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Ester Synthesis Catalyzed by *Mucor miehei* Lipase Immobilized on Magnetic Polysiloxane–Polyvinyl Alcohol Particles

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Abstract

Mucor miehei lipase was immobilized on magnetic polysiloxane–polyvinyl alcohol particles by covalent binding with high activity recovered. The performance of the resulting immobilized biocatalyst was evaluated in the synthesis of flavor esters using heptane as solvent. The impact on reaction rate was determined for enzyme concentration, molar ratio of the reactants, carbon chain length of the reactants, and alcohol structure. Ester synthesis was maximized for substrates containing excess acyl donor and lipase loading of 25 mg/mL. The biocatalyst selectivity for the carbon chain length was found to be different concerning the organic acids and alcohols. High reaction rates were achieved for organic acids with 8 or 10 carbons, whereas increasing the alcohol carbon chain length from 4 to 8 carbons gave much lower esterification yields. Optimal reaction rate was determined for the synthesis of butyl caprylate (12 carbons). Esterification performance was also dependent on the alcohol structure, with maximum activity occurring for primary alcohol. Secondary and tertiary alcohols decreased the reaction rates by more than 40%.

Index Entries: Lipase; sol-gel matrix; polysiloxane–polyvinyl alcohol; esterification; activity.

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Introduction

The use of lipases in organic media is now of major commercial importance, and this interest is expected to grow over the coming years as a wider range of lipase catalysts becomes available *(1–4)*. For example, a range of fatty acid esters is now being synthesized on a commercial scale using lipases in essentially nonaqueous media *(1,2)*. To expand further their synthetic utility, efficient methods for immobilizing lipases are needed because immobilization allows enzyme reuse and thus reduces overall process costs *(2,5)*. Several methods have been reported, such as deposition on solid supports *(6,7)*, covalent binding *(8,9)* and entrapment within a polymer matrix or hydrophobic sol-gel material *(10,11)*. The latter method can be applied to a variety of lipases, yielding immobilized systems with 80-fold esterification activity compared with the free enzyme *(11)*.

The sol-gel process involves the transition of a system from a liquid "sol" (mostly colloidal) into a solid "gel" phase *(11)*. By applying this methodology, it is possible to fabricate ceramic or glass materials in a wide variety of forms: ultrafine or spherical-shaped powders, thin film coatings, ceramic fibers, microporous inorganic membranes, monolithic ceramics and glasses, or extremely porous aerogel materials.

The sol-gel technique is also an excellent method to prepare hybrid material. The low temperature synthesis enables organic or inorganic species to be incorporated into rigid silicon oxide matrices without degradation. The resulting composite combines the chemical and physical properties of the guest with the excellent optic, thermal, and chemical stability of the host silicon oxide matrices *(12)*. Recently we reported a novel procedure for covalent immobilization of antigens from *Yersinia pestis* for antibody detection onto small disks of a polysiloxane–polyvinyl alcohol (POS-PVA) composite prepared by the sol-gel technique *(12)*. This simple but effective procedure gave an immobilized antigen preparation with greatly improved activity and stability *(12)*. The procedure used tetraethoxysilane (TEOS) and PVA for the matrix formation followed by activation with glutaraldeyde to render a more biocompatible surface for covalent immobilization *(12,13)*.

Here, we report the application of this procedure for immobilizing *Mucor miehei* lipase. A catalytic test was aimed at producing esters by direct esterification reactions with a large range of carboxylic acids (from C4 to C16), and a diversity of alcohols (from C4 to C8). Several reaction model systems are analyzed in order to illustrate the kind of products that can be made by using an experimental preparation of lipase immobilized on POS-PVA particles.

Materials and Methods

Chemicals

Mucor miehei lipase (4.79 ± 0.21 U/mg of protein) was kindly donated by Novozymes (Araucaria, PR, Brazil). TEOS was from Aldrich (Milwau-

kee, WI). Glutaraldehyde (25% [w/v]), HCl (minimum 36%), ethanol (minimum 99%), and PVA (MW mol wt. = 72,000) were supplied by Reagen (Rio de Janeiro, RJ, Brazil). Alcohols (*n*-butanol, *sec*-butanol, *tert*-butanol, pentanol, hexanol, and octanol) and organic acids (butyric, octanoic, lauric, palmitic) were purchased from Merck (Darmstadt, Germany). Solvents were standard laboratory grade. Heptane was dried with metallic sodium and used as the solvent for all experiments. Substrates for esterification reactions were dehydrated with 0.32 molecular sieves (aluminum sodium silicate, type 13X; BHD Chemicals, Toronto, Canada) previously activated in an oven at 350°C for 6 h.

