0273-2289/02/98-100/0863/$13.00

Intensification of Lipase Performance for Long-Term Operation by Immobilization on Controlled Pore Silica in Presence of Polyethylene Glycol

Cleide M. F. Soares,[1,2] Heizir F. De Castro,*[,1] M. Helena A. Santana,[2] and Gisella M. Zanin[3]

[1] Department of Chemical Engineering, Faculdade de Engenharia Química de Lorena, PO Box 116, 12600-970, Lorena-SP, Brazil, E-mail: heizir@dequi.faenquil.br; and [2] Department of Biotechnological Processes, Faculdade de Engenharia Química, UNICAMP, PO Box 6066, 13081-900, Campinas-SP, Brazil; and [3]Department of Chemical Engineering, Universidade Estadual de Maringá, 87020-900, Maringá-PR, Brazil

Abstract

In agreement with previous studies, promising results were obtained when lipase was immobilized on controlled pore silica (CPS) in the presence of polyethylene glycol (PEG 1500). This methodology rendered immobilized derivatives with higher operational stability than those lacking PEG 1500. This article extends the scope of this approach by evaluating the combined effects of PEG concentration and lipase loading employing a multivariate statistical approach. A 2^2 factorial design with center point was adopted for a full understanding of these effects and their interactions. Conditions that maximize the immobilization yield were different from those attained for the biocatalyst's operational stability. Possible reasons for the increase in both activity and stability of lipase immobilized on CPS in the presence of PEG 1500 are discussed in light of the influence of surface hydrophilic/hydrophobic balance.

Index Entries: Immobilization; lipase; controlled pore silica; stability; experimental design.

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

Introduction

The selection of an immobilization strategy or modification procedure is based on process specifications for the biocatalyst, which include such parameters as overall enzymatic activity, effectiveness of utilization, deactivation and regeneration characteristics, cost of the immobilization procedure, toxicity of immobilization reagents, and the desired final properties of the immobilized lipase *(1,2)*. The current price of lipases is about one order of magnitude higher than the energy costs associated with standard processes. An immobilized lipase preparation with a high operational stability reduces the operational costs and makes lipase-catalyzed reactions more competitive. By appropriate choice of the immobilization support, it is possible to protect the lipase against inactivation effects ascribed to the organic solvent, impurities, substrates, and/or reaction products *(3)*.

Because of their good mechanical strength and chemical functionality, silica-based carriers and their derivatives with well-defined pore size are popular matrixes for immobilizing several enzymes *(4–6)*. This support has also been used successfully for the immobilization of microbial lipase by covalent binding *(7)*. In accordance with this methodology, silica is functionalized via silane coupling with γ-aminopropyltriethoxysilane (γ-APTS) followed by glutaraldeyde activation *(5–7)*. The surface activation process with γ-APTS seems to reduce the original activity on the carrier, and the addition of stabilizing additives is usually recommended to avoid enzymes from aggregation effects or denaturation that occurs owing to the presence of silane precursors used in the formation of the matrix *(8–10)*.

Studies involving silica as support for immobilizing microbial lipase in the presence of several additives were recently developed in our laboratory *(11,12)*. Proteins (albumin and lecithin) and organic molecules (β-cyclodextrin and polyethylene glycol, [PEG]) were screened in order to select the most effective additive, and the best stabilizing effect was found when PEG was used. The immobilized system performed well in esterification reactions under repeated batch cycles (for the synthesis of butyl butyrate as a model), and the biocatalyst half-life was found to be 147 h, five fold higher than for the control (immobilized lipase lacking PEG) *(8)*.

Similar results were found by Stark and Holmberg *(11)*, who compared the behavior of lipase from *Rhizopus* sp. covalently bonded on activated silica in the presence and absence of PEG 1500. Their results show that PEG 1500 had an important effect on the hydrolytic activity. Direct coupling gave an activity of immobilized lipase of 28–34% of that of the free enzyme, whereas coupling with the PEG 1500 exhibited 56–57% activity.

