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Immobilization of *Thermomyces lanuginosus* Lipase in PVA-alginate Beads

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Abstract. *Thermomyces lanuginosus* lipase was immobilized in PVA-alginate beads, obtaining immobilization % in the range of 94.4-98.4% using PVA concentrations ranging from 11% to 12.5%, and with cross linking times of 45 and 60 min using boric acid. Initial reaction rate was determined in free and immobilized state by hydrolysis of p-nitrophenol palmitate. Operational stability at different pH (4-7), agitation (100-500 r.p.m.), and temperature (40-80 °C) was investigated. Results showed that pH 6 and 7 no considerable loss of enzyme activity or enzyme was observed. At temperatures over 70 °C, enzyme suffers physical damage and showed a considerable loss of activity. No significant difference was observed when agitation was varied from 100 to 500 r.p.m.
Keywords: lipase, immobilized, PVA-alginate, *Thermomyces lanuginosus*.

Resumen. Se inmovilizó la lipasa de *Thermomyces lanuginosus* en esferas de PVA-alginato, obteniendo un porcentaje de inmovilización del 94.4% al 98.4% utilizando concentraciones de PVA del 11% al 12.5%, y con tiempos de entrecruzamiento con ácido bórico de 45 y 60 min. Se determinó la velocidad inicial de forma libre e inmovilizada mediante la hidrólisis del p-nitrofenol palmitato. Se llevó a cabo un estudio de estabilidad operacional a diferente pH (4-7), velocidad de agitación (100-500 r.p.m.) y temperatura (40-80 °C). Los resultados mostraron que a pH 6 y 7 no se observó una considerable pérdida de actividad enzimática o de enzima. A temperaturas arriba de los 70 °C el biocatalizador sufrió daño físico y la enzima mostró una considerable pérdida de actividad enzimática. En la velocidad de agitación no se observó una diferencia significativa cuando se varió la velocidad de agitación de 100 a 500 r.p.m.
Palabras clave: lipasa, inmovilizada, PVA-alginato, *Thermomyces lanuginosus*.

Introduction

Enzymes present remarkable features, like unusual diversity and versatility. They catalyze specific reactions involving only one or a few compounds that are structurally related and distinguish almost completely between isomers and regioisomers. This property enables biotransformation reactions to yield a wide range of useful biological and chemical compounds as tools for pharmaceutical, food, and agrochemical derivatives. In addition, the fraction of unwanted intermediates and byproducts is reduced giving products of high purity and improved quality [9].

Lipases (triacyl glycerol acyl hydrolases, E.C. 3.1.1.3), which are typically thought of in terms of their hydrolysis of triacylglycerols to glycerol and free fatty acids, possess the unique feature of acting at an interface between aqueous and non-aqueous phases. Furthermore, they have exceptional characteristics for catalysis of various types of reaction with high specificity, including those of hydrolysis of esters and organic synthesis reactions such as esterification, transesterification and regioselective acylation, these synthesis reactions requiring water-restricted environments in order to occur [12].

To fully exploit the technical and economical advantages of lipases, it is recommended to use them in an immobilized state to reduce the cost and the poor stability of the soluble form [10]. Immobilization also facilitates the separation of products, enhances lipase properties such as thermostability and activity and provides more flexibility with enzyme/substrate contact by using various reactor configurations [13].

Several methods have been reported, such as deposition on solid supports [22, 24, 25], covalent binding [3, 2] and entrapment within a hydrophobic sol-gel material [16, 26] or within a polymer matrix. The latter method has been more widely used to variety of lipases [8, 25].

Recently, a synthetic polymer, polyvinyl alcohol (PVA), which is cheap and non-toxic to microorganisms, has been used for cell and enzyme immobilization [3, 25, 7]. A new method of immobilization of activated sludge using PVA cross-linked with boric acid was developed [14]. This immobilization method has also been used for biomass fixation (activated sludge and pure cultures) for wastewater treatment such heavy metals and sulphate oxidation [18, 21, 15].

