0273-2289/02/98-100/0977/$12.50

# Esterification Activity and Stability of *Candida rugosa* Lipase Immobilized into Chitosan

**Ernandes B. Pereira,[1] Heizir F. de Castro,[2] Flávio F. De Moraes,[1] and Gisella M. Zanin*,[1]**

*[1]Chemical Engineering Department, State University of Maringá, Av. Colombo 5790, BL D-90, 87020-900, Maringá-PR, Brazil, E-mail: gisellazanin@maringa.com.br; and [2]Department of Chemical Engineering, Faculty of Chemical Engineering of Lorena, P.O. Box 116; 12600-970; Lorena-SP, Brazil*

## Abstract

Microbial lipase from *Candida rugosa* immobilized into porous chitosan beads was tested for esterification selectivity with butanol and different organic acids (C2–C12), and butyric acid and different aliphatic alcohols (C2–C10). After 24 h, the acids tested achieved conversions of about 40–45%. Acetic acid was the only exception, and in this case butanol was not consumed. Different alcohols led to butyric acid conversions >40%, except for ethanol, in which case butyric acid was converted only 26%. The system's butanol and butyric acid were selected for a detailed study by employing an experimental design. The influence of temperature, initial catalyst concentration, and acid:alcohol molar ratio on the formation of butyl butyrate was simultaneously investigated, employing a $2^3$ full factorial design. The range studied was 37–50°C for temperature ($X_1$), 1.25–2.5% (w/v) for the catalyst concentration ($X_2$), and 1 and 2 for the acid:alcohol molar ratio ($X_3$). Catalyst concentration ($X_2$) was found to be the most significant factor and its influence was positive. Maximum ester yield (83%) could be obtained when working at the lowest level for temperature (37°C), highest level for lipase concentration (2.5% [w/v]), and center level of acid:alcohol molar ratio (1.5). The immobilized lipase was also used repeatedly in batch esterification reactions of butanol with butyric acid, revealing a half-life of 86 h.

**Index Entries:** Lipase; immobilization; chitosan; esterification; activity; stability.

*Author to whom all correspondence and reprint requests should be addressed.

## Introduction

There is a growing interest in using enzymes, especially lipases, in organic media. Because of the inhibitory effects of organic solvents, biocatalyst immobilization is often recommended *(1,2)*. Immobilization also enables the easy separation of the enzyme catalyst from the reaction mixture and can lower the costs of enzymes dramatically *(3)*. In view of the current high cost of some available commercial matrixes, the possibility of using cheap and/or alternative supports for immobilizing lipases such as rice husk *(4)*, $CaCO_3$ powder *(5)*, cellulose *(6)*, chitin, and its derivatives *(7,8)* has been recently considered. Of these, chitosan, a natural product derived from chitin, has several advantages for use as a support, including its lack of toxicity; its chemical reactivity, which allows easy fixation of enzymes; and multiple physical forms *(7)*.

The feasibility of using chitosan as matrix for immobilizing microbial lipases has been previously demonstrated in several studies *(8–11)*, including those developing in our laboratory *(10,11)*. According to Pereira et al. *(11)*, porous chitosan beads showed a favorable configuration for immobilizing *Candida rugosa* lipase by maintaining the enzyme conformation and its active site since no chemical species were involved in the adsorption step. The resulting immobilized lipase on chitosan beads exhibited characteristics that were considered suitable for hydrolysis of oil and fats. Here we report additional results dealing with the characterization of *C. rugosa* lipase immobilized on chitosan in organic medium. The catalytic test was performed with the aim of producing esters by direct esterification reactions between a large range of carboxylic acids (from C2 to C12) and a diversity of alcohols (from C2 to C10). In esterification, the reaction efficiency is dictated by environmental parameters such as enzyme hydration, reaction temperature, substrate concentration, substrate size, mass of lipase, substrate molar ratio, and chemical structure. We have analyzed a number of reaction model systems in order to illustrate the kind of products that can be made by using an experimental preparation of lipase immobilized on chitosan beads. The operational stability of the immobilized lipase was also investigated in batch reactions reusing the enzyme.

