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# Entrapment of lipase into K-carrageenan beads and its use in hydrolysis of olive oil in biphasic system

P.D. Desai[b], A.M. Dave[b], Surekha Devi[a,*]

[a] *Chemistry Department, Faculty of Science, The M.S. University of Baroda, Vadodara 390002, India*
[b] *GSFC Science Foundation, Vigyan Bhavan, Fertilizernagar 391750, Vadodara, India*

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**Abstract**

The *porcine pancrease* lipase was immobilized by entrapment in the beads of K-carrageenan and cured by treatment with polyethyleneimine (PEI) in the phosphate buffer. The retention of hydrolytic activity of lipase and compressive strength of the beads were examined. The activity of free and immobilized lipase was assessed by using olive oil as the substrate. The immobilized enzyme exhibited a little shift towards acidic pH for its optimal activity and retained 50% of its activity after 5 cycles. When the enzyme concentration was kept constant and substrate concentration was varied the $K_m$ and $V_{max}$ were observed to be $0.18 \times 10^{-2}$ and 0.10, and $0.10 \times 10^{-2}$ and 0.09 respectively, for free and for entrapped enzymes. When the substrate concentration was kept constant and enzyme concentration was varied, the values of $K_m$ and $V_{max}$ were observed to be $0.19 \times 10^{-7}$ and 0.41, and $0.18 \times 10^{-7}$ and 0.41 for free and entrapped enzymes. Though this indicates that there is no conformational change during immobilization, it also shows that the reaction velocity depends on the concentration. Immobilized enzyme showed improved thermal and storage stability. Hydrolysis of olive oil in organic–aqueous two-phase system using fixed bed reactor was carried out and conditions were optimized. The enzyme in reactor retained 30% of its initial activity after 480 min (12 cycles).


*Keywords:* Lipase; Immobilization; K-carrageenan; Hydrolysis; Olive oil

## 1. Introduction

The microbial lipase has been used as biocatalyst for a variety of reactions such as hydrolysis of fats, synthesis of esters and glycerides, and modification of lipids [1–3]. Among these reactions, the hydrolysis of fats is the primary reaction of the lipase as the nature designed lipases mainly to cleave fatty acids from their glyceride backbones for lipolysis in an organism and the same has been studied extensively.

Though immobilization of enzymes has several advantages, the selection of support material and the method of immobilization are prominent factors influencing the enzymatic reactions. The lipase has been immobilized on natural support such as alginate [4], chitosan [5] and cellulose acetate [6]. K-carrageenan is a naturally occurring polysaccharide isolated from the seaweed. It is readily available, non-toxic and high molecular weight polymer composed of repeating units of β-D-galactose sulfate and 3,6-anhydro-α-D-galactose units. K-carrageenan can easily be converted into gel in the presence of metal ions, amines, amino acid derivatives and water soluble organic solvents. Jang et al. [7] have immobilized *Zymomonas mobilis* in K-carrageenan and the rigidity of beads was reported to increase two-fold with 90% conversion efficiency on the addition of polyols/glycerol. The immobilized cells were further employed for sorbitol production. Immobilization of *fumarase* in K-carrageenan mixed gel was carried out which was further used for the production of L-malic acid by Yonghong et al. [8]. Lipase from *Pseudomonas fluorescens* biotype I was immobilized in K-carrageenan gel and the strength of the gel was enhanced by treatment with

* Corresponding author. Tel.: +91 265 2795552.
*E-mail address:* surekha_devi@yahoo.com (S. Devi).

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potassium chloride and glutaraldehyde solution by Kosugi et al. [9].