Thermomyces lanuginosus (previously *Humicola lanuginosa*) lipase (TLL) is the enzyme responsible for the lipolytic activity of Lipolase®, a commercial lipase preparation of Novozymes Corp. that has important industrial applications. In aqueous media the enzyme is very stable, being active over the pH range of 7-11. It maintains activity reasonably well at 55-60 °C although the recommended temperature for applications is between 30 and 40 °C [12]. However, the immobilization of this lipase in beads of PVA-Alginate has been little explored.

In this work *Thermomyces lanuginosus* lipase immobilized in beads of PVA-Alginate using boric acid as crosslinking agent were screened for hydrolysis of p-Npp (p-Nitrophenol palmitate) at different pH (4-7), agitation (100-500 rpm), and temperature (40-80 °C).

Results and discussion

Hydrolysis rate of p-NPP at different concentrations of free enzyme

Figure 1 shows initial hydrolysis rate of p-NPP using free enzyme at different concentrations. An increase in initial hydrolysis rate was observed in the range of 0.05 to 0.25 mg ml^{-1}, with maximum hydrolysis rate of 9.8×10^{-3} mmol/min. Enzyme concentrations higher than 0.25 mg mL^{-1} did not improve hydrolysis rate, further more at a concentration of 0.65 mg hydrolysis rate decrease as low of 5.4×10^{-3} mmol/min.

Such behavior is quite different from the typical aqueous systems, in which the reaction rate is normally first-order in enzyme concentration. This type of behavior was also found by Fernandes *et al*. (2004), reporting that a high enzyme concentration in the system led to an aggregation of the molecules, which hinders catalysis and consequently decreases the specific activity.

Immobilization of Thermomyces lanuginosus lipase in PVA-alginate beads

Thermomyces lanuginosus lipase was immobilized by the PVA-alginate-boric acid method. The enzyme gets entrapped in a PVA-boric acid gel grille. After the enzyme-polymer mixture were dropped in the treatment mixture (saturated boric acid solution containing 2% calcium hydroxide), spherical gel beads were formed without agglomeration, which exhibited rubber like elastic properties. PVA contributed strength and durability to the beads, whereas calcium alginate improved the surface properties, reducing the tendency to agglomerate. Three different ratio percentage of PVA:alginate were used 11:1, 12:1 and 12.5:1.5 (Tables 1 and 2). In the preparation of the lipase immobilized PVA beads, contact time with the boric acid affects gel strength greatly, so two different crosslinking times were tested (45 and 60 min). Boric acid is consumed during the PVA-gelling reaction. Thus, an excess amount of the boric acid is required for a rapid progression of PVA polymerization. First, the gelling reaction occurred immediately on the surface of the immobilized beads. Subsequent gelling reaction inside the beads was accomplished with the further diffusion of the boric acid into the beads. Results shows that using 11% PVA and 1% alginate, with a crosslinking time of 45 minutes has the highest initial hydrolysis rate of p-NPP (7.1×10^{-3} mmol/min $\pm 2 \times 10^{-4}$), and with percentages of 12% and 12.5% of PVA provides greater immobilization percentage but lower initial hydrolysis rate. For the case of 60 minutes crosslinking time, similar results were obtained for immobilization percentage and initial hydrolysis rate of p-NPP. For this reasons 11%:1% of PVA:alginate with 45 minutes of crosslinking was selected for operational stability evaluation.

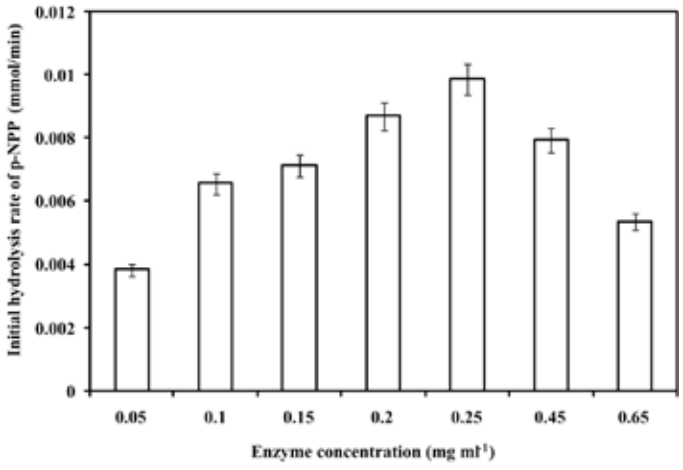

Figure 1. Initial hydrolysis of p-NPP rates at different concentrations of free enzyme (pH 7 and 40°C).