Stabilization of proteins with PEG is well known. This polymer has been shown to stabilize several enzymes with an effect, that increases with increasing concentration and chain length *(12)*. The terminal hydroxyl groups of PEG molecule also provide a ready site for covalent attachment to other molecules and surfaces. In this case, PEG molecules can be used to increase the stability of the immobilized enzyme on support. The PEG of

low molecular weight, in particular, is a good stabilizing additive for lipase immobilization *(9,11)*, because of its ability to create a favorable microenvironment for the catalysis of synthesis reactions with hydrophobic substrates. Moreover, it shows high affinity for the lipase from crude aqueous solutions and high levels of activity retention after immobilization, with a successful application as biocatalysts in the synthesis of esters from carboxylic acids and in the interesterification of triglycerides *(2,11)*.

Based on previous studies, lipase loading and PEG concentration were identified as the major factors affecting the activity and stability of the immobilized lipase on controlled pore silica (CPS) *(8)*. The present article explores the effect of introducing a very long hydrophilic spacer arm, PEG 1500, on the lipase immobilization process on CPS with the aim of improving the lipase performance for long-term operation. Statistical optimization was carried out to attain optimal values for these experimental factors. A 2^2 full factorial design was adopted in this investigation *(13)*. The coupling yield and biocatalyst half-life were evaluated as a function of PEG 1500 concentration and lipase loading.

Material and Methods

Materials

Commercial *Candida rugosa* lipase (Type VII) was purchased from Sigma (St. Louis, MO). The lipase was a crude preparation with a nominal specific activity of 1440 U/mg of protein based on the Bradford method *(14)*. CPS was supplied by Corning, with the following characteristics: average particle porosity (ε) of 0.566, particle matrix density (ρs) of 2.178 g/cm^3, particle density (dry) (ρp) of 0.948 g/cm^3, particle size of 30 mesh (0.59 mm) containing pores of 375Å *(7,8)*. Silane γ-APTS, glutaraldehyde (25% solution), and PEG 1500 were from Sigma. Olive oil (low acidity) was purchased at a local market. Substrates for esterification reactions (*n*-butanol and butyric acid were from Merck) were dehydrated, with 0.32-cm molecular sieves (aluminum sodium silicate, type 13; X-BHD Toronto, Canada). Solvents were standard laboratory grade, and other reagents were purchased from either Aldrich (Milwaukee, WI) or Sigma.

Immobilization of Lipase on CPS

Lipase was immobilized by being covalently bound on CPS previously treated with γ-APTS, followed by the reaction of the pretreated beads with glutaraldehyde solution, according to the procedure previously described *(7)*. For each gram of CPS (dry wt), suitable amounts of enzyme (0.05–0.13 g) and PEG 1500 (2.5–7.5 mg) were dissolved in distilled water and simultaneously mixed with the support under low stirring for 2 h at room temperature. Then, 10 mL of hexane was added, and the mixture of enzyme-support-additive was incubated overnight at 4°C *(8)*. The immobilized lipase was filtered (Whatman filter paper 41) and thoroughly rinsed

with hexane. Analyses of hydrolytic activities carried out on the lipase loading solution, and immobilized preparations were used to determine the coupling yield (η%) according to Eq. 1:

$$\eta\ (\%) = \frac{Us}{U_0} \times 100 \quad (1)$$

in which *Us* is the total activity recovered on the support, and U_0 is the units offered for immobilization.

Hydrolytic Activities

Hydrolytic activities of free and immobilized lipase were assayed by the olive oil emulsion method *(7)*. The substrate was prepared by mixing 50 mL of the 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 either free (1 mL of lipase, 5 mg/mL) or immobilized (100–250 mg) lipase was incubated for 5 min at 37°C. The reaction was stopped by adding of 10 mL of acetone:ethanol solution (1:1). The liberated fatty acid was titrated with 25 m*M* 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).

Protein Assay

Protein was determined according to Bradford's method *(14)* using bovine serum albumin as the standard. The amount of bound protein was determined indirectly by calculating the difference between the amount of protein introduced into the coupling reaction mixture and the amounts of protein in the filtrate and washing solutions.

Esterification Reactions

Reaction systems consisted of heptane (20 mL), *n*-butanol (250 m*M*), butyric acid (250 m*M*), and immobilized lipase (1.0 g, dry wt). The mixture was incubated at 37°C for 24 h with continuous shaking at 150 rpm. The remaining butanol and the butyl butyrate formed were determined by gas chromatography using a 6-ft 5% DEGS on a Chromosorb WHP, 80/10 mesh column (Hewlett Packard, Palo Alto, CA) and hexanol as internal standard. Esterification activity was expressed as micromoles of butyl butyrate formed/(min·g of dry support) *(15)*.

