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Production and immobilization of partially purified lipase from *Penicillium chrysogenum*

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ABSTRACT

An extracellular lipase from *Penicillium chrysogenum* produced maximal activity 225 U/mL after four days at pH 6.5. It was partially purified 4.1 fold by ammonium sulphate precipitation (70%). The enzyme was immobilized on various carriers viz. alginate, k-carrageenan and polyacrylamide gel. The immobilization yield of enzyme immobilized in k-carrageenan and polyacrylamide gel (63.41% and 48.93% respectively) was low in comparison to that immobilized with alginate (81.57%). Different concentrations of alginate were tried to study their effect on lipase production. Maximum immobilization yield was observed with 3% alginate. The optimal pH of the partially purified lipase was 7.5 and the optimum temperature was 35 °C. At 60 °C the immobilized enzyme retained 62.79% of its activity. Broader pH tolerance and higher heat stability could be achieved by this method. Immobilized lipase retained 72.09% relative activity after six hydrolysis cycles.

Keywords: lipase, *Penicillium chrysogenum*, immobilization, alginate, k-carrageenan, polyacrylamide

INTRODUCTION

In their natural environment, lipases (EC 3.1.1.3) catalyze the hydrolysis of esters formed from glycerol and long-chain fatty acids (Sharma *et al*., 2001; Rodrigues *et al*., 2008). However, under appropriate experimental conditions, these enzymes are also very active biocatalysts for the esterification of fatty acids, alcoholysis and transesterification reactions (Balcão *et al*., 1996; Pandey *et al*., 1999). Many lipases from microbial sources have been investigated and found to be promising catalysts for the hydrolysis and synthesis of fats and oils. Some of the commercially important lipase producing fungi are: *Rhizopus arrhizus, Rhizopus japanicus, Mucor miehei, Aspergillus niger, Rhizopus niveus, Candida rugosa* (Benjamin and Pandey, 1998; Ellaiah *et al*., 2004), *Mucor* sp. (Abbas *et al*., 2002), *Aspergillus terreus* (Gulati *et al*., 1999), *Penicillium* sp. (Pimentel *et al*., 1997; Lima *et al*., 2003).

Versatility of lipase leads to multiple industrial applications in food and flavor making, pharmaceuticals, synthesis of carbohydrate esters, amines and amides biodetergents and recently cosmetics and perfumery. In order to use them more economically and efficiently in aqueous as well as in non-aqueous solvents, their activity, selectively and operational stability can be modified by immobilization (Hung *et al.*, 2003).

Immobilization of enzymes is the key to expand the applications of these natural catalysts by enabling easy separation and purification of products from reaction mixtures and efficient recovery of enzyme proteins. The most explored approach to immobilize enzymes has involved the use of solid supports via a variety of mechanisms such as adsorption on hydrophobic supports (Palomo *et al*., 2007; Blanco *et al*., 2007) or entrapment encapsulation (Kawakami and Yoshida, 1996; Wang and Hsieh, 2008) or covalent attachment to solid supports (Knezevic *et al*., 2006; Lozano *et al*., 2007; Foresti and Ferreira, 2007).

With immobilized enzymes, improved stability, reuse, continuous-operation, possibility of better control of reaction, high purity and product yields and hence more favourable economic factors can be expected (Ruckenstein and Wang, 1993).

Entrapment, one of the immobilization techniques, can be defined as physical restriction of enzyme within a confined space or network. Gelation of polyanionic or polycationic polymers by the addition of multivalent counter-ions is a simple and common method of enzyme entrapment. Alginates are one of the most frequently used polymers due to their mild gelling properties and non-toxicity. Alginate is an anionic linear copolymer composed of 1,4-linked β-D-mannuronic acid and α-L-gluronic acid in different proportions and sequential arrangements (Won *et al*., 2005).

Most lipases used in biotechnological applications are produced by fungi and bacteria. Filamentous fungi are interesting sources of lipases because they produce extracellular lipases. In this regards, the lipase of *Penicillium* sp. is among the well-known lipase producers mainly used in the dairy industry (Pimentel *et al*., 1994; 1997).

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The present paper deals with the production, partial purification and the immobilization of *P. chrysogenum* lipase using different entrapment techniques with matrices calcium alginate, *k*-carrageenan and polyacrylamide gel.