The conventional oil hydrolysis process, carried under pressurized steam at high temperature, produces undesirable coloured impurities, which need to be removed from the products. In contrast, the enzymatic splitting of fats is carried out at ambient temperatures under normal atmospheric pressure. It is well known that the water-immiscible organic solvents facilitate the lipase-catalyzed hydrolysis of lipid, probably serving as a reservoir both for substrate and product. Among various kinds of solvents tested, iso-octane has been reported to be a preferred organic solvent for olive oil hydrolysis [10]. Although the batch wise hydrolysis of olive oil using immobilized lipase in isooctane-buffer two-phase system and in organic solvents has been extensively studied [11–13]. However, batch wise operation posed serious limitations of depletion of water and accumulation of glycerol.

To the best of our knowledge there is no report on the entrapment of *porcine pancrease* lipase in K-carrageenan beads hardened with polyethyleneimine (PEI) in potassium phosphate buffer. Hence attempts are made to optimize the conditions for the entrapment of *porcine pancrease* lipase in K-carrageenan and to use it for the hydrolysis of olive oil in two phase system using a fixed bed reactor.

## 2. Experimental

### 2.1. Materials

*Porcine pancrease* lipase (EC 3.1.1.3) was obtained from Sigma Chemicals Co., USA. K-carrageenan was obtained as a gift from Central Salt and Marine Research Institure, Bhavnagar, India. Polyethyleneimine was obtained from Fluka AG, Switzerland. Triethanolamine hydrochloride, copper nitrate and di-octyl sodium sulphosuccinate (Aerosol OT or AOT) were obtained from S.D. Fine-Chemicals Ltd., Mumbai, India. All other reagents and chemicals used were of analytical grade.

### 2.2. Entrapment of lipase

The entrapment of lipase in K-carrageenan was carried out by modifying the method reported by Shukla et al. [14]. K-carrageenan (3% (w/v)) solution in water was prepared by heating up to 60 °C and on cooling to 35 °C subsequently mixed with the enzyme solution (1–20 mg) for 1–4 h. The mixture was extruded dropwise through a syringe into 1–4% (w/v) PEI solution in potassium phosphate buffer of pH 7. Beads were left in the solution for 2 h for hardening. The beads were washed several times with water for removal of excess PEI. The hydrolytic activity of free and immobilized enzyme was determined spectroscopically as per the reported method [15] using olive oil as substrate.

### 2.3. Bead strength and size

Lipase entrapped in K-carrageenan gel was converted into cubes of 1.5 cm × 1.5 cm × 1.5 cm dimensions and was cured by immersing in various curing agents for different time intervals. The gel strength of these cubes was measured as the critical compression force using the Hounsfield test equipment (model: H 10 KS), at a fixed speed of 10 mm/min.

The size of the bead was measured using occular microscope (model: METZ–877), Metzer-opto-Electronic Instrument (P) Ltd., India and selecting 200 beads randomly.

### 2.4. pH activity profile

The activity of free and immobilized lipase was measured by incubating them at 35 °C for 10 min in buffer solutions of pH 2.0–12.0 and olive oil as a substrate.

### 2.5. Thermal stability

The free and immobilized enzymes were placed in the buffer solution of optimum pH and incubated at various temperatures (30–60 °C) for different time intervals. The activity of the enzyme was determined as described earlier. The thermodeactivation constant ($K_d$) was calculated using following equation [16]:

$$\ln A_t = \ln A_o - K_d(t)$$

where $A_o$ is the initial activity, and $A_t$ is the activity after heat treatment for $t$ minutes.

### 2.6. Storage stability

The residual activities of the free and immobilized enzymes stored at room temperature (∼35 °C) were determined and the activities were expressed as the percentage retention of their activities at various time intervals.

### 2.7. Reusability of immobilized lipase

To evaluate the reusability of the immobilized lipase, the beads were washed with water and buffer solution after use and then suspended again in a fresh aliquot of a substrate to measure the enzymatic activity. This procedure was repeated until the enzyme lost 50% of its original activity. The turn over number of the enzyme catalyzed process was calculated.

