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Optimized Conditions for *In Situ* Immobilization of Lipase in Aldehyde-silica Packed Columns

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Abstract Optimal conditions for the *in situ* immobilization of lipase in aldehyde-silica packed columns, *via* reductive amination, were investigated. A reactant mixture, containing lipase and sodium borohydride (NaCBH), was recirculated through an aldehyde-silica packed column, such that the covalent bonding of the lipase, *via* amination between the amine group of the enzyme and the aldehyde terminal of the silica, and the reduction of the resulting imine group by NaCBH, could occur inside the bed, *in situ*. Mobile phase conditions in the ranges of pH 7.0~7.8, temperatures between 22~28°C and flow rates from 0.8~1.5 BV/min were found to be optimal for the *in situ* immobilization, which routinely resulted in an immobilization of more than 70 mg-lipase/g-silica. Also, the optimal ratio and concentration for feed reactants in the *in situ* immobilization: mass ratio [NaCBH]/[lipase] of 0.3, at NaCBH and lipase concentrations of 0.75 and 2.5 g/L, respectively, were found to display the best immobilization characteristics for concentrations of up to 80 mg-lipase/g-silica, which was more than a 2-fold increase in immobilization compared to that obtained by batch immobilization. For tributyrin hydrolysis, the *in situ* immobilized lipase displayed lower activity per unit mass of enzyme than the batch-immobilized or free lipase, while allowing more than a 45% increase in lipase activity per unit mass of silica compared to batch immobilization, because the quantity of the immobilization on silica was augmented by the *in situ* immobilization methodology used in this study.

Keywords: lipase, *in situ* immobilization, aldehyde-silica, tributyrin hydrolysis

INTRODUCTION

Enzymes are often used in an immobilized form in bioconversion and biotransformation processes, as this allows the re-use or continuous use of the biocatalyst. The catalytic activity of an immobilized enzyme can be gradually lost during repeated use in a packed column due to a number of factors [1-3]: physical damage due to shear stress or carrier attrition, compression or fouling, microbial contamination, and denaturation or deactivation by long-term contact with unfavorable environments, such as temperature, pH, ionic strength and various chemical agents. Also, the enzyme can leak out of the column, due to flagging strength of the coupling or bonding of the enzyme used in its immobilization onto the support.

Loss of enzyme activity, due either to deactivation or leaking, is an inevitable consequence of long-term repeated use in artificial environments, and results in decreased product yields or productivity [3,4]. Therefore, when the overall enzyme activity and productivity are lowered to unacceptable levels, the old enzyme in a packed column must be rejuvenated, either by replacement or supplementation with fresh enzyme [5,6]. The usual protocol in such circumstances is to repack the column with a newly immobilized enzyme following the discharge of all or part of the old one from the column. The new enzyme is conventionally prepared by batch immobilization in a separate, usually liquid phase, reaction. Although this batch immobilization and column repacking is the simplest method for the recovery of activity, it is not easy to reproduce identical packed-bed conditions to those that had previously existed with the old immobilized enzyme. Therefore, time and effort are often required to figure out the physicochemical and hydraulic conditions of a newly packed-bed, and to readjust the operating conditions of the system for achieving optimized yields and productivity [3,7]. This problem may be intensified in a large-scale immobilized enzyme system. If it is possible to immobilize fresh enzyme *in situ*, directly into the packed bed, without emptying and repacking the column, such inconveniences of batch immobilization and repacking could be obviated. A few trials have reported the *in situ* immobilization of enzymes [8-10], most of which involved small-scale HPLC systems.