## Materials and Methods

### Materials

Microbial (*C. rugosa*) lipase (Type VII) was purchased from Sigma (St. Louis, MO). The chitosan was supplied by SP Chemical Farma (São Paulo, Brazil) with the following characteristics: 93% purity, 6% moisture, and 40-mesh granulometry. Olive oil (low acidity) was purchased at a local market. Alcohols (ethanol, butanol, pentanol, hexanol, heptanol, octanol, and R/S citronellol) and organic acids (acetic, butyric, oenantic, caprylic, pelargonic, capric, and lauric) were purchased from either Merck or Aldrich (Milwaukee, WI). Solvents were standard laboratory grade. Heptane was

dried with metallic sodium and used as solvent for all experiments. Substrates for esterification reactions were dehydrated, with 0.32-cm molecular sieves (aluminum sodium silicate, type 13; X-BHD Chemicals, Toronto, Canada), previously activated in an oven at 350°C for 6 h.

### *Immobilization of Lipase into Porous Chitosan Beads*

Chitosan (20 g) previously soaked in hexane was mixed with 50 mL of lipase aqueous solution (0.1 g/mL) under agitation for 3 h at room temperature, followed by an additional 18 h under static conditions at 4°C. The derivative was filtered and thoroughly rinsed with hexane.

### *Hydrolytic Activities*

Hydrolytic activities of the immobilized lipase were assayed by the olive oil emulsion method *(11)*. The substrate was prepared by mixing 50 mL of olive oil with 50 mL of emulsification reagent. The reaction mixture consisting of 5 mL of the emulsion, 2 mL of 100 m*M* sodium phosphate buffer (pH 7.0), and immobilized lipase (250 mg) was incubated for 5 min at 37°C. The reaction was stopped by adding 10 mL of acetone:ethanol solution (1:1). The liberated fatty acid was titrated with 0.02 *N* potassium hydroxide solution using phenolphthalein as an indicator. One unit of enzyme activity was defined as the amount of enzyme that produces 1 μmol of free fatty acid/min under the assay conditions (37°C, pH 7.0). Hydrolytic activity of the immobilized lipase assayed by this method was found to be 32.6 U/mg (dry support).

### *Esterification Reactions: Influence of Fatty Acid and Alcohol Chain Lengths*

Ester syntheses took place in 100-mL closed flasks containing 20 mL of dry *n*-heptane containing either 0.30 *M* butanol and a 0.45 *M* concentration of several organic acids (acetic, butyric, oenantic, pelargonic, caprylic, and lauric) or a 0.30 *M* concentration of several alcohols (ethanol, *n*-butanol, hexanol, and R/S citronellol) and 0.45 *M* butyric acid. Substrates were preconditioned by incubating the substrate for 30 min with agitation at the reaction temperature prior to the addition of immobilized lipase. The mixtures were incubated with 0.5 g of immobilized lipase (dry wt), under agitation (150 rpm) at 37°C for 24 h. The molar conversion was calculated with reference to butanol or butyric acid taking into account the initial and final concentrations of these reactants as expressed by Eq. 1:

$$\text{Molar Conversion (\%)} = \frac{C_0 - C}{C_0} \times 100 \quad (1)$$

in which $C_0$ is the initial reactant concentration and $C$ is the reactant concentration at a given time.

### Experimental Design

The influence of temperature, initial catalyst concentration, and acid:alcohol molar ratio on the formation of butyl butyrate was simultaneously investigated employing a $2^3$ full factorial design *(12)*. The range studied was 37–50°C for temperature ($X_1$), 1.25–2.5% (w/v) for the catalyst concentration ($X_2$), and 1 and 2 for the acid:alcohol molar ratio ($X_3$). Three experiments were carried out at the center-point level, for estimation of experimental error. All runs were performed at random. The ester yield ($Y$%) was taken as the response of the design experiments. The results were analyzed using Statistica (version 5.0) software.

### Batch Operational Stability Tests

The operational stability of the immobilized system was assayed by using 1.0 g of immobilized lipase (dry wt) and 20 mL of substrate containing 0.30 *M* butyric acid and 0.25 *M* butanol in successive batches (37°C for 24 h at 150 rpm). At the end of each batch, the immobilized lipase was removed from the reaction medium and washed with hexane, in order to remove any substrate or product eventually retained in the matrix. One hour later (the length of time required for evaporation of the solvent), the immobilized lipase was introduced into a fresh medium. Esterification activity was estimated daily and expressed as micromoles of butyl butyrate formed per minute per gram of catalyst. The biocatalyst half-life time ($t_{1/2}$) was determined by applying the inverted linear decay model *(13)*.