### 2.8. Determination of kinetic constants

The Michaelis constant ($K_m$) and the maximum reaction velocity ($V_{max}$) for the free and immobilized lipase were determined by measuring the velocity of the reaction at enzyme concentrations ranging from $0.51 \times 10^{-10}$ to $4.42 \times 10^{-10}$ M at constant substrate concentration ($4.42 \times 10^{-5}$ M) and also by varying substrate concentration from $0.88 \times 10^{-5}$ to $8.85 \times 10^{-5}$ M at constant enzyme concentration ($1.51 \times$

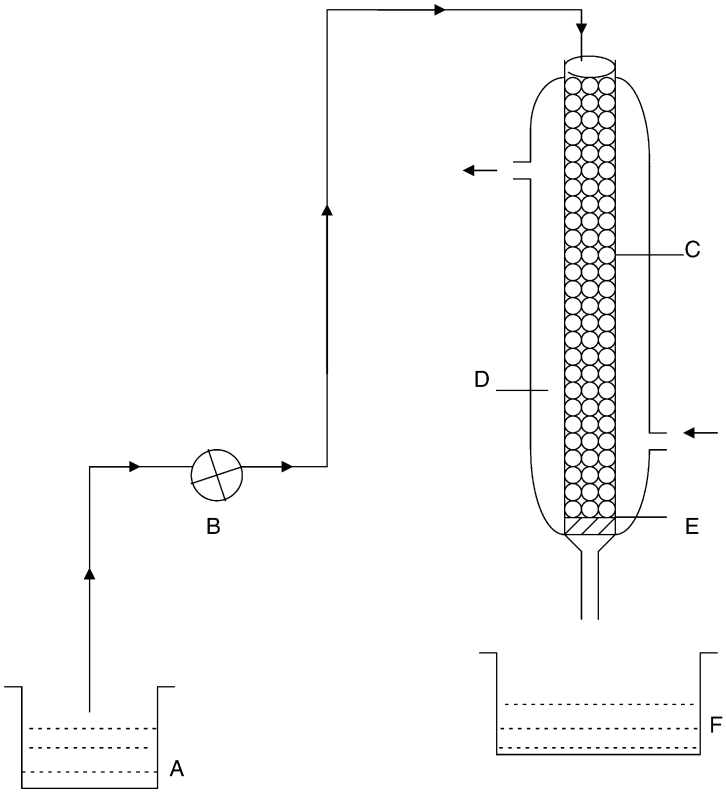


Fig. 1. Schematic diagram of fixed bed reactor. (A) Reactant, (B) peristaltic pump, (C) beads with immobilized enzyme, (D) temperature controller, (E) glass frit and (F) product collector.

$10^{-10}$ M). The free and immobilized enzymes at optimum pH were incubated with substrate for 10 min at 35 °C. From the measured activity of the enzymes, $K_m$ and $V_{max}$ were calculated using Lineweaver–Burk plot of 1/*S* versus 1/*V*.

### 2.9. Hydrolysis of olive oil

Jin et al. [17] have reported the hydrolysis of olive oil catalyzed by surfactant-coated *candida rugosa* lipase in isooctane–aqueous two-phase system and reported 52% conversion after 32 h. Yoshitsugu et al. [18] immobilized lipase from *Pseudomonas fluorescens* biotype I by adsorption on the anion exchange resin and carried out a continuous hydrolysis of beef tallow oil in a counter current reactor using a non-emulsion substrate. They carried out the hydrolysis in fluidized as well as fixed bed reactors and found that the fluidized bed reactor was superior to the fixed bed one.