Operational Stability

Operational stability was assayed by using 1.0 g (dry wt) of immobilized lipase in successive batches under the same conditions as described for esterification reactions. Twenty-four hours after starting each batch, the immobilized lipase was removed from the reaction medium and rinsed with heptane, in order to extract any substrate or product eventually

Table 1
Experimental Range and Levels of Independent Process Variables According to 2^2 Full Factorial Design.

		Levels		
Variable	Symbols	–1	0	+1
PEG 1500 (mg/g)	x_1	2.5	5.0	7.5
Lipase loading (U/g)	x_2	90	165	230

retained in the matrix. One hour later (length of time required for the solvent to evaporate), the immobilized derivative was introduced into a fresh medium *(7,8)*. The same lipase preparation was reused for 10 consecutive days. Esterification activities were daily evaluated and plotted as a function of the operational time. Half-life ($t_{1/2}$) for each immobilized preparation was estimated according to Eq. 2 *(4)*:

$$t_{1/2} = \frac{0.693}{K_d} \tag{2}$$

in which $t_{1/2}$ is the half-life, and K_d is the deactivation constant.

Experimental Design

The influence of PEG concentration (x_1) and lipase loading (x_2) was studied using a 2^2. full factorial design. The coupling yield (η%) and biocatalyst half-life ($t_{1/2}$) were taken as responses of the design experiments. The range and levels of the studied variables are given in Table 1. Runs were performed at random. Two experiments were carried out at the center-point level, for estimation of experimental error. Data processing and calculations were carried out using Statistica (version 5.0) software. The statistical significance of the regression coefficients was determined by student's *t*-test *(13)*; the model equation was determined by Fisher test *(13)*; and the proportion of variance explained by the model obtained was given by the multiple coefficient of determination, R^2.

Results

The levels of PEG 1500 and lipase loading studied were 2.5–7.5 mg/g and 90–230 U/g, respectively (Table 1). The levels of the chosen variables were made based on our previous results *(8)*. Coupling yields (η%) were calculated according to the data given in Table 2, and values for biocatalyst half-life were estimated by Eq. 2 taking into account the results plotted in Fig. 1.

The experimental matrix and both response factors are shown in Table 3. The coupling yield and biocatalyst half-life varied strongly (from 30 to 59.9% and from 119 to 417 h). Table 3 also shows that, independently of the PEG concentration, increasing the lipase loading from 90 to 230 U/g

Table 2
Coupling Yield and Catalytic Activities
of *C. rugosa* Lipase Immobilized on CPS in Presence of PEG[a]

Run	Lipase loading (U/g support)	Bound protein (%)	Coupling yield (%)	Hydrolytic activity (μmol/[mg·min])	Esterification activity (μmol/[g·min])
1	90	97.2	58.5	53.9	56
2	90	93.8	59.9	49.2	60
3	230	95.3	30.7	69.8	165
4	230	94.0	48.2	117.6	154
5	165	81.1	52.9	78.6	135
6	165	78.0	55.3	92.5	134

[a]Esterification activities are expressed as micromoles of butyl butyrate per minute per gram of dry support by following the rate of product formation from the reaction mixture containing butanol and butyric acid.

Table 3
Experimental Design and Results According to the 2^2 Full Factorial Design

	Variable		Response	
Run	x_1 (mg/g support)	x_2 (U/g support)	Coupling yield (%)[a]	Biocatalyst half-life (h)[b]
1	2.5	90	58.5	119
2	7.5	90	59.9	154
3	2.5	230	30.7	170
4	7.5	230	48.2	417
5	5.0	165	52.9	201
6	5.0	165	55.3	164

[a]Calculated according to Eq. 1.
[b]Calculated according to Eq. 2.

decreased the coupling yield. High coupling yield values (>58%) were attained when a minimum level of lipase loading was used (runs 1 and 2). By contrast, the highest biocatalyst half-life value (417 h) was attained when a maximum level of both PEG concentration and lipase loading was used (run 4).