In this study, the feasibility of an *in situ* immobilization method, with the immobilization of lipase taking place on a modified silica support, for the purpose of possible use in a low-pressure preparative system was investigated. Various conditions of the *in situ* immobilization reaction were optimized in order to increase the quantity of im-

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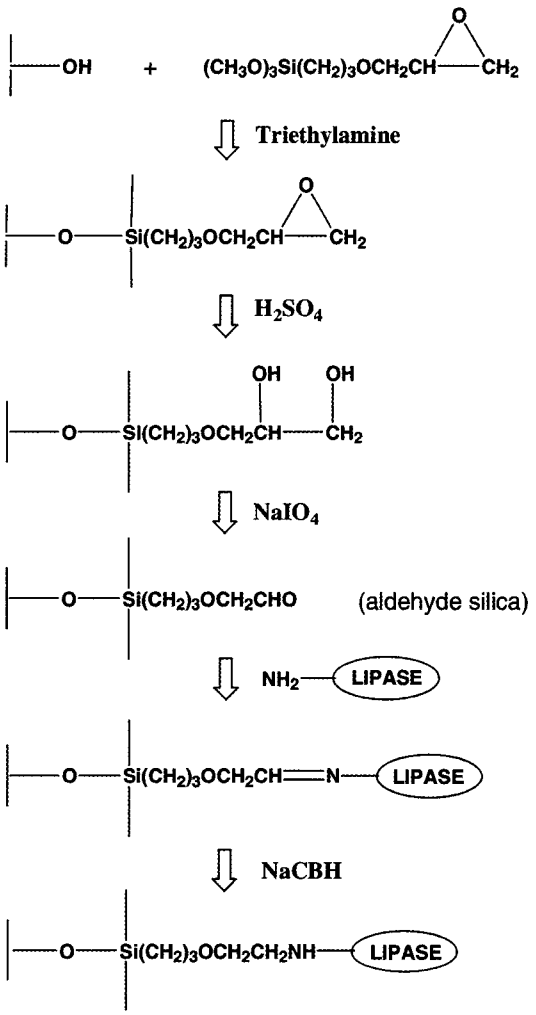

Fig. 1. The reaction scheme of silica derivatizations and lipase immobilization *via* reductive amination to aldehyde-silica.

mobilized lipase per unit mass of silica support. These conditions included the reactant composition and concentration, pH of the mobile phase, buffer strength, temperature and flow rate. The hydrolytic properties of the *in situ* immobilized lipase were then compared with those of free lipase and batch-immobilized lipase.

MATERIALS AND METHODS

Materials

Porcine pancreatic lipase (Sigma L3126) which is known to have a hydrolytic activity of 30~90 units/mg for triacetin and 100~400 units/mg for olive oil [11], was used in this study. One unit of this enzyme hydrolyzes 1 μmole equivalent of fatty acids in 1 min.

The Davisil 663XWP (extra wide pore) spherical silica gel (35~75 μm diameter, 500 Å pore size) was purchased from Supelco (Bellefonte, PA, USA). The reagents for the silica derivatization and lipase immobilization, such as 3-glycidoxy-propyltrimethoxysilane, toluene, trimethylamine, periodic acid and sodium cyanoborohydride (NaCBH), were all obtained from Aldrich (St. Louis, MO, USA).

Derivatization of Silica

Aldehyde-silica, which was used as a support material for the lipase immobilization, was prepared, essentially according to Larsson's method [12]. Twenty grams of dried silica was first converted to epoxy-silica, by epoxidation with 3-glycidoxypropyltrimethoxysilane (10 mL) in the presence of trimethylamine (0.25 mL) and toluene (250 mL). The resulting epoxy-silica was further converted to diol-silica by treatment with sulfuric acid (10 mM, 500 mL), and the resulting diol-silica was then oxidized to aldehyde-silica using 0.8% periodic acid (Fig. 1). After vacuum evaporation and drying, 22~24 g of aldehyde-silica was routinely obtained.

