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Evaluation of the catalytic activity of lipases immobilized on chrysotile for esterification

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ABSTRACT

In the present work, the ester synthesis in organic media catalyzed by lipases immobilized on chrysotile was studied. Lipases of different sources (*Mucor javanicus*, *Pseudomonas cepacia*, *Rhizopus oryzae*, *Aspergillus niger* and *Candida rugosa*) were immobilized on chrysotile, an inexpensive magnesium silicate, and used for esterification of hexanoic, octanoic and lauric acid with methanol, ethanol, 1-butanol and 1-octanol at 25°C in hexane as solvent. The best results were obtained with *Mucor javanicus* lipase and lauric acid giving yields of 62-97% of ester.

Key words: chrysotile, lipases, esters.

1 INTRODUCTION

Lipases (triacyglicerol acylhydrolase, EC 3.1.1.3) are enzymes that catalyse hydrolysis of acylglicerides and other fatty acid esters. They are present in several organisms, including animals, plants, fungi and bacteria. They have been attracting the attention of chemical organic synthesis mainly because of their enantioselective properties. Lipases have been widely used in organic synthesis due to their catalytic versatility, commercial availability, low cost and non request cofactors (Jesus et al. 1995, Faber 1997). On the other hand, biocatalysis in non-aqueous media has emerged as a powerful tool for the production of fine chemicals, pharmaceuticals and food ingredients. Advantages commonly associated with the use of organic solvents as reaction media include improved solubility and chemical stability of the organic substrates and facile product recovery (Green et al. 1996).

The immobilization of enzymes in a solid matrix has been widely investigated and a variety of supporting media has been tested (Lima et al. 1996, Oliveira et al. 2000). Efforts have been directed towards the finding of good supports for the immobilization of lipases. Immobilization methods have been developed to supply stability for the lipases in organic media and to facilitate their recovery and reuse. Among the different types of supports that were studied in literature chrysotile appeared to be a further alternative. Chrysotile, one of the main asbestos sources used extensively in the civil construction, has shown excellent adsorption properties due to the high specific area. It is a magnesium silicate of the serpentine group, presenting the molecular formula $Mg_3(Si_2O_5)(OH)_4$, with 43% of MgO, 44,1% of SiO_2, 12,9% of H_2O (Jesus et al. 1998).

Moran et al. studied the immobilization of

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Baker's yeast on chrysotile and its application for the stereoselective reduction of carbonyl compounds such as α-azidopropiophenone. They obtained the azidoalcohols, the corresponding optically active products, with good yields and enantiomeric excess (Moran et al. 1994). Other works used Baker's yeast immobilized on chrysotile for the reduction of phenylketones (Sorrilha et al. 1992), α-haloacetophenones (Carvalho et al. 1991, Aleixo et al. 1993), and the enantioselective synthesis of (R)-(-)-1-phenylethanolamines (Brenelli et al. 1992). Lipase from *Candida cylindracea* was also immobilized on chrysotile and used successfully in organic synthesis (Lima et al. 1996).

In the present work we used chrysotile as a support for the immobilization of different lipases (lipase from *Mucor javanicus*, *Pseudomonas cepacia*, *Rhizopus oryzae*, *Candida rugosa* and *Aspergillus niger*) in the production of esters. They are very important flavour compounds and are widespread in nature in a great variety of foodstuffs.

2 EXPERIMENTAL

2.1 Materials and Methods

The lipases used in this work were from *Pseudomonas cepacia* (Amano PS) with specific activity of 30,000 U/g solid, from *Candida rugosa* (AY Amano 30) with 30,000 U/g solid, from *Aspergillus niger* (Amano 12) with 12,000 U/g solid, from *Mucor javanicus* (Amano 10) with 10,000 U/g solid and from *Rhizopus oryzae* (F-AP 15) with 150,000 U/g solid. Enzymes were kindly supplied by Amano Enzymes Pharmaceutical Co. The alcohols methanol, ethanol, 1-butanol, 1-pentanol, 1-octanol and hexanoic, octanoic and lauric acid were from Vetec (Brazil). All solvents and reagents were analytical grade. The chrysotile type 5RL was supplied by the SAMA'S mine (Goiás – Brazil). The ^{1}H NMR spectra were recorded in $CDCl_3$-solutions with TMS as reference on a Bruker AC 200 spectrometer. Infrared spectra were obtained by using a Perkin-Elmer model 16PC FTIR spectrophotometer. The amount of lipase adsorbed by chrysotile was estimated by UV spectra, on a Metrolab 1700 spectrometer.

2.2 Immobilization Process

The preparation of chrysotile for immobilization of the enzymes has been described previously (Jesus et al. 1998). The lipases from *Pseudomonas cepacia*, *Candida rugosa*, *Aspergillus niger*, *Mucor javanicus* and *Rhizopus oryzae* were immobilized on chrysotile as follows: 0.2g of enzyme in 100 mL of sodium phosphate buffer (pH 7.2) was added to 1.0g of chrysotile and the resulting suspension was shaken for 24h at 25°C. The suspension was filtered under vacuum and the solid was air dried.