The experimental results shown in Table 3 were employed to estimate the main effects of variables and their interactions. The statistical analyses for both response variables evaluated are summarized in Table 4.

According to the student's *t*-test results, the most important factor was the lipase loading (x_2), since it presented a significant effect (95% confidence level) for both responses. For coupling yield, the effects of PEG concentration (x_1) and interaction (x_1x_2) were also significant, although their p values were high ($0.1 < p < 0.2$). According to Silva and Roberto *(16)*, the

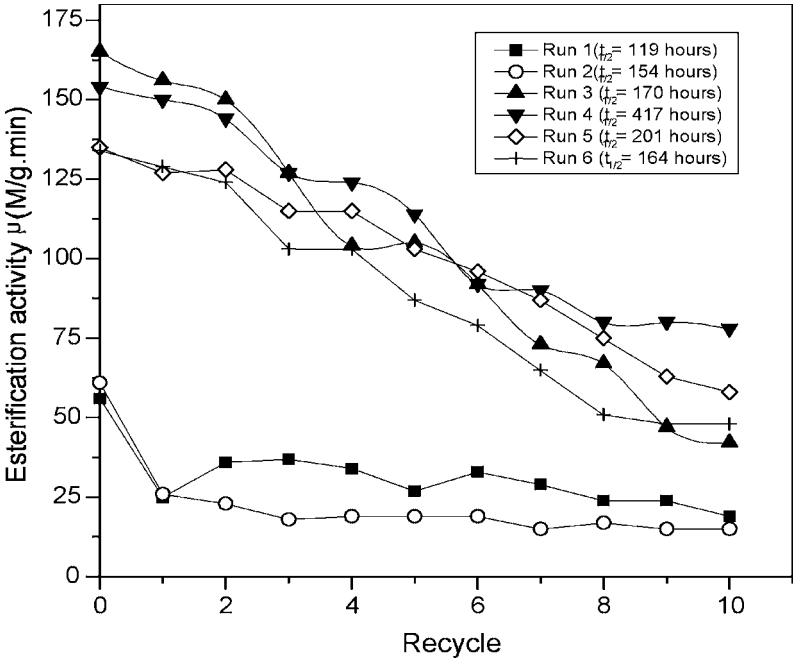

Fig. 1. Batch operational stability test of lipase immobilized on CPS in presence of PEG 1500. Esterification assay was carried out with substrate containing 250 m*M* butanol and 250 m*M* butyric acid in heptane. Initial activities were shown in Table 2 (last column).

Table 4
Estimated Effects, SEs and Student's *t*-test for Immobilization Yield and Biocatalyst Half-life Using 2^2 Full Factorial Design

	Coupling yield (%)			Biocatalyst half-life (h)		
Variable	Effects	SEs	*t*-Value	Effects	SEs	*t*-Value
Mean	50.9	±1.7	30.5	204.2	±13.2	15.5
x_1	9.4	±4.1	2.3[a]	141	±32.4	4.4[b]
x_2	−19.8	±4.1	−4.84[a]	157	±32.4	4.9[b]
$x_1 \cdot x_2$	8.1	±4.1	1.97[a]	106	±32.4	3.3[c]

[a] $p < 0.20$.
[b] $p < 0.05$.
[c] $p < 0.10$.

selection of effects with higher *p* values (*p* value up to 0.2) provides a reasonable assurance of detecting an important effect (protection against a type II error, which results in a false negative).

An important finding in this experiment was that the PEG concentration (x_1) did not have a significant effect on the coupling yield but exerted a positive influence on the biocatalyst half-life. Table 4 also reveals that

Table 5
Analysis of Variance for Model Regression
Representing Coupling Yield and Biocatalyst Half-life

Source	df	Sum of square		Mean square	
		Coupling yield	Biocatalyst half-life	Coupling yield	Biocatalyst half-life
Model	3	1295.8	5576.0	431.9	18588.7
Residual	2	66.9	2092.8	33.4	1046.4
Total	5	1362.7	7688.8		
F ratio		12.91	17.76		
p value		0.07	0.05		
Lack of fit		0.19	0.38		
R^2		0.95	0.96		

lipase loading (x_2) had a significantly positive effect ($p < 0.05$) only on biocatalyst half-life. Although both PEG concentration and lipase loading have significant effects on biocatalyst half-life, their interaction was not significant. Thus, conditions that maximize the coupling yield were different from those attained for biocatalyst stability.