The present work reports an attempt to hydrolyze olive oil in organic–aqueous two-phase medium using lipase entrapped on K-carrageenan. The schematic diagram of the fixed bed reactor used in the study is given in Fig. 1. A fixed bed reactor of column length 20 cm and diameter 1.2 cm containing immobilized lipase (3 gm K-carrageenan) was used. Organic–aqueous system containing 1–5% olive oil substrate in 50 mM AOT in isooctane and 25 mM triethanolamine buffer (pH 7) was used keeping volume ratio of substrate to buffer 3:1. The operational conditions of reactor such as flow rate, substrate concentration, and reactor temperature were varied and their respective effects on conversion were examined. The efficiency of reactor for continuous operation was studied by passing organic–aqueous system at optimum temperature, flow rate and substrate concentration. At the end of each cycle, a sample was withdrawn for the analysis of fatty acid generated using 0.1 M KOH in ethanol [19].

Table 1
Effect of enzyme concentration on immobilization of lipase

| Enzyme taken (mg) | Protein (mg)/g of support | Protein entrapped (%) | Active protein (mg)/g of support | Active protein (%) | Retention of enzyme activity on bead (%) |
|---|---|---|---|---|---|
| 1.0 | 0.56 | 56 | 0.184 | 18 | 33 |
| 5.0 | 2.79 | 56 | 0.960 | 19 | 32 |
| 10.0 | 4.68 | 47 | 1.930 | 19 | 41 |
| 20.0 | 7.60 | 38 | 2.962 | 15 | 39 |

(Dry wt. of K-carrageenan: 60 mg, 2% PEI in 0.1 M potassium hydrogen phosphate buffer, curing time: 2 h).

Table 2
Effect of curing agents on stability and enzyme loading during immobilization

| Gel curing agent | Time of curing (h) | Critical compression force (N) | Immobilized enzyme based on dry mass (mg/gm) |
|---|---|---|---|
| 2% KCl solution | 2 | 12.7 | 20.3 |
| 100 mM Potassium hydrogen phosphate buffer | 2 | 11.6 | 11.4 |
| 2% PEI in distilled water | 2 | 18.6 | 14.0 |
| Effect of PEI concentration in potassium hydrogen phosphate buffer | | | |
| 1% PEI | 2 | 26.1 | 30.2 |
| 2% PEI | 2 | 32.5 | 32.0 |
| 3% PEI | 2 | 34.3 | 26.4 |
| 4% PEI | 2 | 37.8 | 26.0 |
| Effect of time on 2% PEI in potassium phosphate buffer | | | |
| 2% PEI | 1 | 28.3 | 30.0 |
| 2% PEI | 2 | 32.5 | 32.0 |
| 2% PEI | 4 | 33.6 | 29.2 |
| 2% PEI | 12 | 34.7 | 27.9 |
| 2% PEI | 24 | 37.2 | 18.1 |
| Effect of type of buffer | | | |
| 2% PEI in potassium phosphate buffer | 2 | 32.5 | 32.0 |
| 2% PEI in sodium hydrogen phosphate buffer | 2 | 19.3 | 28.3 |
| 2% PEI in calcium acetate buffer | 2 | 17.8 | 13.5 |
| Effect of other hardening agents | | | |
| 2% PEI in distilled water | 2 | 18.6 | 14.0 |
| 2% Hexamine in distilled water | 2 | 17.5 | 10.2 |

## 3. Results and discussion

### 3.1. Entrapment of lipase

The most crucial factors in this study are the quantity of the enzyme, which could be loaded on the beads and its activity imparted. Retention of enzyme activity on entrapment was calculated from the ratio of active protein to the total protein entrapped in beads.

The effect of enzyme concentration on the extent of enzyme entrapped in beads was studied using 1.0–20.0 mg enzyme and 3.0 ml of 3% K-carrageenan solution. The results are given in Table 1. It is observed that with the increasing concentration of enzyme, the amount of protein and active protein in beads increase. However, the per cent protein in beads decreases. The percentage protein entrapped and retention of enzyme activity are observed to be optimal at the enzyme concentration of 10 mg. The maximum entrapment of lipase was observed when beads were cured in 2% PEI in phosphate buffer for 2 h (Table 2). The protein coupled to the support is observed to be 32 mg/g of dry support. The method suggested by Shukla et al. [14] for curing is compared with the one suggested here using PEI in Phosphate buffer. Almost two-fold increase in entrapment of lipase is observed when beads are hardened in 2% PEI in potassium hydrogen phosphate buffer than in 2% PEI in water (Table 2). This indicates that the potassium ions play an important role in curing. This can be supported by the references cited for curing of beads in potassium chloride [20]. From the results in Table 2 it is observed that hardening of beads in 2% PEI in potassium hydrogen phosphate buffer is much higher than that in sodium hydrogen phosphate or calcium phosphate buffer.