POS-PVA Synthesis

A POS-PVA hybrid composite was prepared by the hydrolysis and polycondensation of TEOS as described by Lima Barros et al. *(12)*. The reagents TEOS (5 mL), ethanol (5 mL), and PVA solution 2% (w/v) (6 mL) were carefully mixed and stirred for 5 min at 60°C, followed by addition of two or three drops of concentrated HCl in order to catalyze the reaction. After an incubation period of 40 min, the material was transferred to microwells of tissue culture plates and kept at 25°C until complete gel solidification (formation of the interpenetrated network of POS-PVA). Then the spheres were ground in a ball mill to attain with 37-µm-diameter particles, which was magnetized according to the methodology described by Carneiro-Leão et al. *(8)* based on the coprecipitation from a solution of $FeCl_3 \cdot 6H_2O$ and $FeCl_2 \cdot 4H_2O$. Activation of POS-PVA particles was carried out with 2.5% (w/v) glutaraldehyde at pH 7.0 for 1 h at room temperature, and the particles were thoroughly washed with distilled water.

Lipase Immobilization onto Magnetic POS-PVA Particles

Magnetic POS-PVA particles were incubated with lipase solution containing 0.05 U/mg of protein in phosphate buffer (0.1 *M*, pH 7.0) under low stirring for 16 h at room temperature. The immobilized lipase derivative was recovered by applying a magnetic field (6000 Oe), washed with 1 *M* NaCl, and maintained in 50 m*M* Tris-HCl buffer (pH 8.0) at 4°C. Immediately before the esterification reaction, the immobilized derivative was washed with hexane and vacuum dried to remove water. The resulting preparation had a specific activity of 3.31 U/(mg protein·g of dry support) using *p*-nitrophenol as substrate, and the degree of lipase immobilization was 70% *(13)*.

Esterification Reactions

Esterification reactions were carried out in a closed reactor with 10 mL of dried n-heptane containing suitable amounts of alcohol and acid. A molecular sieve (aluminum sodium silicate, type 13X; BHD Chemicals) was used to removal water. The mixture was incubated at 37°C for 24 h with continuous shaking at 150 rpm. The effects of concentration of immobilized lipase (5–50 mg/mL); molar ratio of reactants (0.5–2.0), acid chain length

(C4–C16), alcohol chain length (C4–C8), and alcohol structure (e.g., primary or secondary) were studied.

Analytical Methods

Water concentrations in liquid and solid phases were measured by the Karl Fisher method using the Karl Fisher Titrator (Mettler DL 18). Butanol and butyl butyrate 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. Acid consumption was monitored by volumetric titration of samples diluted in ethanol employing 0.02 *M* KOH alcoholic solution and phenolphthalein as pH indicator. Esterification was expressed as molar percent of consumed reactant, according to Eq. 1:

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

in which C_o is the initial molar concentration of reactant, and C is the reactant molar concentration at a given time.

Estimation of Partition Coefficients

The partition coefficients (POS-PVA/ external organic solvent) of butanol and butyric acid were estimated according to Eq. 2 as previously described by Dias et al. *(14)*.

$$\text{Partition coefficient} = \frac{C_o - C}{C} \times \frac{V_o}{V - V_o} \quad (2)$$

in which P is the reactant partition coefficient, C_o is the reactant concentration of organic phase, C is the reactant concentration after contact with support, V_o is the total volume (organic phase + support), and V is the organic-phase volume.

Partition experiments were conducted under the same conditions as their reaction counterparts. To estimate the support volume ($V - V_o$) a calibration curve of volume vs support mass was established (matrix volume [cm^3] = 1.72 × support mass [g] – 0.07; $R^2 = 0.999$). The equilibrium concentrations were attained 2 h after the immobilized lipase was added to the organic medium.

