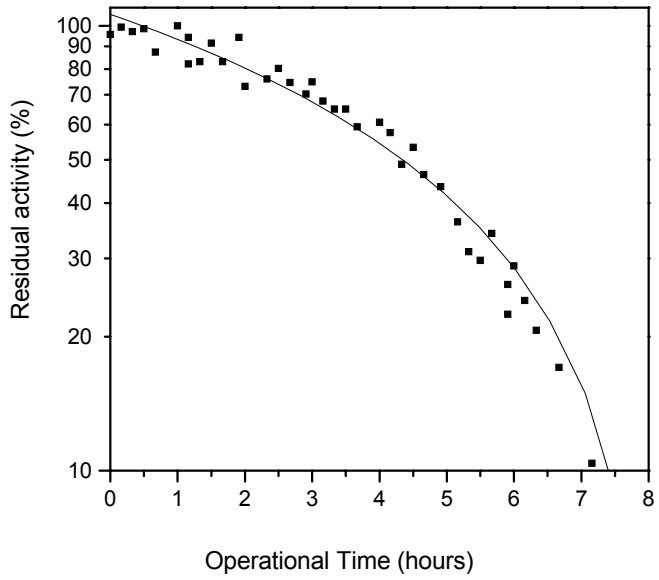


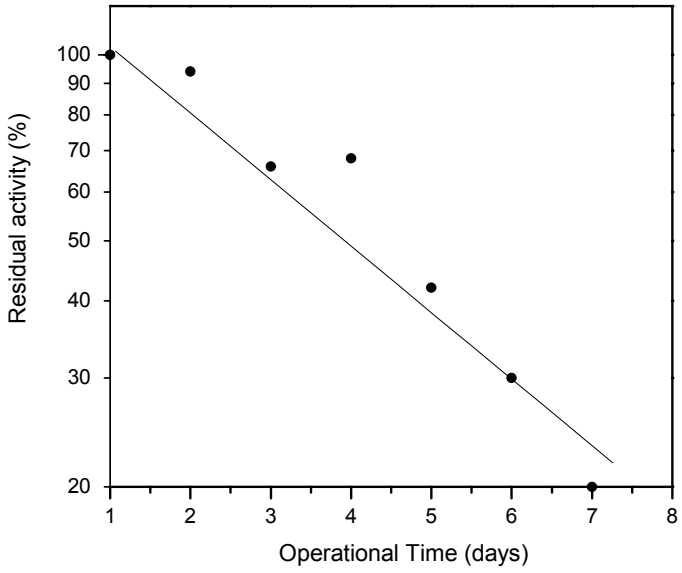
Figure 6: Response surface described by the model $\hat{y}$ that represents the ester yield in the formation of butyl butyrate catalyzed by PCB lipase as a function of lipase of amount (x_2) and molar ratio (x_3).

Table 7: Percentage of molar conversion of n-butanol with carboxylic acids and of butyric acid with aliphatic alcohols

Molar Conversion (%)	**Carbon number**						
	Carboxylic acid						
Butanol	C2	C4	C7	C8	C9	C10	C12
	--	40.74	40.80	45.10	43.87	45.70	45.12
Butyric acid	**Alcohol**						
	C2	C4	C6	C8		C10	
	26.41	49.10	47.15	47.35		45.04	



(7a)



(7b)

Figure 7: Batch operational stability tests for PCB lipase in aqueous (A) and organic (B) media. Initial activities A (68.4 units.mg^{-1}) and B (250 μmol.mg^{-1}.min^{-1}) were defined as 100%.

CONCLUSIONS

Selection of an immobilization strategy is based on process specifications for the catalyst, including such parameters as overall enzymatic activity, effectiveness of lipase utilization, deactivation and regeneration characteristics, cost of the immobilization procedure, toxicity of immobilization reactants and the desired final properties of the immobilized derivative. Some types of support may impose limitations for application of the immobilized lipase on either hydrolysis or esterification reactions.