Batch Immobilization

A standard procedure for the lipase immobilization to aldehyde-silica, *via* the batch mode method (aqueous phase immobilization), was as follows: five grams of aldehyde-silica, in 200 mL of a sodium phosphate buffer (pH 7.3), was mixed with 150 mg of NaCBH, and sonicated for 5 min with an Ikasonic U50 sonicator (IKA Labotechnik, Germany). 500 mg of lipase was added, and stirred gently for 5 days. NaCBH is a reducing agent that links the amine group of the enzyme to the aldehyde terminal of the silica surface *via* reductive amination (Fig. 1). The resulting mixture was filtered and washed with phosphate buffer at pH 7.3. The lipase concentration in the silica-bonded phase was estimated by comparing the UV absorbance, in the aqueous phase, at a wavelength of 254 nm, before and after the immobilization reaction.

In Situ Immobilization

An LC column, 15 mm in diameter (Kontes, NJ, USA), was used to prepare a bed packed with aldehyde-silica. The bottom of the column was filled with glass wool, a polypropylene sintered disk positioned as a bed support, upon which 5 g of dried aldehyde silica was slurry-packed. The standard procedure for the *in situ* immobilization of lipase to aldehyde-silica in the packed column was as follows: 200 mL of reactant mixture solution, containing 2.5 g/L lipase and 0.75 g/L NaCBH in 100 mM phosphate buffer (pH 7.3), was recirculated for 25 h at a rate of 1.0 BV/min. 1 bed volume (BV) was equivalent to 6.5 cm^3. The bed was washed to remove un-immobilized lipase, by flowing phosphate buffer (pH 7.3) for 60 BVs, before measuring the activity.

Measurement of Hydrolytic Activity

Lipase activity was measured using tributyrin (glyceryl tributyrate) hydrolysis [13]. One molecule of tributyrin produces a triol compound, and three molecules of bu-

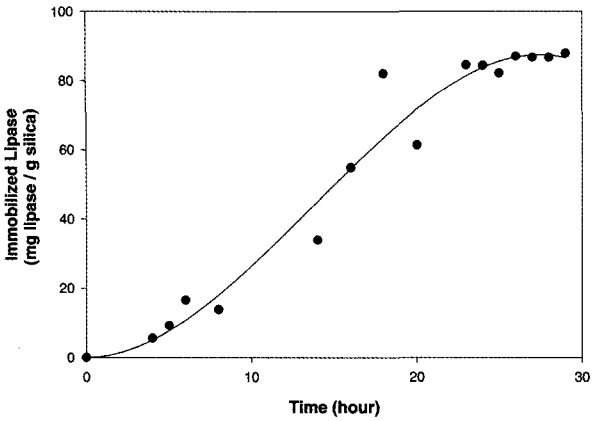

Fig. 2. Time course of the *in situ* lipase immobilization in aldehyde-silica packed columns. Feed: lipase 2.5 g/L and NaCBH 0.75 g/L, in 200 mL of 0.1 M phosphate buffer at pH 7.3. Flow rate: 1 BV/min. Temperature: 22°C.

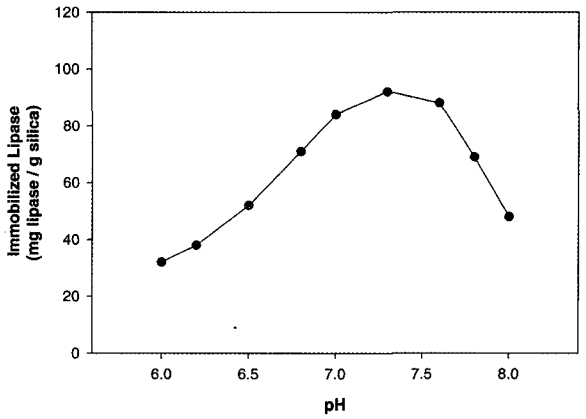

Fig. 3. The effect of pH on *in situ* immobilization. Feed: lipase 2.5 g/L, NaCBH 0.75 g/L, in 200 mL of 0.1 M phosphate buffer. Flow rate: 1 BV/min. Temperature: 22°C.

tanoic acids, through lipase-mediated ester hydrolysis. The resulting butanoic acids were titrated with NaOH, and thus 1 unit of lipase activity in the current experiments was defined as the quantity consuming 1 μmole of NaOH in 1 min.