2.3 Esterification Reaction

1g of chrysotile with immobilized enzyme (140 mg of lipase) was transferred to an erlenmeyer with 25 mL of hexane. Equimolar amounts (0,01 mol each) of the reactants (acids: hexanoic, octanoic and lauric; alcohols: methanol, ethanol, 1-butanol, 1-pentanol and 1-octanol) were added to the erlenmeyer. The mixtures were shaken in an incubator at 25°C for 48 hours. The reactions were monitored by ^{1}H NMR and Thin-Layer Chromatography (TLC) analysis of samples taken periodically from the reaction mixtures. Isolation of the esters was performed by column chromatography with silica gel 60 (70-230 mesh) and hexane:ethylacetate 15:1 as eluent (Figure 1).

Control reactions were done under the same experimental conditions using chrysotile without adsorbed enzyme.

3 RESULTS AND DISCUSSION

Five different lipases (*Rhizopus oryzae*, *Aspergillus niger*, *Candida rugosa*, *Mucor javanicus* and *Pseudomonas cepacia* lipases) were immobilized on chrysotile and used in the esterification of hexanoic, octanoic and lauric acid with methanol, ethanol, 1-butanol, 1-pentanol and 1-octanol as shown in Figure 1. The amount of lipase adsorbed by chrysotile was 70%, with the exception of *Aspergillus niger*

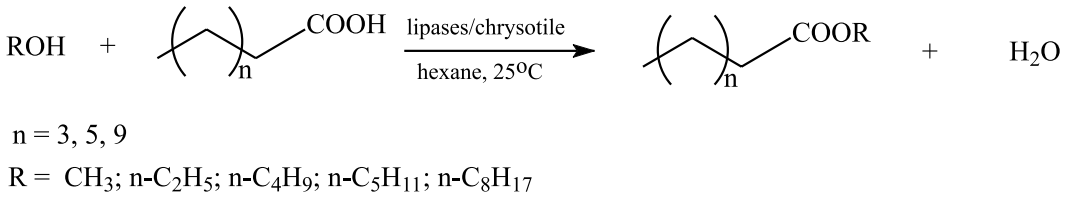

Fig. 1 – Esterification reaction catalyzed by lipases immobilized on chrysotile.

lipase with 30% immobilized. The results obtained for the esterifications with the different lipases immobilized on chrysotile are shown in Table I and II.

TABLE I

Esters obtained by the esterification catalyzed by *Mucor javanicus* lipases immobilized on chrysotile at 25°C.[(a)]

Acids	Ester	Yields (%)[b]
hexanoic	methyl hexanoate	49
	ethyl hexanoate	41
	n-butyl hexanoate	64
	n-pentyl hexanoate	66
octanoic	methyl octanoate	64
	ethyl octanoate	69
	n-butyl octanoate	76
	n-pentyl octanoate	77
	n-octyl octanoate	78
lauric	methyl laurate	62
	ethyl laurate	65
	n-butyl laurate	97
	n-pentyl laurate	82
	n-octyl laurate	84

(a) reaction conditions: time 48 hours, 0,01 mol substrate (1:1), solvent hexane (under agitation); (b) Yields of esters isolated by chromatography using as eluent hexane:ethylacetate (15:1).

It can be observed that the best results were achieved with lauric acid, with yields from 62% to 97%. Carboxylic acids with more then 8 carbons have been shown to be a better substrate for lipases (Jesus et al. 1997). From Table II it can be seen that, except *Aspergillus niger* lipase, all lipases exhibited good catalytic activity in the esterification reaction, differing in the yields of esters. The poor catalytic performance of *Aspergillus niger* lipase is probably due to its low amount adsorbed on chrysotile (60 mg) and its low catalytic activity. The adsorption process can influence the conformation of the lipase and therefore its catalytic activity.

The *Mucor javanicus* lipase showed to be more efficient than the other lipases when immobilized on chrysotile. Concerning the different acids employed, it can be seen from Table I that the most lipophilic lauric acid reacted with higher yields than the other acids. With lipases from *Pseudomonas cepacia* and *Mucor javanicus* n-butyl laurate was obtained with yields superior to 80%.

The esters formed by the lauric acid are, in most cases liposoluble, due to their long carbon chains. Short and long chain esters produced by enzymatic synthesis may be applied as flavors, solvents, lubricants or plasticisers (Rocha et al. 1999). Concerning the different alcohol substrates, it can be seen from Table I that the best yield was achieved with 1-butanol. The reaction was done using chrysotile without adsorbed enzyme and no reaction was observed.