Enzyme immobilization by physical adsorption traditionally refers to binding of the enzymes via weak attraction forces to an inert carrier that has not been chemically derived. Since the carrier is directly involved in binding to the enzyme, both morphological and physical characteristics play important roles. In the present work, flakes and porous chitosan beads were used as the support for immobilizing lipase from different sources. PCB showed a favorable configuration for immobilizing both lipase preparations by maintaining the enzyme´s conformation and its active site, since no chemical species were involved in the adsorption step.

The successful adsorption of microbial lipase on PCB was achieved by placing the powder enzyme on chitosan polymer previously soaked in hexane. The loading 120 units.g^{-1} support was shown to be effective, resulting in a uniform load of enzyme with satisfactory degree of enzyme fixed. The experimental results show that free lipase has an optimum pH of 7.0 at 40°C temperature, while the immobilized enzyme at the same temperature has an optimum pH of 6.0. The thermal stability of the immobilized lipase was higher than that of the free one. Kinetic parameters obtained based on the activity curve as a function of substrate concentration indicated that the free enzyme obeys the Michaelis-Menten equation, while PCB lipase obeys a typical substrate inhibition kinetics. In addition, reduction in activity can still be related to diffusion limitations.

The experimental design has been demonstrated to be effective in the study of the variables in the formation of butyl butyrate. A response equation has been obtained for the ester yield. From this, it was possible to predict the operation conditions required to obtain a defined amount of ester. Initial amount of biocatalyst and substrate molar ratio have positive influences on synthesis, while temperature and molar ratio-temperature interaction have negative influences on the process. The most significant effect was that of initial amount of biocatalyst, although there are other factors, such as temperature and substrate molar ratio, which should be considered.

The immobilized system with an average activity of 50 U.mg^{-1} dry support was shown to have suitable catalytic properties for carrying out both hydrolysis (hydrolysis of olive oil) and esterification reactions (butyl butyrate synthesis or similar esters) as summarized in Table 8.

Table 8: Properties of *Candida rugosa* immobilized on porous chitosan beads

Parameters	Values
Activity ($U.mg^{-1}$)	51.4
Water content (%)	20
Optimum pH	6.0
Optimum temperature (°C)	45
Energy of activation ($kcal.mol^{-1}$)	7.26
Thermal inactivation constant (K_d, h^{-1}) at 50°C	0.63
Energy of deactivation ($kcal.mol^{-1}$)	29.62
K_m (mM)	288.5
V_{max} ($\mu M.mg^{-1}.min$)	54.5
Olive oil hydrolysis, half-life (h)	4.34
Esterification reaction, half-life (h)	86

ACKNOWLEDGMENTS

The authors gratefully acknowledge the financial support received from CAPES and FAPESP.

REFERENCES

Box, G.E.P., Hunter, W.G. and Hunter, J.S. in Statistics for Experimenters: An Introduction to Design, Data Analysis and Model Building, Wiley & Sons Inc., New York, 653 (1978).

Bradford, M. M.A., Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein utilizing the Principle of Protein-Dye Binding, Analytical Biochemistry, 72, 248-254 (1976).

Carneiro da Cunha, M.G., Rocha, J. M. S., Garcia, F.A.P. and Gil, M. H., Lipase Immobilization on to Polymeric Membranes, Biotechnology Techniques, 13, 403-409 (1999).

Castro, H.F., Oliveira, P.C., Soares, C.M.F. and Zanin, G. M., Immobilization of Lipase on Celite for Application in the Synthesis of Butyl Butyrate in Nonaqueous System, Journal American Oil Chemist's Society, *76*, 147-152 (1999).

Castro, H.F., Oliveira, P.C. and Soares, C.M.F., Parâmetros Reacionais para a Síntese Enzimática do Butirato de Butila em Solventes Orgânicos, Ciência e Tecnologia de Alimentos, 17 (3), 237-241 (1997).