MATERIALS AND METHODS

Microorganism

P. chrysogenum used in the present work was kindly obtained from Natural and Microbial Products Chemistry Dept., National Research Centre, Cairo, Egypt.

Media and growth conditions

Culture media

P. chrysogenum was cultured in the following media (g/L in distilled water): yeast extract 5, olive oil 10, KNO_3 2, $MgSO_4 \cdot 7H_2O$ 0.5, K_2HPO_4 1, $ZnSO_4 \cdot 7H_2O$ 0.44, $FeSO_4 \cdot 7H_2O$ 1.1, $MnSO_4 \cdot 7H_2O$ 0.2; and $(NH_4)_2SO_4$ 10 at pH 7.0 using phosphate buffer.

Cultivation

Cultures were carried out in Erlenmeyer flasks (250 mL) with 50 mL of medium. The inoculum was prepared in 100 mL Erlenmeyer flasks with 20 mL of medium. The cultures were inoculated with 1 mL of spores suspension (10^8 m/L) and the flasks were agitated at 120 rpm at 30 °C. After 48 h, the whole volume was transferred into 250 mL Erlenmeyer flasks with 50 mL of liquid using the same conditions of temperature and agitation. At time intervals, samples were withdrawn from the growth medium and then filtered to get the sample without cells (Lima *et al*., 2003).

Protein estimation

The protein content of the enzyme preparations was determined according to the method described by Lowry and coworkers (1951).

Biomass estimation

The mycelial biomass obtained after growth, was separated by filtration, washed by distilled water and dried. It was dried in an oven 60 °C and reweighed till constant weight.

Partial purification by *P. chrysogenum* lipase

The culture broth harvested at the end of cultivation period mycelia was centrifuged at 10,000 x *g* for 10 min at 4 °C. The supernatant obtained was fractionated with 70% ammonium sulphate and kept standing overnight at 4 °C. The precipitate was collected by centrifuginge at 10,000 x *g* for 5 min at 4 °C, dialysed against distilled water. The dialyzed enzymatic fraction was subjected to protein and enzyme immobilization studies (Kanwar and Goswami 2002).

Immobilization of partially purified lipase

Calcium alginate beads

Two milliliter of lipase solution was mixed with 20 mL of sodium (1, 2, 3 and 4% w/v) and then the mixture was stirred thoroughly to ensure complete mixing. As soon as the mixed solution was dripped into 40 mL of $CaCl_2$ solution with a syringe, Ca-alginate beads were formed. After 20 min of hardening the beads were collected by filtration, then washed with tris-HCl buffer (0.2 M pH 7.5) several time to remove the unbound enzyme (Won *et al*., 2005).

k-Carrageenan entrapment method

Eighteen milliliter of k-carrageenan (4% w/v) maintained at 40–50 °C was mixed with 2 mL of lipase solution, poured into sterile 4 inch diameter Petri plates and allowed to solidify. It was then cut into equal small blocks. The k-carrageenan was cured in 2% KCl for 1 h in the refrigerator. These blocks were washed thoroughly two or three times with sterile distilled water (Ellaiah *et al*., 2004).

Polyacrylamide gel entrapment method

Two milliliter of lipase solution was added to 10 mL chilled sterile water. To 10 mL sterile potassium phosphate buffer (pH 7.0, 0.2 M) the following chemicals were added: acrylamide 2.85 g, bisacrylamide, 0.15 g; ammonium persulphate, 10 mg and 1 mL N,N,N^1,N^1-tetramethyl ethylenediamine (TEMED). The chilled lipase solution and chilled potassium phosphate buffer were mixed well and poured into sterile flat bottom 4 inch diameter Petri plates. After polymerization the acrylamide gel was cut into equal size cubes. The acrylamide cubes were cured in sodium phosphate buffer (pH 7.0, 0.2 M) for 1 h in the refrigerator. These cubes were washed thoroughly two to three times with sterile water (Ellaiah *et al*., 2004).