In order to measure the lipase activity in the free aqueous state, 400 mg of free lipase was suspended in 10% tributyrin solution and emulsified in 100 mL of phosphate buffer (pH 7). After 15 min of enzyme reaction under mild agitation, the mixture was filtered and titrated with 10 mM NaOH. To measure the immobilized lipase activity, 10% tributyrin solution in phosphate buffer (pH 7) was passed, at a rate of 1 BV/min, through a packed column containing 5 g of aldehyde silica, where the lipase had been immobilized at a concentration of 80 mg-lipase/g-silica (400 mg lipase per bed). The effluent was not recirculated to the column at this time. The enzyme activity was determined using 10 mM NaOH solution, after the collection of 100 mL of effluent (*ca.* 15.4 min for passage).

RESULTS AND DISCUSSION

Immobilization Capacity

The *in situ* immobilization of lipase was performed by circulating a feed mixture, containing lipase and NaCBH, through the aldehyde-silica packed column, as is shown in the reaction scheme in Fig. 1. Fig. 2 shows the change in the immobilized lipase per unit mass of the aldehyde-silica packed into the column, with respect to circulation time. The composition of the feed mixture was 2.5 g/L of lipase and 0.75 g/L of NaCBH. The mobile phase flow rate was 1 BV/min, or 390 mL/h. The amount of immobilized enzyme increased steadily with time, and reached a plateau, indicating saturation, at about 90 mg-lipase/g-silica after 25 h. This was equivalent to about 1,500 BVs of circulated passage. The pressure drop during the immobilization was negligible, probably because the pore size of the silica particles used in this study was relatively large compared to the molecular size of the lipase, and also because the lipase concentration in the feed was not high enough to induce severe bed clogging.

By way of comparison, a batch immobilization was performed with the same amount of silica and feed composition in a suspended liquid phase, as described in the Materials and Methods section. The amount of immobilized lipase after 5 days, *via* batch mode method, was only about 35 mg-lipase/g-silica, even though the contact time was much longer than in the *in situ* mode of immobilization (data not shown). This comparison demonstrated that the *in situ* immobilization technique used in this study enhanced the immobilization efficiency through close and repeated contact between the enzyme and silica particles in the packed-bed environment.

Temperature and pH

By changing the pH of the feed buffer used to dissolve the lipase and NaCBH, the effects of the mobile phase pH on the *in situ* immobilization reaction. Fig. 3 shows that a neutral pH range was optimal for immobilization, and that more than 80 mg-lipase/g-silica of immobilization efficiency was routinely obtained within the pH range 7.0~7.8. Such dependence implies that acidic and alkaline conditions are not suitable for the catalysis of the reductive amination reaction that utilizes the NaCBH between the amine group of lipase and the aldehyde terminal of the silica surface, which is required for covalent immobilization of lipase onto the aldehyde-silica. Many lipases prefer an alkaline pH, usually between 7.5 and 9.0, for their hydrolytic or esterifying activity, although the optimal activity range of some microbial lipases stretches from pH 4.5 to 6.5 [14]. Therefore, sufficient washing and equilibration would be necessary for pH adjustment, prior to using lipase immobilized by the present method, depending upon its required use.

Fig. 4 shows the influence of temperature on *in situ*

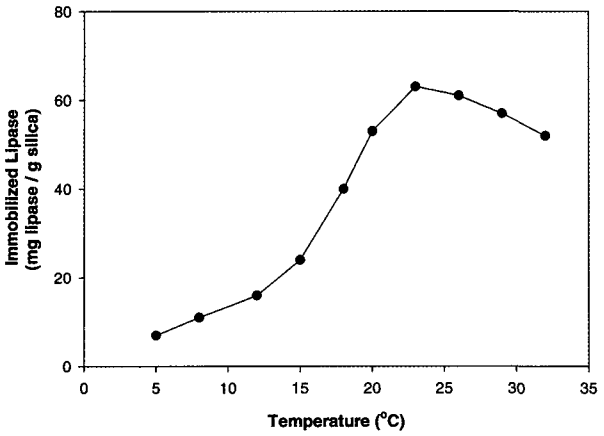

Fig. 4. Temperature dependence of the *in situ* immobilization. Feed: lipase 2.5 g/L, NaCBH 0.75 g/L, in 200 mL of 0.1 M phosphate buffer at pH 7.3. Flow rate: 1 BV/min.