Table 1. Initial hydrolysis rate of p-NPP and immobilization percentage at different ratios of PVA: sodium alginate with 45 minutes of crosslinking.

PVA:sodium alginate	Initial reaction rate (mmol/min)	Immobilization Percentage
11:1	$7.1 \times 10^{-3} \pm 2.1 \times 10^{-4}$	94.5% ± 0.1
12:1	$6.5 \times 10^{-3} \pm 4.5 \times 10^{-4}$	97.1% ± 0.4
12.5:1.5	$5.4 \times 10^{-3} \pm 2.6 \times 10^{-4}$	98.3% ± 0.1

Table 2. Initial hydrolysis rate of p-NPP and immobilization percentage at different ratios of PVA: sodium alginate with 60 minutes of crosslinking.

PVA:sodium alginate	Initial reaction rate (mmol/min)	Immobilization percentage
11:1	$4.3 \times 10^{-3} \pm 2.0 \times 10^{-4}$	94.5% ± 0.1
12:1	$6.5 \times 10^{-3} \pm 1.5 \times 10^{-4}$	97.4% ± 0.2
12.5:1.5	$6.5 \times 10^{-3} \pm 3.8 \times 10^{-4}$	98.4% ± 0.2

Operational stability of immobilized lipases

Effect of pH on the initial hydrolysis rate of p-NPP

Different pH levels were assessed to determine the effect over initial hydrolysis rate of p-NPP. During this stage it was observed that at pH 4 and 5 support structure suffered damage, resulting in a transparent sphere and lowering resistance, this could be attributed to the buffer. This observation was confirmed by Nunes et al. 2010, where PVA was dissolved almost completely after transferring the beads into acetate buffer. Also are reports which mention that phosphate and citrate buffers have a negative effect on the stability of alginate beads [11].

Initial hydrolysis rate of p-NPP was higher for case of pH 7 with an initial hydrolysis rate of 4.8×10^{-3} mmol/min, as initial pH lowered also the initial hydrolysis rate did it, being 4.3×10^{-3} mmol/min for pH 6 and observing an important decrease as low as 7.2×10^{-4} mmol/min and 9.1×10^{-4} mmol/min for the cases of 4 and 5 respectively (Figure 2).

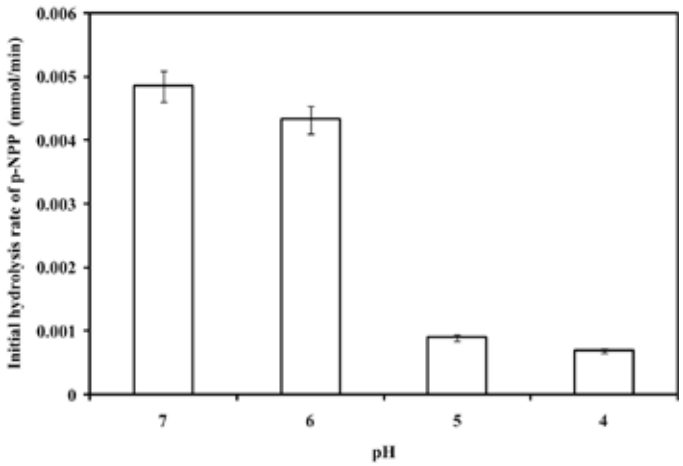

Figure 2. Effect of pH on the initial hydrolysis rate of p-NPP at 40°C and 100 r.p.m.