Castro, H.F., Oliveira, P.C. and Pereira, E.B., Evaluation of different Strategies for Lipase Catalysed Synthesis of Citronellyl Acetate, Biotechnology Letters, 9, 229-232 (1997).

Dordick, J.S., Enzymatic Catalysis in Monophasic Organic Solvents, Enzyme and Microbial Technology, 11 (4), 55-56 (1989).

Faber, K., Biotransformation in Organic Chemistry: A Textbook, Springer-Verlag, Berlin (1997).

Felse, P.A. and Panda, T., Studies on Applications of Chitin and its Derivatives, Bioprocess Engineering, 20, 505-512 (1999).

Fonseca, L.P., Cardoso, J.P. and Cabral, J.M.S., Immobilization Studies of an Industrial Penicillin Acylase preparation on a Silica Carrier, Journal Chemical Technology and Biotechnology, 58 (1), 27-37 (1993).

Frense, D., Lange, U. and Hartmeier, W., Immobilization of *Candida rugosa* lipase in Lyotropic Liquid Crystals and Some Properties of the Immobilized Enzyme, Biotechnology Letters, 18, 293-298 (1996).

Itoyama, K., Tokura, S. and Hayashi, T., Lipoprotein Lipase Immobilization onto Porous Chitosan Beads, Biotechnology Progress, 10, 225-229 (1994).

Krajewska, B., Chitin and its Derivatives as Supports for Immobilization of Enzymes, Acta Biotechnologica, 11 (3), 269-277 (1991).

Malcata, F.X., Reyes, H.R., Garcia, H.S., Hill, C.G. and Amundson, C.H., Immobilized Lipase Reactors for Modification of Fats and Oils – A Review, Journal American Oil Chemist's Society, 67 (12), 890-910 (1990).

Oguntimein, G.B., Anderson, W.A. and Moo-Young, M., Synthesis of Geraniol Esters in a Solvent-Free System Catalyzed by *Candida antarctica* Lipase, Biotechnology Letters, 17, 77-82 (1995).

Pereira, E.B., de Castro, H.F., de Moraes, F.F. and

Zanin, G.M., Esterification Activity and Stability of *Candida rugosa* Lipase Immobilized into Chitosan, Applied Biochemistry Biotechnology, 98/100, 977-986 (2002).

Pereira, E.B., de Castro, H.F., de Moraes, F.F. and Zanin, G.M., Kinetic Studies of Lipase from *Candida rugosa:* A Comparative Study of the Free and the Immobilized Enzyme on Porous Chitosan Beads, Applied Biochemistry Biotechnology, 91/93, 739-752 (2001).

Pereira, E.B., Lipase Livre e Imobilizada em Quitosana: Caracterização e Potencial de Aplicação em Reações de Hidrólise e Síntese, Master´s thesis, Universidade Estadual de Maringá, 127p (1999).

Rosu, R., Iwasaki, Y., Shimizu, N., Doisaki, N. and Yamane, T., Intesification of Lipase Performance in a Transesterification Reaction by Immobilization on $CaCO_3$ Powder, Journal of Biotechnology, 66, 51-59 (1998).

Soares, C.M.F., de Castro, H.F., de Moraes, F.F. and Zanin, G. M., Characterization and Utilization of *Candida rugosa* Lipase Immobilized on Controlled Pore Silica, Applied Biochemistry and Biotechnology, 77/79, 745-757 (1999).

Tantrakulsiri, J., Jeyashoke, N. and Krisanangkura, K., Utilization of Rice Hull Ash as Support Material for Immobilization of *Candida cylindracea* Lipase, Journal of American Oil Chemist's Society, 74, 173-175 (1997).

Tischer, W. and Wedekind, F., Immobilized Enzymes: Methods and Applications, Biocatalysis from Discovery to Application Topics in Current Chemistry, 200, 95-126 (1999).

Yahya, A. R. M., Anderson, W. A. and Moo-Young, M., Ester Synthesis in Lipase-catalyzed Reactions, Enzyme and Microbial Technology, 23, 438-450 (1998).