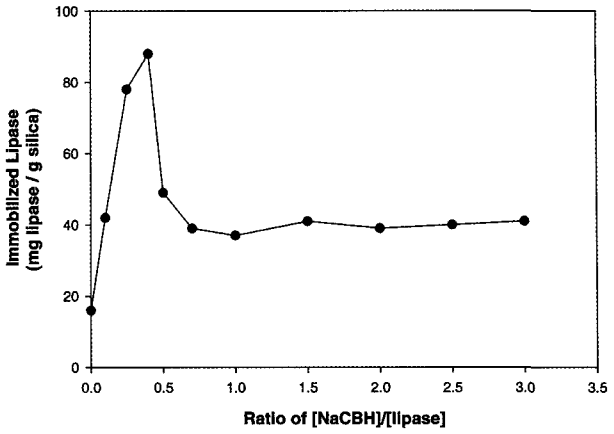

Fig. 5. The effect of the feed composition on the *in situ* immobilization. Feed: mixture of lipase and NaCBH in 200 mL of 0.1 M phosphate buffer at pH 7.3. Flow rate: 1 BV/min.

immobilization. The best immobilization was obtained within the temperature range 22~28°C, at which more than 60 mg-lipase/g-silica of immobilization was achieved. A drastic decrease in the immobilization efficiency was observed within a lower temperature range, while a lesser decrease was observed within the higher temperature range. It is generally known, in the field of organic synthesis, that the reductive amination of an imine group using reducing agents is better achieved at a high temperature, up to 60°C, and in a pressurized state [15]. However, these extreme ranges were avoided here to prevent enzyme deactivation. For the routine *in situ* immobilization of lipase, 22°C was used as the standard reaction temperature throughout this study.

Effects of Feed Composition

The effects of the feed composition (lipase and NaCBH) on the *in situ* immobilization were examined. Firstly, the lipase concentration was kept constant, at 2.5 g/L, and the NaCBH concentration was varied, from 0 to 3.0 g/L. As shown in Fig. 5, more than 80 mg-lipase/g-silica of immobilization was achieved with a feed mass ratios of [NaCBH]/[lipase] within the range of 0.3~0.4. The initial 15 mg/g of immobilization with no NaCBH was probably due to the physical sorption of lipase onto the aldehyde-silica surface. When the ratio of [NaCBH]/[lipase] was greater than 0.5, the immobilization efficiency dropped rapidly, and thus 40 mg/g of immobilization was reached, on average, with ratios greater than 0.7. The reason for this decreased immobilization at high NaCBH concentrations, relative to that of the lipase, was not clear, but it was suspect that some unwanted chemical modification or structural change, in either the enzyme or the silica functional group, might have occurred in the presence of such high concentrations of the reducing agent, NaCBH.

Fig. 6 illustrates the effects of variations in the feed concentration, but still maintaining the [NaCBH]/[lipase] ratio at 0.3. An optimal concentration of NaCBH and lipase, 0.75 g/L and 2.5 g/L, respectively, resulted in the best immobilization, up to 90 mg-lipase/g-silica. Even when the [NaCBH]/[lipase] ratio was at the optimal 0.3, as in Fig. 5, the use of a higher feed concentration did not improve the *in situ* immobilization, perhaps because such high concentrations of the feed components may have increased the non-specific interactions between silica and lipase, and thus, would possibly hinder the anticipated proper reductive amination necessary for stable immobilization.