Effect of agitation on the initial hydrolysis of p-NPP rate
Effect of agitation rate over initial hydrolysis rate of p-NPP was investigated at pH 7. No significant difference was observed for all cases studied, initial hydrolysis rate of p-NPP was $4.7 \times 10^{-3} \pm 3.7 \times 10^{-4}$ mol/min, and moreover enzyme released after 24 hours was the same amount for all cases (6.2 ± 0.1%) regardless of agitation rate. This phenomenon can be attribute to immobilized method used on this study, enzyme encapsulation in the polymer matrix of PVA-alginate offers an advantage avoiding the release of enzyme due to high agitation rates. Figure 3 shows results obtained on this experimental stage.

Effect of temperature on the initial hydrolysis of p-NPP rate
The effect of temperature of immobilized enzyme system over the initial hydrolysis rate of p-NPP was investigated; temperature range used on this stage was 40-80°C and pH 7. The immobilized enzyme presented maximum initial hydrolysis rate of p-NPP at 40 °C (3.7×10^{-3}). At higher temperatures initial hydrolysis rate of p-NPP decreased importantly, being 60 °C (2.5×10^{-3}) maximum temperature at which operational stability of the PVA-alg beads were viable. Temperature higher than 60°C caused severe damage to physical structural PVA-alg beads, spherical shape was completely deformed and virtually dissolved in the buffer, thereby preventing the subsequent determination of protein in order to determine the rate of release of the immobilized enzyme after 24 hours at these temperatures. Figure 4 shows effect of temperatures over initial hydrolysis rate of p-NPP at different temperatures.

Conclusions

Results obtained on the present study showed that PVA-alginate-boric acid method is an efficient method for entrapment of *Thermomyces lanuginosus* lipase. Highest hydrolysis rate of p-NPP (7.0×10^{-3} mmol/min $\pm 2 \times 10^{-4}$) was obtained when using 11% PVA and 1% alginate, with a crosslinking time of 45 minutes. Operation stability of immobilized enzyme was optimum when conditions were as follow: pH in the range of 6 to 7 and temperature 40 to 60 °C. For the case when agitation rate was varied, the operation stability didn't show any significant differences for (100-500 rpm). Results obtained from present study have lead to undergoing research by our group to optimize fatty acids methyl esters (FAME's) production using immobilized lipase under reaction condition mentioned above.

Experimental Part

Materials

Thermomyces lanuginosus lipase, polyvinyl alcohol (PVA) (98-99% of hydrolysis, average MW 14,000), sodium alginate and

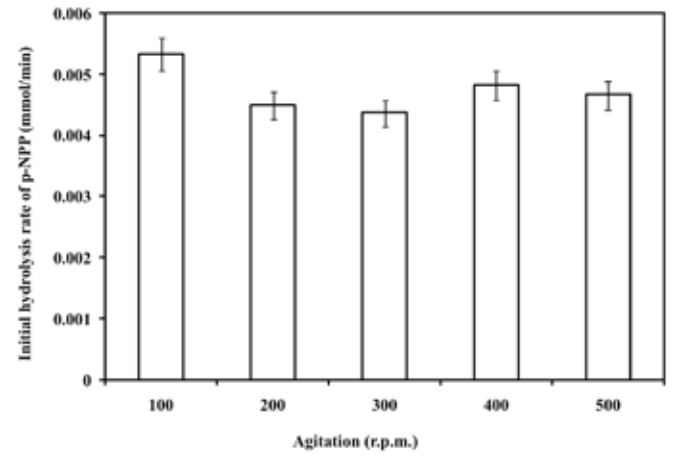

Figure 3. Effect of agitation on the initial hydrolysis of p-NPP rate at 40°C and pH 7.