### 3.2. Effect of hardening agent on the physical strength of gel

Potassium chloride solution has been widely reported as a curing agent for hardening of K-carrageenan beads [20]. Chibata has used PEI as a curing agent and claimed it as a better hardening agent [21]. Hence, we have examined extent of hardening of beads in potassium chloride, PEI in water as well as in potassium hydrogen phosphate buffer and hexamine in water. From the results given in Table 2 the compressive

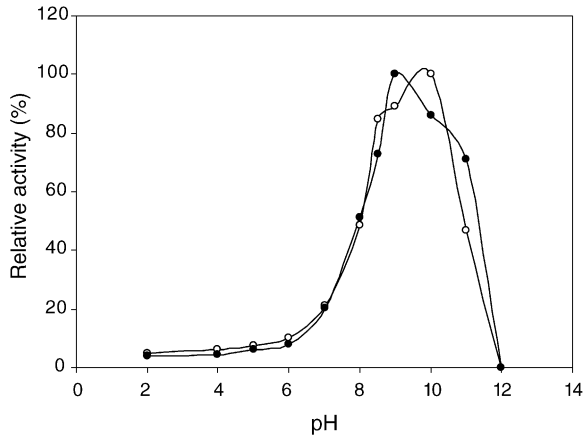


Fig. 2. Effect of pH on the activity of free and immobilized lipase at 35 °C. Free enzyme (◯) and immobilized enzyme (●).

Table 3
Thermodeactivation constant for free and entrapped enzyme at 40, 50 and 60 °C at different time interval

| Time (min) | Thermodeactivation constant × $10^2$ | | | | | |
|---|---|---|---|---|---|---|
| | 40 °C | | 50 °C | | 60 °C | |
| | Free | Immobilized | Free | Immobilized | Free | Immobilized |
| 30 | 1.82 | 0.90 | 4.29 | 1.36 | 9.32 | 3.80 |
| 60 | 1.53 | 0.69 | 3.65 | 1.15 | – | 2.76 |
| 90 | 1.49 | 0.57 | – | 0.99 | – | – |
| 120 | 1.20 | 0.48 | – | 0.84 | – | – |

strength of the beads cured in PEI–potassium hydrogen phosphate buffer was observed to be 2.3 times higher than that of the PEI–water cured gel indicating that the PEI and potassium ions of phosphate buffer jointly enhance the curing reaction. Hence it is proposed that the combination of PEI and potassium hydrogen phosphate buffer is a superior curing agent than the other agents tried earlier.

From the curing studies it is observed that use of 2% PEI in potassium hydrogen phosphate buffer in place of 2% PEI in distilled water for curing increases two-fold retention of enzyme in beads. Hardening of beads with 2% potassium chloride retained more enzymes in beads than hardening of beads in 2% PEI solution in water. Increase in time of curing in 2% PEI solution in phosphate buffer decreases enzyme content in beads. Also increase in concentration of PEI for hardening does not show considerable effect on the retention of enzyme. Use of hexamine as hardening agent decreases extent of enzyme content in beads.

From the size distribution profile it has been observed that 80% of the beads have 2.6 ± 0.2 mm diameter. The bulk density of beads was observed to be 0.6621 gm/cm$^3$.