Determination of pH and temperature effects on the lipolytic activity

The optimal pH for free and immobilized enzyme activity was determined by incubating the enzyme-substrate at various pH from 5–6.5 using 0.1 m phosphate buffer and from 7–8.5 using 0.1 m Tris-HCl buffer. The effect of pH stability on lipase activity was measured with the enzyme (free and immobilized) previously incubated during 1 h at the desired pH and the residual activity was assayed at pH 7.5. The optimal temperature for both free and immobilized enzymes was determined by incubating the reaction mixture at 25, 30, 37, 40, 45, 50 °C. Controls were performed with boiled enzyme. For thermal stability study, the enzyme (free and immobilized) was incubated at various temperatures for 1 h and the residual activity was measured.

Assay for lipase activity

A suitable volume (1 mL) of the culture filtrate was mixed with 2.5 mL of deionized water and 1 mL of to 0.1 M Tris-HCl buffer (pH 7.5). 3 mL of 10% (v/v) triglyceride emulsion prepared in 10% (w/v) gum arabic by homogenizing in top drive homogenizer for 10 min were then added.

The mixture was incubated for 2 h at 35 °C in shaking water bath after which 10 mL of 99% acetone (absolute) was added. The resulting mixture was then titrated against 0.05 N NaOH using thymolphthalein indicators. It should be noted that NaOH was previously standardized against standard 0.05 N HCl using phenolphthaleine indicators. Blanks obtained on using boiled enzyme samples and the activities were obtained in terms of U/mL of enzyme solution. The lipase unit was defined as that produces 1 μmol of free fatty acids under assayed conditions (Parry *et al*., 1966).

Repeated use of lipase immobilized in the alginate beads

In order to test the stability of lipase entrapped in the ca-alginate beads, the beads were used several times for the hydrolysis reaction. Each run lasted for 2 h after which the beads were separated and washed with 0.1 M Tris-HCl (pH 7.5). The reaction medium was the replaced with fresh medium. The activity of freshly prepared beads in the first run was defined as 100%.

RESULTS AND DISCUSSION

Production of extracellular lipase from *P. chrysogenum* and the effect of pH

P. chrysogenum produced an extracellular lipase during growth on a medium containing olive oil as the sole carbon source.

The results recorded in Figure 1 indicated that the production of lipase increased steadily with the cultivation time and the best enzyme production, about 205 U/mL was reached at 4.50 g/100 mL dry weight after 4 days of cultivation. Maximum lipase production was observed during the iodophase of growth and then the activity declined. In our study, the secretion of lipase was induced by olive oil, as no other organic nutrient or carbon substrate other than olive oil was supplemented in the growth medium for the production of lipase. Lipase production is generally induced by the lipid substrates. The favorable effect of olive oil and oleic acid on lipase production has been observed by various researchers (Chahinian *et al*., 2000; Fadiloglu and Erkmen, 2002; Lima *et al*., 2003). However, the enzyme production decreased on increasing the incubation period showing lipase activity of 155 U/mL after 7 days of incubation. This may be attributed to the fact that lipase production has been frequently inhibited by the end products namely, glycerol and fatty acids (del Rio *et al*., 1990; Kanwar and Goswami, 2002).

With regard to the effect of different buffer values of the fermentation medium on lipase production (Table 1), phosphate buffer was used to adjust the fermentation medium for lipase production.

P. chrysogenum was able to grow and secrete lipase over a wide range of pH values, ranging from 4.0 to 9.0. Growth was maximal between pH 6.0–7.0 and lipase production was maximal between pH 6.5–7.0. A relatively small amount of the enzyme was detected at pH 9.0 (120 U/mL). The pH optima for extracellular lipase activity for a variety of *Penicillium* sp. have been investigated. The results agreed with those of Lebyreva and Marchenkova (1983) who adjusted the pH of the fermentation media at 7.0 for lipase production using *Penicillium roquefortii* BKMF-2019. Similar results were obtained by Sztajer and Maliszewka (1989) and Pimentel *et al*. (1994) who found that the best pH for lipase production was 7.2 and 7.5 respectively using *Penicillium citrinum*.