Results and Discussion

Influence of Immobilized Lipase Concentration

Butanol conversion to butyl butyrate employing different concentrations of immobilized lipase was examined in the presence of butyric acid and n-butanol at a 1.25:1 molar ratio. Syntheses were performed at 37°C, and immobilized lipase concentration varied from 5 to 50 mg/mL. As expected, completion of the reaction was very dependent on the enzyme

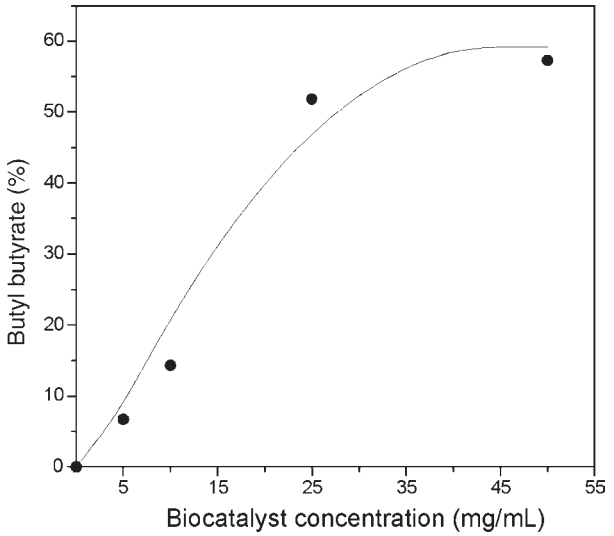

Fig. 1. Effect of immobilized lipase contents on the butyl butyrate synthesis at 37°C for 24 h. A molar ratio between butyric acid and butanol of 1.25 was used for enzyme contents of 5, 10, 25, and 50 mg/mL.

concentration (Fig. 1). Molar conversions > 50% were achieved in 24 h when high enzyme concentrations were used (25 and 50 mg/mL). Reactions carried out with lower enzyme concentrations (5 and 10 mg/mL) achieved lower molar conversions (6.7 and 14.7%, respectively). Such performance could be improved by modifying other variables that also affect the reaction yield, such as increasing the incubation temperature up to the optimum (45°C) for this lipase preparation *(13)* or using a higher butyric acid:*n*-butanol ratio. Acid in excess allows acyl group complex formation, leading to ester formation and reducing the competition between acceptors, an important parameter for esterification reaction kinetics *(15)*.

Effect of Substrate Molar Ratio

Lipase is known to catalyze esterification through an acyl-intermediate formed between the fatty acid substrate and the enzyme. Free enzyme can bind fatty acid to produce either this intermediate or the ester product. With a high concentration of alcohol, the acyl-intermediate will be consumed, and the enzyme may then start to bind product and catalyze its hydrolysis, thereby reversing the reaction. When present in an excess of fatty acid, however, most of the enzyme is found in the acylated form, preventing it from binding the product *(15,16)*.

Table 1
Partition Coefficients Matrix/Heptane of Reactants Estimated According to Eq. 2 at Initial Bulk Concentration of 100 m*M* in Heptane at 37°C and 150 rpm

	Partition coefficients	
Reactants	POS-PVA lipase	STY-DVB lipase[a]
n-Butanol	2.68	2.32
Butyric acid	1.28	1.66

[a]From ref. *7*.

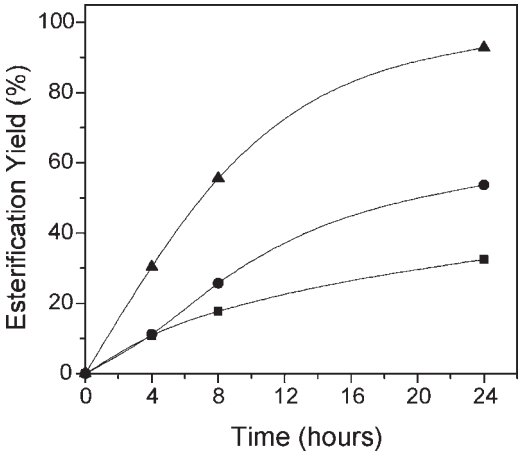

Fig. 2. Influence of butyric acid:butanol molar ratio on butyl butyrate yield. Molar ratios of 2:1 (▲), 1:1 (●), and 0.5:1 (■) were used at 37°C and 50 mg/mL of POS-PVA lipase.

For immobilized lipase preparations, a more complex mechanism is expected to occur since esterification efficiency is also highly dependent on the hydration state of the enzyme preparation, which can be greatly modified by the nature of the substrate and the support *(1,4)*. In the case of butyl butyrate synthesis, analysis of substrate polarity measured as partition coefficient (Table 1) showed a higher value for butanol than for butyric acid, favoring butanol migration to the solid phase (immobilized lipase). Thus, there should be more alcohol than acid at the active site of the immobilized lipase, requiring an excess of acid in the reaction medium to provide equimolar amounts of reactants and satisfactory yields *(7)*.