### 3.3. Optimum pH

The pH is one of the important parameters capable of altering enzymatic activities in aqueous solution. Relative activity as a function of pH is depicted in Fig. 2. It was observed that maximum enzyme activity is exhibited at pH 10.0 and 9.0 by free and immobilized lipase respectively indicating a little effect of hydrophobicity of support on the optimum pH of immobilized enzyme activity.

### 3.4. Thermal stability

The knowledge on thermal stability of an immobilized enzyme is useful in exploring the potential applications of the enzyme. Relative activity as a function of time and temperature are illustrated in Fig. 3. It is observed that entrapped enzyme shows better thermal stability at all temperatures and times. However, marked reduction in activity for both free and entrapped enzyme is observed at 60 °C. Thermodeactivation constants calculated are given in Table 3. The thermal deactivation constants for immobilized lipase are lower than those for free ones at all temperatures, indicating thereby that the immobilized lipase is less prone to denaturation due to temperature.

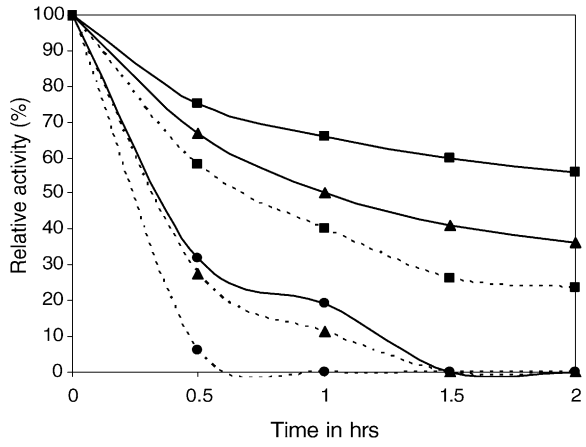


Fig. 3. Effect of temperature on the activity of free and immobilized lipase at pH 8.5 at 40 °C (■), 50 °C (▲) and 60 °C (●). Free enzyme (- - - -) and immobilized enzyme (—).

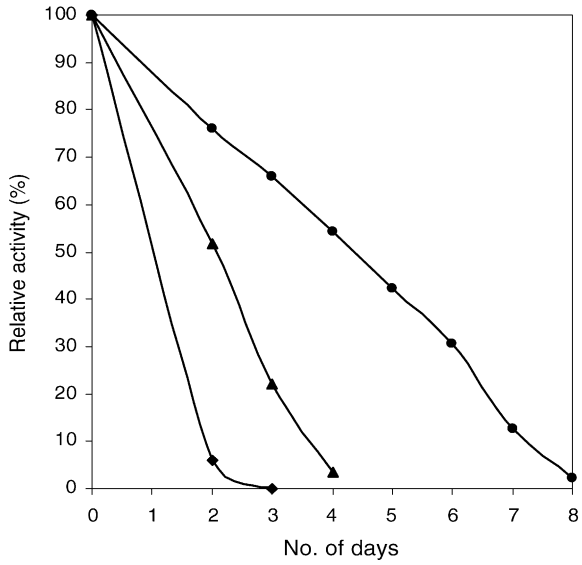


Fig. 4. Storage stability of free and immobilized enzyme at 35 °C. Free enzyme (♦), immobilized enzyme (in water) (▲) and immobilized enzyme (dry) (●).

### 3.5. Storage stability

The stability of an enzyme is of significant importance for scheduling its application in a particular reaction. The immobilized and free enzymes were stored in distilled water as well as immobilized enzyme was also stored in dry state for various time periods at 35 °C. The residual activity of the enzyme was determined as a function of time using olive oil emulsion as substrate and results are given in Fig. 4. It is observed that the free enzyme looses its 50% activity within 1 day, whereas the immobilized one in dry state could retain its 50% activity for 4 days. The immobilized enzyme stored without any medium of suspension shows higher stability than stored in distilled water, which indicates that the enzyme slowly leaches from the support in aqueous medium.