Partial purification of *P. chrysogenum* lipase

A summary of *P. chrysogenum* lipase partially purified is presented in Table 2. The lipase was partially purified about 4.10 fold over the crude extract with 60.44%

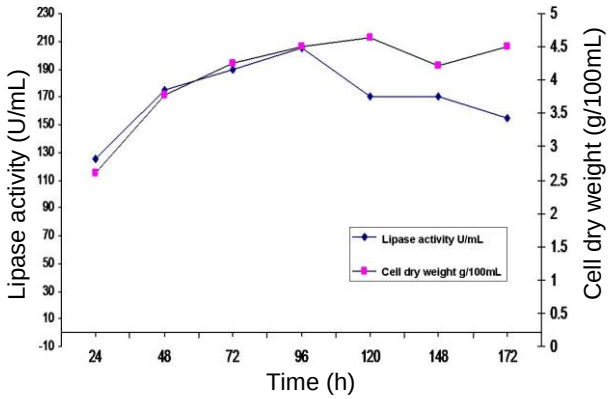

Figure 1: Effect of different incubation period on lipase activity by *P. chrysogenum*

Table 1: Effect of different buffered pH medium on lipase activity by *P. chrysogenum*

pH	Lipase activity (U/mL)	Cell dry weight (g/100mL)
4.0	140	2.84
4.5	165	3.01
5.0	175	3.25
5.5	190	3.82
6.0	210	4.00
6.5	225	4.20
7.0	205	4.50
7.5	160	4.41
8.0	155	4.33
9.0	120	4.30

Table 2: A profile of partial purified *P. chrysogenum* lipase

Purification steps	Collected volume (mL)	Protein (mg/mL)	Lipase activity (U/mL)	Total protein (mg)	Total activity units	S.E.A. (U/mg)	Recovery (%)	Purification fold
Crude filtrate	100	25	225	2500	22500	9.0	100	1.0
ppt by 70% ammonium sulphate	40	9.2	340	368	13600	36.95	60.44	4.10

Table 3: Immobilization of *P. chrysogenum* lipase using different carriers

Carriers	Enzyme added (U/g carrier A)	Unbound enzyme (U/g carrier B)	Bound Enzyme (U/g carrier I)	Immobilization yield %
Ca-alginate	280	90	155	81.57
k-Carrageenan	280	75	130	63.41
Polyacryl amide	280	45	115	48.93

Immobilization yield % = $\frac{I}{A-B}$ x 100

Table 4: Effect of alginate concentration using immobilized *P. chrysogenum* lipase

Alginate concentration (%)	Enzyme added (U/g carrier)	Unbound enzyme (U/g carrier)	Bound enzyme (U/g carrier)	Immobilization yield %
1	280	100	120	66.66
2	280	90	155	81.57
3	280	75	175	85.36
4	280	105	120	62.85

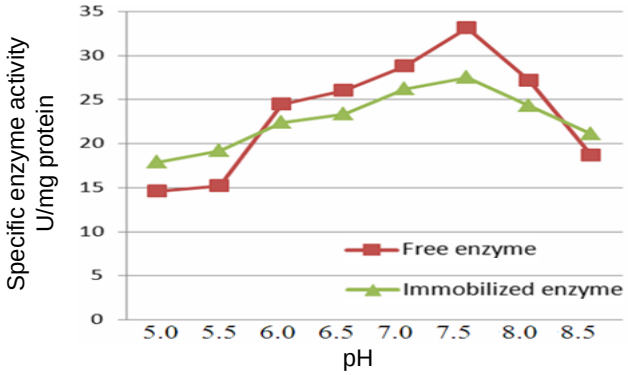

Figure 2: Effect of substrate pH on the specific enzyme activity of free and immobilized lipase

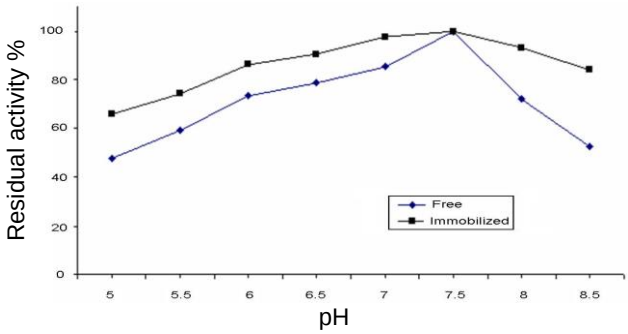

Figure 3: pH stability of the free and immobilized *P. chrysogenum* lipase

recovery using 70% ammonium sulphate for precipitation. The specific enzyme activity of the partially purified lipase increased to 36.95 U/mg proteins. Similar results were obtained by Shu *et al*. (2006) and Saxena *et al*. (2003) who used 70% ammonium sulphate in the partial purification of lipase.