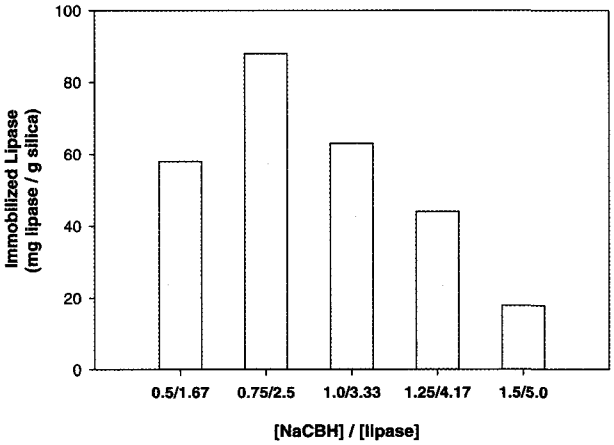

Fig. 6. The effect of the feed concentration on the *in situ* immobilization with a [NaCBH]/[lipase] ratio maintained at 0.3. Feed: mixture of lipase and NaCBH in 200 mL of 0.1 M phosphate buffer at pH 7.3. Flow rate: 1 BV/min.

Flow Rate of Mobile Phase

The influence of varying the flow rates of the feed mixture, from 0.1 to 2.5 BV/min, through the aldehyde-sili-

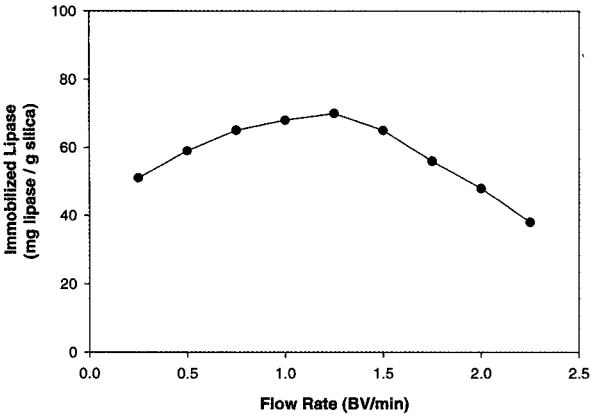

Fig. 7. The effect of the mobile phase flow rate on the *in situ* immobilization. Feed: lipase 2.5 g/L, NaCBH 0.75 g/L, in 200 mL of 0.1 M phosphate buffer at pH 7.3. Flow rate: 1 BV/min at 20°C.

ca packed column was investigated. The total passage of the feed was kept constant, at 1,000 BVs. Fig. 7 shows that immobilization proceeded most efficiently within the flow rate range 0.8 and 1.5 BV/min, which resulted in an immobilization rate of more than 70 mg-lipase/g-silica. The immobilization performance was lowered with both slower and faster flow rates than those mentioned above. This kind of flow rate dependency is quite universal in packed-bed reactions, such as adsorption or ion exchange. The major reason for lower immobilization at higher flow rates is that the contact time between reactants (lipase and aldehyde-silica) is insufficient, and thus an effective local equilibrium for the reaction may not be attained. Generally, low flow rates are preferred in order to prolong the local contact time, but this may result in a loss of the optimal mobile phase reactant composition (lipase and NaCBH), usually because the extent of axial dispersion increases. In the present study, a flow rate of 1 BV/min was used as a routine value for the *in situ* immobilization of lipase.

Activity of Immobilized Lipase

Liquid phase free lipase, batch immobilized lipase and *in situ* immobilized lipase were compared with regard to their lipase activity for tributyrin hydrolysis (Table 1). When the lipase activity per unit mass of enzyme was compared, the batch-immobilized lipase exhibited the best performance. The superiority of the batch-immobilized enzyme over that of the free liquid phase enzyme is the result of an inherent advantage of the immobilized enzyme system, namely the close and continuous contact between substrates in the mobile phase and the enzyme on a packed solid support. Also, many enzymes exhibit higher stability and activity when immobilized than in their free soluble states. The increased stability could also be the result of reduced conformational variation, as well as reduced attack by reactive solutes, due to steric protection [7,16,17].