### Analytical Methods

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

## Results and Discussion

### Selection of Reaction System

Initial experiments involved the investigation of the effect of chain length of acyl donor on the esterification performance with butanol. Figure 1 displays the molar conversion of butanol as a function of the fatty acid chain length after 24 h. The different tested acids (C4–C12) gave similar molar butanol conversions (40–45%). The only exception was regarding the use of acetic acid, as acyl donor, and in this case there was no conversion of butanol. This was an expected result since acetic acid is considered to be a potent inhibitor of lipase activity. This negative effect can be attributed to

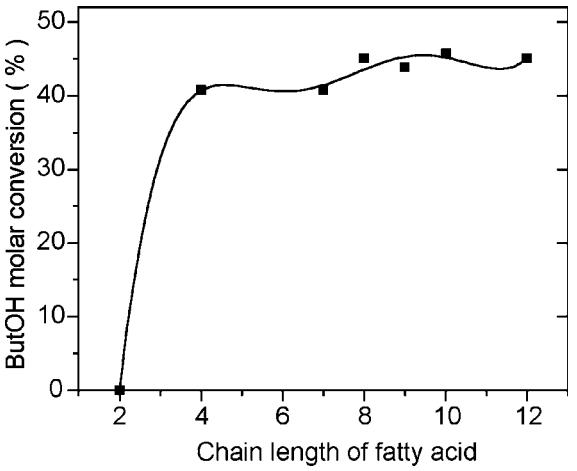


Fig. 1. Influence of fatty acid chain length on molar conversion of butanol (reactions were performed at 37°C for 24 h).

the high polarity of the acetic acid (log $P = -0.23$), and because it is more soluble in aqueous phase than in organic media, most of the acetic acid would be expected to be located in the microaqueous environment of the enzyme. Under such conditions, the acetic acid changes the polarity of the reaction medium, which, in turn, modifies the partitioning of water between the solid phase (enzyme preparation) and the liquid phase (substrate), resulting in its accumulation in the enzyme's solid phase *(14)*. This may reduce the enzyme's local pH. Therefore, the enzyme active site is modified and the reaction becomes nearly impossible. Similar behavior was reported by several researchers *(15,16)* using a commercial immobilized lipase (lipozyme), which supported the dependence of enzyme activity on substrate concentration in the aqueous layer around the catalyst where enzymatic reaction occurs.

The effect of alcohol chain length from ethanol to octanol was verified in the synthesis of butyl esters (Fig. 2). The different alcohols promoted molar conversions of butyric acid >40%, except for the case of ethanol, in which butyric acid presented a molar conversion of only 26%. This result comes probably from the high dehydrating power of ethanol causing similar, but less severe effects than those described previously for acetic acid.

These results likely reflect both the intrinsic selectivity of the enzyme and different accessibility of substrates to the enzyme-active site. In addition, it should be considered that the substrate polarity could also contribute to the reduction in enzyme activity. In such a context, short chain starting materials such as ethanol and acetic acid may display some restrictions to be used on esterification reactions catalyzed by lipase immobilized on chitosan beads. We therefore selected the system consisting of butanol and butyric acid for further study.

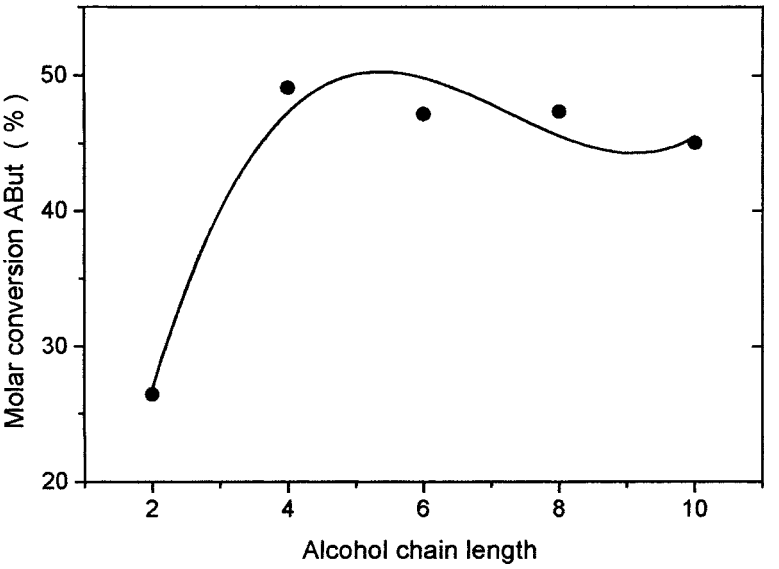


Fig. 2. Influence of alcohol chain length on molar conversion of butyric acid (reactions were performed at 37°C for 24 h).