An important parameter of an immobilized enzyme preparation, in general, is the preservation of its catalytic activity throughout the immobilization procedure and the retention of this activity over prolonged periods of time. This study was accomplished for the esterification reaction of lauric acid and 1-pentanol catalyzed by *Mucor javanicus* lipase and the results can be observed in Table III, where

TABLE II

Esters obtained by the esterification catalyzed by differents lipases immobilized on chrysotile at 25°**C**.[(a)]

Lipase	Acids	Ester	Yields (%)[b]
Rhizopus oryzae	hexanoic	n-butyl hexanoate	76
	octanoic	n-butyl octanoate	98
	lauric	n-butyl laurate	56
Aspergillus niger	hexanoic	n-butyl hexanoate	n.d[c]
	octanoic	n-butyl octanoate	n.d[c]
	lauric	n-butyl laurate	n.d[c]
Candida rugosa	hexanoic	n-butyl hexanoate	53
	octanoic	n-butyl octanoate	70
	lauric	n-butyl laurate	68
Pseudomonas cepacia	hexanoic	n-butyl hexanoate	71
	octanoic	n-butyl octanoate	57
	lauric	n-butyl laurate	85

(a) reaction conditions: time 48 hours, 0,01 mol substrate (1:1), solvent hexane (under agitation); (b) Yields of esters isolated by chromatography using as eluent hexane:ethylacetate (15:1); (c) n.d.= not determined because of the low amount of ester obtained. Formation was evaluated by TLC chromatography.

TABLE III

Reuse of lipase from *Mucor javanicus* **immobilized on chrysotile in the esterification reaction of 1-pentanol with lauric acid at** 25°**C**.[(a)]

Alcohol	Ester	Used	Yields (%)[b]
1-pentanol	n-pentyl laurate	1	82
		2	80
		3	25
		4	24

(a) reaction conditions: time 48 hours, 0,01 mol substrate (1:1), solvent hexane (under agitation); (b) Yields of esters isolated by chromatography using as eluent hexane:ethylacetate (15:1).

it can be seen that after the first reuse the yields of esters lowered by 25%. As for the little reuse of the support, it probably does not result from leaching, as no desorption was observed. Probably, a modification in the enzyme structure related to the reuse process must be affecting the lipase conformation necessary to the catalysis, therefore, inactivating part of the enzyme molecule.

To compare the effect of the immobilization of the lipases the reaction of esterification of the

lauric acid and 1-butanol was repeated in the same experimental conditions, 48 hours at 25°C, using free lipases in hexane. No product formation was observed. Ahmad et al. (1998) have demonstrated that organic solvents may affect the reaction by direct by interacting with the enzyme. Enzymes need a small amount of water to retain their active three-dimensional conformational state.

The catalytic performance of *Mucor javanicus* lipase to produce n-pentyl laurate in different organic solvents was observed, using the log P as the polarity measurement (in which P is the partition coefficient between 1-octanol and water). In most cases, the enzymatic activity is low in relatively hydrophilic solvents, with log P < 2; moderate in solvents with log P between 2 and 4; and high in apolar solvents, where log P > 4 (Chen and Sih 1989). The n-pentyl laurate yield was 82% in hexane (log P = 3,5), 70% in cyclohexane (log P = 3,2), 68% in toluene (log P = 2,5) and no ester formation in dichloromethane (log P = 0,93), acetone (log P = –0,23) and acetonitrile (log P = –0,33) was observed.

4 CONCLUSION

Lipases of different sources were immobilized on chrysotile and applied successfully in the production of esters in organic media. With the exception of *Aspergillus niger* lipase, enzymes kept their catalytic activity. The use of immobilized enzymes on chrysotile may present many advantages such as simple performance and design of the bioreactor, facile recovery of the products, ready reuse of the biocatalyst and operational stability of the enzyme. Lipases act at the interface between hydrophobic and hydrophilic regions and, therefore, the immobilization in good support helps the biocatalysis.

The use of chrysotile with adsorbed lipases is, thus, a very attractive and viable alternative for enzymatic reactions in organic media.

5 ACKNOWLEDGMENTS

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Editor's note: Chrysotile is now banned for any industrial applications in many parts of Brazil, due to concerns with its alleged toxicity.

RESUMO

Neste trabalho foi estudada a síntese de ésteres catalisada por lipases imobilizadas em crisotila (asbesto), em meio orgânico. Lipases de diferentes fontes (*Mucor javanicus*, *Pseudomonas cepacia*, *Rhizopus oryzae*, *Aspergillus niger* e *Candida rugosa*) foram imobilizadas em crisotila, um silicato magnesiano de baixo custo, e utilizadas na esterificação dos ácidos hexanóico, octanóico e láurico com metanol, etanol, 1-butanol, 1-pentanol e 1-octanol em hexano como solvente, a 25°C. Os melhores resultados foram obtidos com a lipase de *Mucor javanicus* e o ácido láurico, obtendo-se ésteres com rendimentos entre 62 e 97%.

Palavras-chave: asbesto, crisotila, lipases, ésteres.

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