This expectation was confirmed by running a set of experiments in which the effect of the butyric acid (BA): butanol (ButOH) molar ratio on the esterification catalyzed by POS-PVA lipase was investigated in the range of 0.5–2.0. As shown in Fig. 2, no inhibition of enzyme activity was

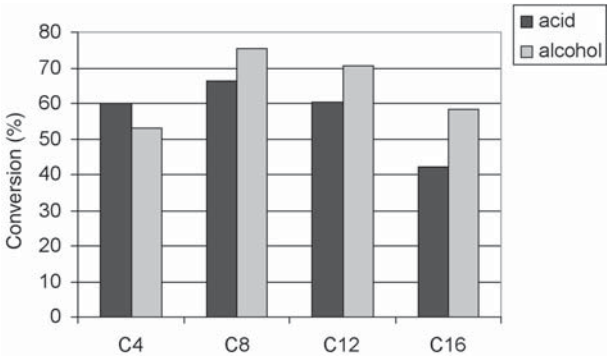

Fig. 3. Effect of acid chain length on ester synthesis catalyzed by POS-PVA lipase Reactions were carried out at 37°C for 24 h using *n*-butanol. C4, Butyric acid; C8, caprylic acid; C12, lauric acid; C16, palmitic acid.

detected when butyric acid was used in excess. Actually, progress of the esterification was limited by the availability of butyric acid in the reactor vessel. When the molar ratio was 0.5 a low esterification yield (33%) was obtained. This percentage increased with the rise in butyric acid concentration, reaching 93% yield, when the acid:alcohol molar ratio was 2.0. At an equimolar ratio, the yield was approximately half that attained when butyric acid was used in excess. The results suggest that the POS-PVA lipase, like other preparations, is greatly influenced by substrate molar ratio in the formation of the product. For example, studying the same reaction system with *Candida rugosa* lipase immobilized on styrene-divinylbenzene copolymer (STY-DVB) as catalyst, Oliveira et al. *(7)* 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. This similarity can be attributed to the hydrophobic character of the supports and partition coefficient values attained for these immobilized derivatives: POS-PVA lipase and STY-DVB lipase (Table 1).

Effect of Acid Chain Length

It has been clearly demonstrated in the literature that in the case of direct esterification, the chain length of acid and alcohol affects the ester yield *(16–19)*. In the present work, we investigated the reactivity of acyl donors of various chain lengths, ranging from butyric (C4) to palmitic acid (C16) in the esterification of butanol. The reaction medium consisted of amounts of the required acid and butanol at a fixed molar ratio (1.5) and 25 mg/mL of POS-PVA lipase. The molar conversion of butanol was calculated after 24 h of reaction, and the values attained for each acyl donor tested are displayed in Fig. 3.

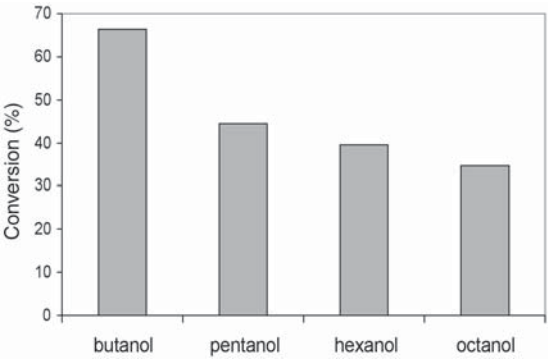

Fig. 4. Effect of alcohol chain length on ester synthesis catalyzed by POS-PVA lipase. Reactions were performed at 37°C for 24 h using caprylic acid as acyl donor.

Reaction rates by POS-PVA lipase gradually increased from C4 to C8, reached a plateau between C8 and C12, and then an activity decrease was observed, probably owing to a substrate-size feature limitation. From this set of results, caprylic acid (C8) may be considered the best acyl donor for the synthesis of butyl esters in heptane by POS-PVA lipase.