## *Factorial Design*

The effects of different experimental variables on the synthesis of butyl butyrate were simultaneously investigated, employing a $2^3$ full factorial design. Three variables (temperature, amount of lipase, and substrate molar ratio) were taken into consideration. The experimental matrix and the results are shown in Table 1.

As can be seen in Table 1, the ester yield ($Y$%) varied strongly (from 26.9 to 73.1%). Table 1 also shows that, independently of the other variables, increasing the lipase level from 1.5 to 2.5% (w/v) substantially increased the ester yield from 26.9 to 57.7% (run 3). A further increase in the 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 (73.1%). Therefore, all three factors studied, temperature ($X_1$), lipase level ($X_2$), and substrate molar ratio ($X_3$), seem to have played a critical role in the synthesis of butyl butyrate. Table 2 summarizes results of the statistical analyses and shows SEs 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 presented 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 2 also reveals that while substrate molar ratio ($X_3$) had a positively significant effect ($p<0.05$), temperature exerted a negative influence on the ester yield. The influence of temperature is in agreement with thermal stability data for this immobilized lipase preparation in aqueous medium *(11)*. The

Table 1
Matrix for a $2^3$ Full Factorial Design and Experiment Results

| Run | $X_1$ | $X_2$ | $X_3$ | $T$ (°C) | E[b] (% w/v) | MR[a] | $Y$ (%) |
|---|---|---|---|---|---|---|---|
| 1 | – | – | – | 37 | 1.5 | 1 | 26.9 |
| 2 | + | – | – | 50 | 1.5 | 1 | 23.1 |
| 3 | – | + | – | 37 | 2.5 | 1 | 57.7 |
| 4 | + | + | – | 50 | 2.5 | 1 | 53.8 |
| 5 | – | – | + | 37 | 1.5 | 2 | 38.5 |
| 6 | + | – | + | 50 | 1.5 | 2 | 26.9 |
| 7 | – | + | + | 37 | 2.5 | 2 | 73.1 |
| 8 | + | + | + | 50 | 2.5 | 2 | 57.7 |
| 9 | 0 | 0 | 0 | 43 | 2 | 1.5 | 48.1 |
| 10 | 0 | 0 | 0 | 43 | 2 | 1.5 | 46.7 |
| 11 | 0 | 0 | 0 | 43 | 2 | 1.5 | 46.7 |

[a]Initial acid:alcohol molar ratio.
[b]Enzyme concentration.

Table 2
Estimated Effects, Standard Errors and Student's *t*-Test for Ester Yield ($Y$%) Using $2^3$ Factorial Design

| Factor | Effect | SE | *t*-Value | *p*-Value |
|---|---|---|---|---|
| Average | 45.4 | ± 0.6 | 74.3 | — |
| Temperature ($X_1$) | −8.7 | ± 1.4 | −6.1 | 0.037[b] |
| Enzyme ($X_2$) | 31.7 | ± 1.4 | 22.1 | 0.000[a] |
| Molar ratio ($X_3$) | 8.7 | ± 1.4 | 6.1 | 0.037[b] |
| $X_1X_2$ | −0.97 | ± 1.4 | −0.7 | 0.53 |
| $X_1X_3$ | −4.7 | ± 1.4 | −3.4 | 0.028[b] |
| $X_2X_3$ | 0.98 | ± 1.4 | 0.7 | 0.33 |

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

results also suggest that the chitosan-immobilized lipase, like other preparations, is strongly influenced by the substrate molar ratio on the formation of the product. The main effects were fitted by multiple regression analysis to a linear model, and the best fitting response function can be written by Eq. 2:

$$\widehat{y} = 45.4 - 4.3\ X_1 + 15.9\ X_2 + 4.3\ X_3 - 2.4\ X_1 \cdot X_3 \qquad (2)$$

in which $\widehat{y}$ is the ester yield (%); and $X_1$, $X_2$, and $X_3$ represent temperature, lipase loading, and molar ratio, respectively.