### 3.6. Reusability of lipase

Immobilized system offers an advantage of reusability. Mojovic et al. [22] immobilized 15.4 mg of *Candida rugosa* lipase on one g of polymeric support, which on repeated use retained 56% of its initial activity after the fifth cycle in batch hydrolysis of palm oil. It is observed that in the present work 59% of enzyme activity is retained after five repeated cycles. The turn over number of immobilized enzyme is observed to be $10.1 \times 10^6$.

### 3.7. Determination of kinetic constant

The effect of the substrate concentration and enzyme concentration on the kinetics of the reaction catalyzed by free and immobilized lipase was studied using olive oil as substrate. From Lineweaver–Burk plot of $1/V$ versus $1/S$. (Fig. 6a and b) Michaelis constant ($K_m$) and the maximum reaction velocity ($V_{max}$) of the free and immobilized enzymes were calculated and results are given in Table 4. No significant change in $K_m$ and $V_{max}$ values for the free and immobilized enzyme is observed when substrate concentration or enzyme concentration is kept constant which indicates that there is almost no possibility of conformational changes during immobilization. However, the reaction is observed to be first order as the $K_m$ and $V_{max}$ values vary with concentration.

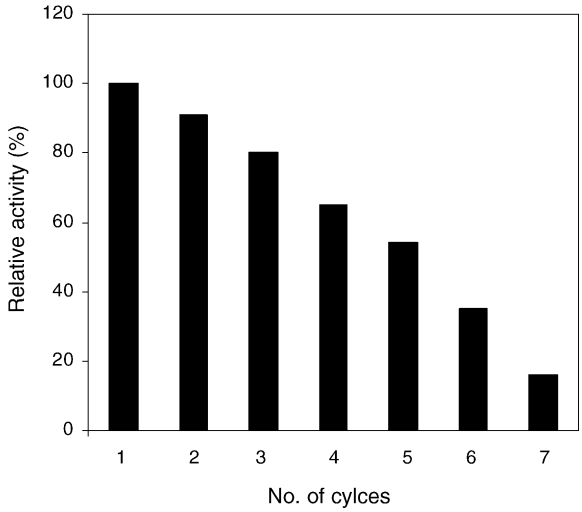


Fig. 5. Reusability of immobilized enzyme at 35 °C and pH 8.5.

### 3.8. Olive oil hydrolysis

In the present study, reusability of entrapped lipase for hydrolysis of olive oil was investigated for seven consecutive process cycles and the results are presented in Fig. 5.

Pahn et al. [23] adsorbed *Chromatobacterium viscosum* lipase on liposome and carried out the continuous glycerolysis of olive oil in a continuous bioreactor with polysulfone membrane. They also studied the effect of flow rate of substrate in isooctane on conversion and reported that the conversion increased with decrease in the flow rate. In the present study also it is found that with increasing flow rate the conversion decreases due to the lesser time of contact between substrate and immobilized enzyme. The maximum conversion is found at 1% substrate concentration (Fig. 7 ). From Fig. 8, it is observed that the effect of temperature is more pronounced at higher flow rates and maximum conversion is observed at 30 °C and 0.25 $cm^3\,min^{-1}$ flow rate. When reactor is tested for the continuous operation under optimum conditions, 30% of its initial activity is found to be retained for 12 cycles (480 min) in aqueous-isooctane two-phase system (Fig. 8).

Lavayre et al. [24] reported only 1% of hydrolytic activity of lipase on propylated spherosil beads compared to free lipase whereas in our case, the entrapped lipase showed 41% hydrolytic activity. Zorica et al. [4] immobilized *candida*