Based on the response evaluated, mathematical models were developed for both response factors. Regression analyses were performed to fit the response functions with the experimental data (Table 5). Models expressed by Eqs. 3 and 4, in which the variables take their coded values, represent the coupling yield and biocatalyst half-life as a function of PEG concentration ($\hat{y}_1$) and lipase loading ($\hat{y}_2$). For biocatalyst half-life factor, only the PEG concentration (x_1) and lipase loading (x_2) were significant at a level of 5% of probability. However, to minimize the determination of error, the effect of their interaction was also kept in the model.

$$\hat{y}_1 = 50.9 + 4.7x_1 - 9.9x_2 + 4.0x_1 \cdot x_2 \quad (3)$$

$$\hat{y}_2 = 204.2 - 70.5x_1 + 78.5x_2 - 53.0x_1 \cdot x_2 \quad (4)$$

By analyzing Table 5, it can be seen that linear models were adequate for describing the relationships between the responses under study and the experimental factors. For coupling yield, the regression is statistically significant ($p = 0.07$) at 10% probability level and also presents a good determination coefficient ($R^2 = 0.95$). The statistical significance of the model equation for biocatalyst half-life revealed that this regression is statistically significant ($p = 0.05$) at a 95% confidence level. In addition, the model did not show lack of fit and presented a high determination coefficient ($R^2 = 0.96$), explaining 96.0% of the variability in the response.

The response surface to estimate the coupling yield over independent variables PEG concentration ($\hat{y}_1$) and lipase loading (x_2) is shown in Fig. 2. The fitted surface has a true maximum, and the coordinates of the maxi-

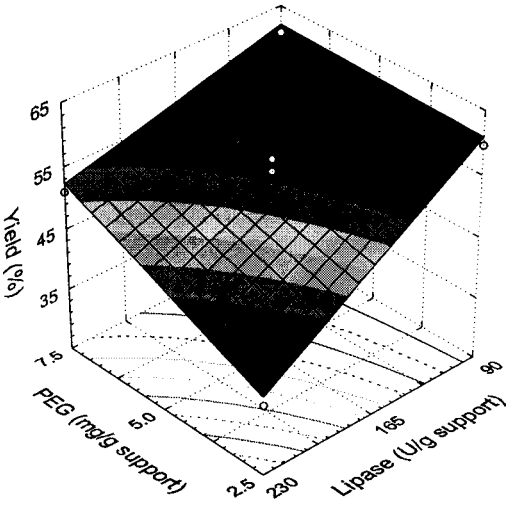

Fig. 2. Response surface described by model $\hat{y}_1$ representing coupling yield for lipase-immobilized CPS in presence of PEG 1500.

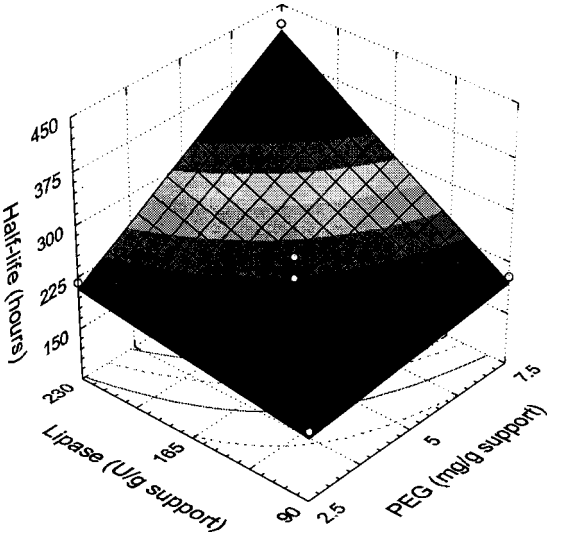

Fig. 3. Response surface described by model $\hat{y}_2$ representing biocatalyst half-life.

mum point were found to be x_1 = and x_2 = corresponding to the optimal of PEG concentration and lipase loading of 2.5 mg/g and 90 U/g, respectively. In these conditions, the model predicted the maximum coupling yield of 60%.