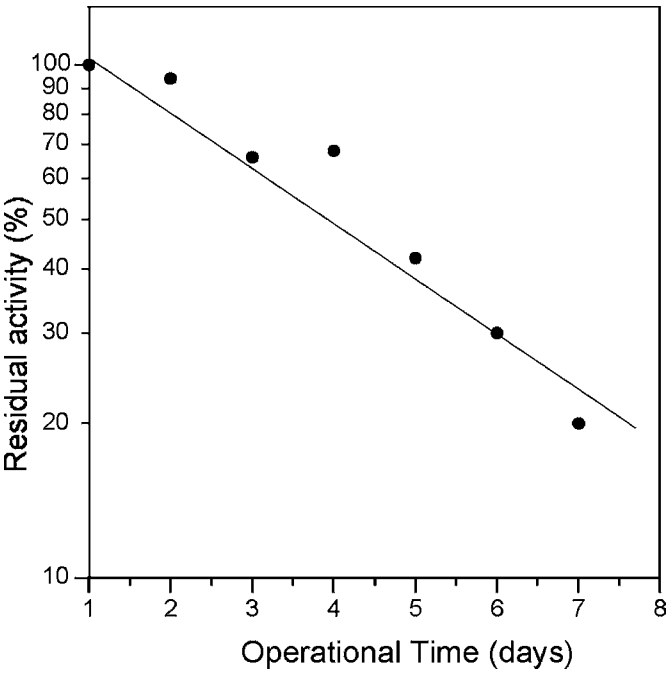


Fig. 3. Batch operational stability test for immobilized lipase preparation. Residual activity is plotted against operational time taking the esterification activity of 109 μmol/(g·m) as 100%. Esterification assay was carried out with substrate containing 250 m*M* butanol and 300 m*M* butyric acid in heptane. Initial hydrolytic activity was 32.8 U/mg.

The statistical significance of this model was evaluated by the F-test, which revealed that this regression is statistically significant at the 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, working at the lowest level for temperature (37°C), highest level for lipase concentration (2.5%), and center level of initial acid:alcohol molar ratio (1.5). Further experiments were performed to study particular conditions arising from the results of the experimental design and a concentration of 26 g of butyl butyrate/L was attained, which corresponds to a yield of 83%.

## *Operational Stability*

The stability of the immobilized system was also assessed by reusing the immobilized lipase seven times in the synthesis of butyl butyrate. The synthesis was followed by evaluating the formation of butyl butyrate per gram of biocatalyst per minute (esterification activity). Figure 3 presents the residual activity as a function of operational time. There was a slow

decrease in the esterification activity with a total reduction of 83% at the end of the seventh recycle, giving a half-life time ($t_{1/2}$) of 86 h. This marked decrease, however, was not observed in relation to hydrolytic activity, which was reduced only 28% (residual hydrolytic activity was 23.8 U/mg, corresponding to a $t_{1/2}$ of 351 h). The contrasted behavior regarding synthetic and hydrolytic activity suggested that no desorption of the enzyme from the support occurred. It is therefore likely that reactants and product bind to the solid enzyme phase, resulting in drastic changes in the enzyme esterification activity for the next cycle. In this sense, techniques allowing the removal of these potential inhibitors from the enzyme's solid phase are expected to improve the operational stability of the immobilized lipase on chitosan beads.

In our work, hexane was used as a solvent based on previous studies that demonstrated the feasibility of this solvent to dehydrate immobilized lipase preparations for consecutive batch runs *(17)*. Further studies must be carried out in order to investigate other dehydration techniques such as air sparging, the use of molecular sieves, or washing with polar solvents (such as acetone).

## Conclusion

The present work was carried out to investigate further the characteristics of an experimental preparation of lipase immobilized on porous chitosan beads. The use of this preparation was extended to the direct esterification of a large range of carboxylic acids (from C2 to C12), with a diversity of alcohols (from C2 to C10). With the exception of acetic acid and ethanol, the size of the carbon chain showed no significant influence on the esterification rates. Acids and alcohols containing four or more carbons were considered to be suitable reactants resulting in high esterification rates. The experimental design was demonstrated to be effective in the study of the variables on the formation of butyl butyrate. A response equation was obtained for the yield of ester. From this, it was possible to predict the operation conditions required to obtain a defined amount of ester. The initial amount of biocatalyst and substrate molar ratio had positive influences on the synthesis, while temperature and molar ratio–temperature interaction had negative influences on the process. The most significant effect was the initial amount of biocatalyst, although there are other factors, such as temperature and substrate molar ratio, that should be considered.

Concerning the operational stability, a rapid decrease in the conversion was observed when immobilized lipase was repeatedly reused using hexane to dehydrate the catalyst prior to the subsequent batch. This decrease appears to be related not only to the water formed, but also to an interaction with the reaction product (ester). The application of a more polar solvent to extract the inhibiting factors should be investigated as a potential solution to the problems associated with the repeated-batch use of immobilized lipase on chitosan beads.

## Acknowledgments

We gratefully acknowledge the Conselho Nacional de Desenvolvimento Científico e Tecnológico, the Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior, and the Fundação de Amparo à Ciência do Estado de São Paulo for their financial support.

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