Table 1. Comparison of immobilized quantities and lipase activities for tributyrin hydrolysis

	Free enzyme	Batch immobilized enzyme	*in situ* Immobilized enzyme
Immobilized quantity (mg lipase/g silica)	-	35[a]	80[b]
Total quantity of lipase (mg)	400	175	400
Lipase activity[d] (units/g silica)	-	18,900	27,800
Lipase activity (units/mg lipase)	410[c]	540	347

[a] Batch immobilization: 5 g aldehyde-silica and 500 mg lipase in 200 mL phosphate buffer (pH 7) for 5 days.
[b] *In situ* immobilization: 5 g aldehyde-silica packed column, 500 mg lipase in 200 mL phosphate buffer (pH 7) recirculated for 25 h, 1.0 BV/min.
[c] Free reaction: 400 mg free lipase in 100 mL phosphate buffer (pH 7) contcining 10% tributyrin, 15 min reaction.
[d] Immobilized reaction: 100 mL of phosphate buffer (pH 7) containing 10% tributyrin was passed at 1.0 BV/min, *ca.* 15.4 min of passage time.

Meanwhile, the *in situ* immobilized lipase displayed the lowest activity per unit mass of enzyme. This result implies that quite a dense immobilization took place with the *in situ* immobilization in the packed aldehyde-silica bed, and thus the active sites for hydrolysis were sterically hindered for some fraction of the immobilized lipase. *In situ* immobilization, however, allowed for more than 45% more lipase activity per unit mass of silica than the batch immobilization. This enhancement in the total activity was due to an increase in the quantity of immobilization onto the silica by the *in situ* immobilization methodology used in this study.

CONCLUSION

The general protocol for the recovery of activity in immobilized enzyme systems involves batch immobilization and repacking, but the reproduction of the physical and hydraulic conditions is not easy in a newly packed-bed, nor is it a simple matter to readjust the operating conditions of a system to regain optimum yields and productivity. Such inconveniences can be circumvented through the adoption of *in situ* immobilization, which allows the rejuvenation of activity in the packed bed without emptying and repacking of the column.

In this study, the optimal conditions for *in situ* immobilization of lipase in an aldehyde-silica packed column were investigated, using sodium borohydride (NaCBH) as a reducing agent. The optimal mobile phase conditions for *in situ* immobilization were within the following ranges: pH 7.0~7.8, temperature 22~28°C and flow rate 0.8~1.5 BV/min. The operation of the system within

these ranges routinely resulted in more than 70 mg-lipase/g-silica immobilization. An optimal ratio of feed reactants also existed, namely a mass ratio [NaCBH]/[lipase] of 0.3 in the mobile phase of the *in situ* immobilization. Maintaining this reactant ratio was very important, as the immobilization efficiency was found to drop rapidly when this ratio was greater than 0.5. A composition of 0.75 g/L NaCBH and 2.5 g/L lipase resulted in the best immobilization, up to 80 mg-lipase/g-silica, which was more than 2 times that obtained by the batch technique. Even when the [NaCBH]/[lipase] ratio was optimal, the use of a higher feed concentration did not improve the efficiency of the *in situ* immobilization.

The liquid phase free, batch immobilized and *in situ* immobilized lipases were also compared with regard to their activity for tributyrin hydrolysis. The *in situ* immobilized lipase exhibited lower activity per unit mass of enzyme than the other two cases, but allowed more than a 45% improvement in the lipase activity per unit mass of silica compared to batch immobilization. The *in situ* immobilization methodology used in this study has been confirmed as a very useful technique for the enhancement of immobilization quantity, which also facilitated the maintenance of higher enzyme activity in packed enzyme reaction systems.

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