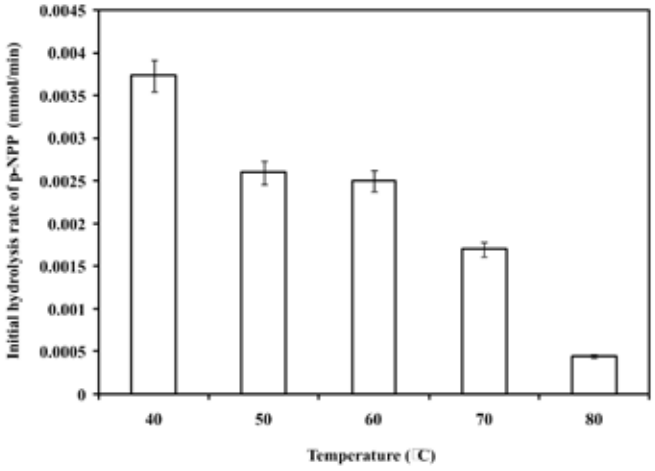

Figure 4. Effect of temperature on the initial hydrolysis of p-NPP rate at pH 7 and 100 r.p.m.

p-NPP (p-nitrophenol palmitate) were purchased from Sigma Aldrich. All other reagents used were of analytical reagent grade unless otherwise indicated.

Hydrolysis of p-Npp rate at different concentrations of free enzyme

Initial rate of hydrolysis of p-NPP at different enzyme concentrations was performed to choose the amount of enzyme which produces the highest initial rate and then continue with the immobilization. Different lipase (*Thermomyces lanuginosus)* concentrations were assessed (0.05, 0.1, 0.15, 0.2, 0.25, 0.45 y 0.65 mg mL^{-1}). Solutions were prepared on 10 mL test tubes, adding 2.5 mL of Tris-HCl buffer (50 mM, pH 7), 20 µL of p-Npp 50 mM in acetonitrile and different enzyme concentrations mentioned above, with a final substrate concentration of p-NPP (0.3 mM) in the reaction mixture. Solutions were incubated at 40 °C and p-NPP concentration released was measured every 5 minutes during the first 30 minutes by spectrophotometer technique (Spectronic Unicam Genesys™ 8) at 400 nm, the method followed was the same as that previously described by Ciuffreda et al. 2001. The variation in the absorbance at 400 nm of the assay against a blank without enzyme was monitored. The concentration of p-nitrophenol (p-NP) released was calculated from the slope of the absorbance curve vs. time by using a millimolar extinction coefficient of 12.5 $mM^{-1}cm^{-1}$ for p-nitrophenol. This value was determined by measuring the absorbance of different standard solutions of p-NP.

Immobilization of Thermomyces lanuginosus lipase in PVA-alginate beads

Immobilization was carried out varying the concentration of PVA, sodium alginate and crosslinking time (Table 3).

The solution (water and PVA) was heated at a temperature of 80 °C to dissolve PVA and sodium alginate solution. The PVA-alginate solution was then cooled to a temperature of around 40 °C, followed by addition of 1 ml of lipase solution (0.25 mg ml^{-1}) and mixed thoroughly. The mixture was dripped into a solution of saturated boric acid neutralized with calcium hydroxide with crosslinking time correspondent.

The beads were washed with distilled water to remove any excess boric acid and stored in Tris-HCl buffer pH 7. Total protein was quantified after washing process.

Operational stability of immobilized lipases

In order to investigate the operational stability of immobilized lipases on the initial hydrolysis of p-NPP rate, different parameters were varied as describe as follow: effects of buffer pH (4, 5, 6 and 7; acetate and tris —HCl 50 mM) Activity determinations at higher pH were not performed because of the difficulties in rate estimation caused by the spontaneous hydrolysis of pNPP, most lipases reported in the literature have optimal activity at neutral or slightly basic pH values (Aryee et al., 2007; Castro-Ochoa et al., 2005; Lima et al., 2004; Segura et al., 2006); agitation (100, 200, 300, 400 and 500 rpm) and temperature (40, 50, 60, 70 and 80 °C). 200 ml jacketed glass bottles were used. Immobilized enzyme (0.25 mg) was placed in reactors (jacketed glass bottles). Total protein released in to the buffer and initial hydrolysis rate were quantified after 24 hours for all cases. Protein was determined according to Lowry modified by Peterson [19].

Table 3. Conditions used during immobilization procedure of *Thermomyces lanuginosus* lipase.

PVA (%)	*Sodium alginate (%)*	*Crosslinking time (min)*
11	1	45, 60
12	1	45, 60
12.5	1.5	45, 60

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