Immobilization of *P. chrysogenum* lipase using different carriers

The experimental results of immobilized *P. chrysogenum* lipase using different various entrapment techniques are shown in Table 3. The immobilization yield of lipase entrapped in calcium alginate (81.57%) was higher than that of enzyme entrapped in k-carrageenan (63.41%) and polyacrylamide (48.93%). The low yield with k-carrageenan and polyacrylamide blocks may be due to the diffusional resistance of nutrients and oxygen into the matrices. These results are in accordance with studies on *A. niger* (Jamil and Omar, 1992; Ellaiah *et al*., 2004) and *Candida rugosa* (Ferrer and Carles, 1992). On the contrary, Hemachander and coworkers (2001) found that polyacrylamide gave the best performance using *Ralstonia pickettii*.

Therefore, alginate was considered to be the best matrix for the production of lipase and the partially purified enzyme entrapped by sodium alginate was used for further investigation.

Effects of alginate concentration

Due to the cross-linking between alginate and Ca^{2+} leads to gelation, alginate concentration is a major parameter for enzyme gel entrapment. Therefore effect of alginate concentration on immobilization yield was investigated. Alginate concentration was increased from 1 to 4% (w/v) maintaining the $CaCl_2$ concentration (2% w/v) As a result, the immobilization yield increased as shown in (Table 4) until it reached 85.36% at 3% alginate concentration. Immobilization yield decreased (62.85%) on increasing alginate concentration (4% w/v). This is due to the conformational changes in the entrapped enzyme and/or limitation of substrate transfer from the bulk phase into the alginate beads as alginate concentration increased (Knezevic *et al*., 2002).

Effect of pH on activity and stability of the free and immobilized *P. chrysogenum* lipase

Effect of pH on the specific enzyme activity of both free and immobilized lipase of *P. chrysogenum* was studied by varying the pH of the reaction medium from 5.0–8.5 and the pH profile is shown in Figure 2.

The lipase was found to be nearly alkaline lipase which showed higher specific enzyme activities for both free and immobilized enzyme at pH 7.5 and it retained nearly 76.74% (compared to pH 7.5) of the activity at pH 8.5 for immobilized lipase while 56.22% for free one. The variation of the residual activity of free and immobilized lipase with pH is shown in Figure 3. The immobilized lipase was stable in the pH range from 6.5 to 8.0. Free lipase was stable in the pH range from 6.5 to 7.5. This indicated that immobilization appreciably improved the stability of lipase in the neutral and alkaline region. Similar results were obtained on using immobilized *Candida rugosa* lipase on chitosan (Hung *et al*., 2003).

Effect of temperature on activity and stability of the free and immobilized *P. chrysogenum* lipase

The temperature dependence of the hydrolytic activity of free and immobilized lipase is shown in Figure 4. The optimum reaction temperature (35 °C) of the lipase was not altered by immobilization. The immobilized lipase demonstrated higher specific enzyme activities above 35 °C as compared to the free lipase. However denaturation of the enzyme occurred at 50 °C for immobilized lipase and at 45 °C for the free enzyme. These results indicate that lipase is more stable when immobilized in a matrix with higher hydrophobicity (Shaw *et al*., 1990).

Heat stability of the lipase entrapped in alginate matrices is much better than that of the corresponding free enzyme (Figure 5). Immobilized enzyme remained active up to 50 °C while the activity of the free enzyme started to decrease from 40 °C. At 60 °C the residual activity of the immobilized lipase was 62.79% compared to 52.45% for free enzyme.