The response surface (Fig. 3) described by the model for biocatalyst half-life showed that the highest half-life (417 h or 17.5 d) can be attained at a PEG concentration and lipase loading of 7.5 mg and 230 U/g of dry support, respectively.

Table 6
Comparison of Recycle Potential for Microbial Lipase (*C. rugosa*) Immobilized on Different Supports in Synthesis of Butyl Butyrate

Support	Hydrolytic activity (U/mg dry support)	Esterification activity (μmol/[g·min])	Half-life (h)	Reference
PNMA[a]	66	180	180	*17*
STY-DVB	125	240	620	*15*
Chitosan	51	100	66	*17*
Chitin	45	98	100	*17*
CPS	119	154	417	This work

[a]Polymethylolacrilamide

The characteristics of the immobilized lipase obtained in this work support data from the literature, indicating that the microbial lipase when immobilized on hydrophobic supports, such as CPS and styrene-divinylbenzene copolymer (STY-DVB), are more stable than those immobilized on hydrophilic supports (chitin and chitosan). Table 6 compares data for the half-life of *C. rugosa* lipase immobilized on hydrophobic and hydrophilic supports when tested in esterification reactions of *n*-butanol with butyric acid under successive batch runs *(15,17)*. Only the lipase immobilized on CPS showed similar operational stability to that obtained by the lipase immobilized on STY-DVB.

Discussion

Enzyme activity can be affected by the process conditions chosen for the preparation of the biocatalyst. Immobilization together with additives can substantially improve the enzyme performance in long-term operation. In agreement with previous studies, promising results were obtained when lipase was immobilized on CPS in the presence of PEG 1500. This methodology rendered immobilized derivatives with higher operational stability than those containing no PEG 1500. We have extended the scope of this approach by evaluating the combined effects of PEG concentration (x_1) and lipase loading (x_2) employing a multivariate statistical approach. A 2^2 factorial design with center point was adopted for a full understanding of these effects and their interactions. The goal was to maximize the coupling yield ($\hat{y}_1$) and increase the biocatalyst half-life ($\hat{y}_2$) for application in the synthesis of butyl butyrate. The response surface methodology was adopted, and empirical linear mathematical models were constructed for the coupling yield and biocatalyst half-life. Conditions that maximize the immobilization yield were different from those attained for the operational stability. According to the mathematical models, the value predicted for maximum coupling yield was found to be 60 for 7.5 mg of PEG 1500 and 90 U/gram of dry support, while the highest half-life (417 h) was achieved for 7.5 mg and 230 U/g of dry support.

This was an expected behavior since different response factors were used in the statistical analyses. In the particular case of the coupling yield, the aim was to attain maximum recovery of the enzyme offered in the immobilization step, while the biocatalyst half-life was also a function of the initial biocatalyst activity. Note that the immobilized lipase preparation having the highest hydrolytic activities (117.6 U/mg) also gave the highest operational stability (run 4).

Note also that the use of PEG 1500 protects the enzyme from denaturation effects, without having interference on the reaction rate. However, because PEG is soluble in heptane, it is probable that this property favors the substrate partition to the solid phase, reducing eventual diffusion limitations of the immobilized enzyme.

The experimental results also suggest that PEG modifies the interactions between lipase and activated silica, producing alterations in the coupling yield and esterification rate. Although it was not performed, a full characterization of the activated support surface in the presence of PEG or the complex enzyme-PEG-support, allowing in this way, the identification of the space distribution of all components involved in the immobilization procedure, some configurations can be expected in this system, such as: 1) direct enzyme linking with the bifunctional agent as spacer arm followed by association with PEG, or 2) direct PEG linking onto the activated support having part of the enzyme occlusion in its internal space.

The second hypothesis seems to be the most probable configuration. PEG coating compromises a balance between attractive linking forces to the support and repulsive forces owing to the steric effect produced by the interaction of the polymeric chains in the solution *(9)*. It is known that in its free form, PEG has a tendency to join proteins in solution, which possibly promote the enzyme occlusion and bind the polymer protective layer onto the support. The hydrocarbon chain length influences directly the repulsive forces within polymeric matrixes and increases the viscosity of media, which has a negative effect on chain flexibility in the matrix formation. This may explain the observed low values for immobilization yields as described by several researchers *(8,18)* when PEG having high molecular weight (PEG 10000) was used as additive. In the case of PEG 1500, there is a balance between repulsive and attractive forces, promoting the occlusion and fixation of the polymeric chain onto the activated support.