Similar results from Langrand et al. *(18)* showed that lipase from *M. miehei* was more active in synthesizing geranyl esters by esterification in the presence of acids with carbon atom numbers 4 to 6. Other researchers *(19)* have demonstrated that for esterification of octanol, this enzyme was most active in the presence of an acid with a carbon atom number of 7. According to these researchers, this effect seems to be related to the fitting of both substrates into the active site of the enzyme: an optimum value of 15 for the sum of carbon chain length of the two substrates was deduced from those results *(19)*. Selmi et al. *(20)* reported a different behavior in triglyceride synthesis catalyzed by a commercial immobilized *M. miehei* lipase: high rates and equilibrium yields were obtained with long chain fatty acids, mainly hexadecanoic and octadecanoic acids. These differences probably can be explained by the diversity among enzyme sources; the type of support; and the methods of immobilization employed in each investigation, since immobilization technique can change fatty acid specificity *(17,21,22)*.

Effect of Alcohol Chain Length

The influence of alcohol chain length was studied by using substrate containing caprylic acid and the required alcohol (C4 to C6 and C8) at a fixed molar ratio (1.5) and 50 mg/mL of POS-PVA lipase. The results of acid molar conversion after 24 h are displayed in Fig. 4. The carbon chain significantly influenced esterification performance. As the length of the alcohol carbonic chain increased, lower molar conversion was detected. The highest value (70%) was attained for butanol and the lowest (40%) for octanol.

Table 2
Influence of Butanol Structure on Ester Synthesis Performed at 37°C for 24 h

Butanol structure	Reaction time (h)	Butyric acid (mg/mL)	Water content (ppm)	Molar conversion (%)
n-Butanol	0	21.25	317	0
	24	8.57	302	59.63
sec-Butanol	0	21.82	343	0
	24	16.51	302	24.33
tert-Butanol	0	22.61	392	0
	24	17.81	423	21.27

Pereira et al. *(17)* did not observe differences on acid conversion with alcohol varying between C4 and C10 in reactions performed with *C. rugosa* lipase immobilized on chitosan. However, Manjon et al. *(23)* working with *M. miehei* lipase immobilized on Celite verified that esterification yields decreased as the chain length of the acid or alcohol increased, with the alcohol length having a higher influence than the acid.

Comparison of Figs. 3 and 4 reveals that alcohol chain length exerted more influence on ester yield than the acid carbon size. For alcohol size varying from C5 to C8, conversion was lower than 40%, and for acid size from C4 to C16, similar esterification yields (60–80%) were found. These results may reflect both the intrinsic selectivity of the enzyme and different accessibility of substrates to enzyme active site *(22)*.

Effect of Alcohol Structure

In addition to the chain length, the effect of branching of the carbon chain was studied. Specificity of POS-PVA lipase was studied by monitoring esterification reactions of *n*-butanol, *sec*-butanol, and *tert*-butanol with butyric acid, as shown in Table 2. The highest rate of conversion to ester (60%) occurred in the presence of *n*-butanol, compared with *sec*-butanol and *tert*-butanol. The branching was found to decrease significantly the esterification yield by a factor of 0.4 for sec-butanol and 0.65 for tert-butanol. Antczak et al. *(24)* reported a similar conversion pattern.

Note that control and maintenance of low water contents during esterification progress was a difficult task only for reactions carried out with *tert*-butanol (Table 2). Besides affecting reaction yields, high water contents favors the reverse reaction (ester hydrolysis) by decreasing even more the amount of ester formed *(1,4)*.

Conclusions

The alkoxysilane sol-gel process is an efficient method to prepare silica glass by the hydrolysis of alkoxysilane precursors and by subsequent con-

densation of the remaining silanols, followed by aging and drying under ambient atmospheres and sintering. The hydrolysis and polycondensation of TEOS is as follows:

Si (OEt)e → Hydrolysis → n(EtO)x Si (OH)y-x → Polycondensation → (SiO_2)n

In our work, particles of POS-PVA were utilized to immobilize *M. miehei* lipase, and the performance of the immobilized derivative was investigated for ester synthesis in organic media. POS-PVA lipase was able to catalyze ester synthesis by proper selection of the environmental parameters enzyme concentration, molar ratio of the reactants, substrate size, and chemical structure. Higher yields were achieved for substrates containing acyl donor in excess and using high loading of immobilized lipase (25 mg/mL). A close relationship between reactant polarity and ester formation was verified. Acid containing eight carbons and primary alcohol with four carbons were considered to be suitable reactants resulting in high esterification rates. Other reaction parameters, such as temperature, could be investigated to improve esterification yields.

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