These results are in accordance with the concept that immobilized lipase provided a more rigid external

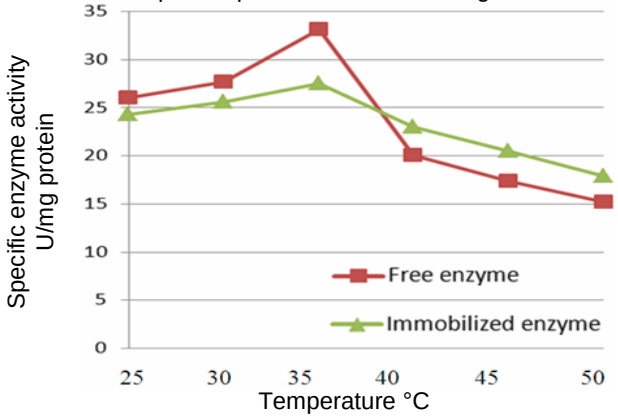

Figure 4: Effect of reaction temperature on the specific enzyme activity of free and immobilized *P. chrysogenum* lipase

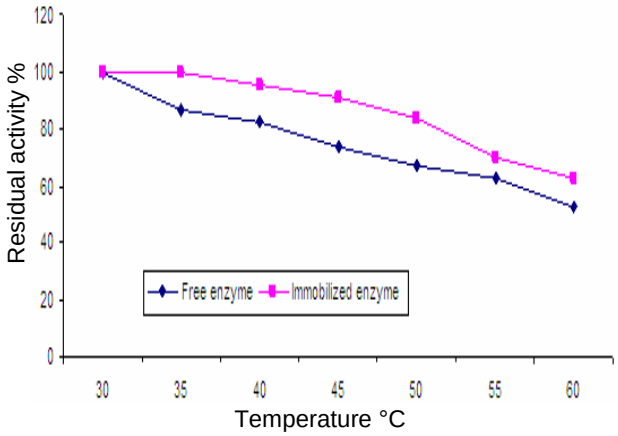

Figure 5: Thermal stability of free and immobilized lipase

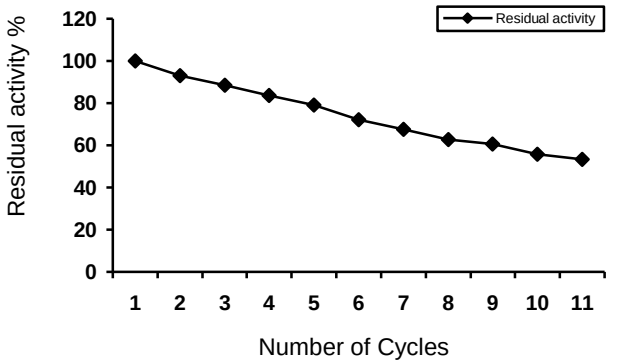

Figure 6: Effect of repeated use of immobilized *P. chrysogenum* lipase on residual activity at pH 7.5 and 35 °C

backbone for lipase molecules; the effect of higher temperature in breaking the interactions responsible for proper globular, catalytic active structure became less

prominent, thus increasing the thermal stability of the immobilized enzyme (Dove and Madamwar, 2006).

Reuse of the immobilized *P. chrysogenum* lipase

One of the important characteristics of an immobilized enzyme is its stability and reusability over an extended period of time.

The repeated use of the immobilized *P. chrysogenum* lipase in hydrolysis of olive oil was studied. The residual activity of the immobilized enzyme at repeated use is shown in Figure 6. After 6 reuses, immobilized lipase retained 72.09% of its activity. Hung and coworkers (2003) found that the repeated use for immobilized *C. rugos*a lipase on chitosan retained 74% after 10 reuses. Similarly Yi and coworkers (2009) showed that the activity of immobilized lipase of *C. rugosa* on alanine chitosan beads relatined 77% of the initial activity after 10 times of reuse. On the contrary Won and coworkers (2005) found that the immobilized lipase of *C. rugosa* entrapped in Ca-alginate gel beads retained 72% after three uses.

CONCLUSIONS

The main objective of the present study was to increase the capacity of the local isolate *P. chrysogenum* to produce lipase through the application of the immobilization technique. *P. chrysogenum* lipase was partially purified about 4.1 fold with a specific activity of 36.95 u/mg protein. The immobilized enzyme was found to be stable over PH range 6.5–8. It was clarified to be tolerant within the temperature range of up to 60 °C with maximal activity at 35 °C. The enzyme can be reused up to 6 runs which is a promising technique for large-scale preparation of immobilized lipase for industrial applications.

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