However, in agreement with the literature, PEG is soluble in water, toluene, and other solvents, but insoluble in hexane, ether, and ethylene glycol *(19)*. Based on the immobilization procedure adopted in our study, the removal of unbound enzyme from the immobilized system by hexane-washing steps attained its objective without promoting the enzyme leakage from the support owing to the additive protection. The solubility property of PEG in another solvent also favored the reaction system in which heptane was used as an organic medium. This may help to improve biocatalyst half-life and suggests that internal diffusion limitations are less

pronounced when the immobilization of lipase on CPS is performed in the presence of PEG 1500.

The results of the present study indicate that the proposed strategy was efficient, promoting a 13-fold increase in the biocatalyst half-life when compared with the immobilized lipase preparation lacking PEG *(7,8)*. These finding could be of considerable practical importance since enzyme stability is vital to all industrial processes.

References

1. Balcão, V. M., Paiva, A. L., and Malcata, F. X. (1996), *Enzyme Microb. Technol.* **18,** 392–416.
2. Villeneuve, P., Muderhwa, J. M., Graille, J., and Haas, M. J. (2000), *J. Mol. Cataly. B: Enzymat.* **9,** 1–20.
3. Dias, F. S., Correia, A. C., and Baptista, F. O. (1999), *Bioprocess Eng.* **21,** 517–524.
4. Fonseca, L. P., Cardoso, J. P., and Cabral, J. M. S. (1993), *J. Chem. Tech. Biotechnol.* **58,** 27–37.
5. Baron, M., Florêncio, J. A., Zanin, G. M., Ferreira, A.G., Ennes, R., and Fontana, J. D. (1996), *Appl. Biochem. Biotechnol.* **57–58,** 605–615.
6. Zanin, G. M. and de Moraes, F. F. (1998), *Appl. Biochem. Biotechnol.* **70–72,** 383–394.
7. Soares, C. M. F., de Castro, H.F., Moraes, F. F., and Zanin, G. M. (1999), *Appl. Biochem. Biotechnol.* **77–79,** 745–748.
8. Soares, C. M. F., de Castro, H. F., Santana, M. H. A., and Zanin, G. M. (2001), *Appl. Biochem. Biotechnol.* **91–93,** 703–718.
9. Rocha, J. M. S., Gil, M. H., and Garcia, F. A. P. (1998), *J .Biotechnol.* **66,** 61–67.
10. Reetz, M. T., Zonta, A., and Simpelkamp, J. (1996), *Biotechol. Bioeng.* **49,** 527–534.
11. Stark, M. and Holmberg, K. (1989), *Biotechnol. Bioeng.* **34,** 942–950.
12. Gray, C. J. (1987), *Biocatalysis* **1,** 187–196.
13. Box, G. E. P., Hunter, W.G., and Hunter, J. S. (1978), in *Statistics for Experimenters: An Introduction to Design, Data Analysis and Model Building*, Bradley, R. A., Hunter, J. S., Kendall, D. G., and Watson, G. S., eds.,Wiley Series in Probability and Mathematical Statistics, Wiley & Sons, NY, pp. 306–317.
14. Bradford, M. M. A. (1976), *Anal. Biochem.* **72,** 248–254.
15. Oliveira, P. C., Alves, G. M., and de Castro, H. F. (2000), *Biochem. Eng. J.* **5,** 63–71.
16. Silva, C. J. S. M. and Roberto, I. C. (1999), *Biotechnol. Technol.* **13,** 743–747
17. Pereira, E. B. (1999), MSc thesis, University of Maringa State, Parana, Brazil.
18. Soares, C. M. F. (2000), MSc thesis UNICAMP, São Paulo, Brazil.
19. Harris, J. M. (1992), *Poly (Ethylene Glicol) Chemistry: Biotechnical and Biomedical Applications*, Plenum, NY, p. 228.