Table 4
Kinetic parameters for free and immobilized enzyme

| Conditions | Parameters | Free enzyme | Immobilized enzyme |
|---|---|---|---|
| (A) | | | |
| Substrate conc. = $0.88 \times 10^{-5}$ M–$8.85 \times 10^{-5}$ M | $K_m$ (mM) | $0.18 \times 10^{-2}$ | $0.10 \times 10^{-2}$ |
| Enzyme conc. = $1.51 \times 10^{-10}$ M (constant) | $V_{max}$ (mM/min) | 0.10 | 0.09 |
| (B) | | | |
| Enzyme conc. = $0.51 \times 10^{-10}$ M–$4.42 \times 10^{-10}$ M | $K_m$ (mM) | $0.19 \times 10^{-7}$ | $0.18 \times 10^{-7}$ |
| Olive oil conc. = $4.42 \times 10^{-5}$ M (constant) | $V_{max}$ (mM/min) | 0.41 | 0.41 |

A at constant enzyme concentration; B at constant substrate concentration.

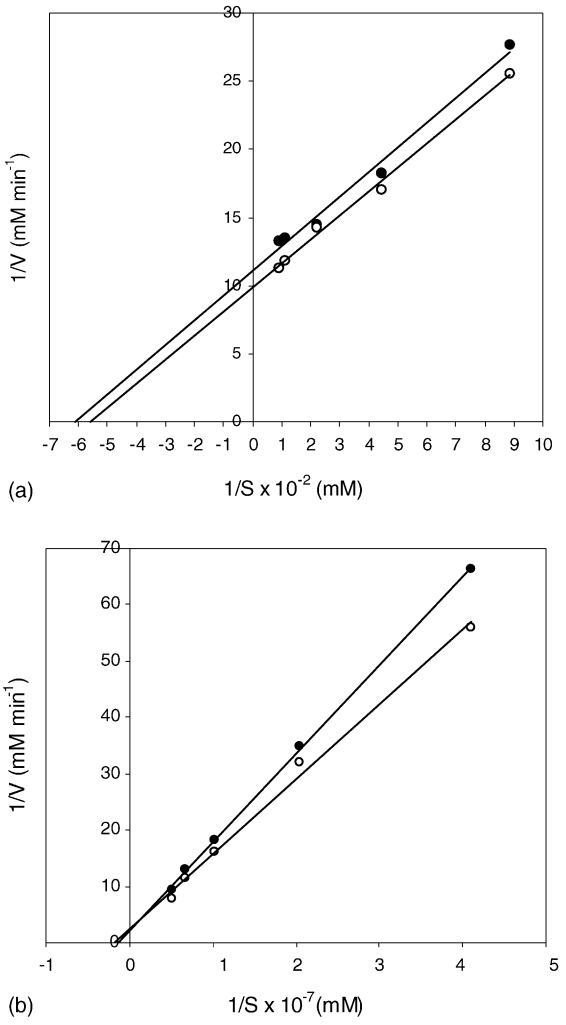


Fig. 6. Lineweaver–burk plots for olive oil hydrolysis (temperature 35 °C, pH 8.5, time 10 min), free enzyme (○) and immobilized enzyme (●). (a) When enzyme concentration kept constant and (b) when substrate concentration kept constant.

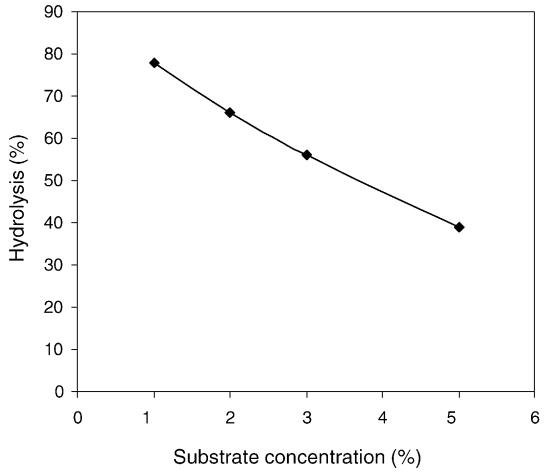


Fig. 7. Effect of substrate concentration on hydrolysis at 0.25 $cm^3$ $min^{-1}$, flow rate at 35 °C.

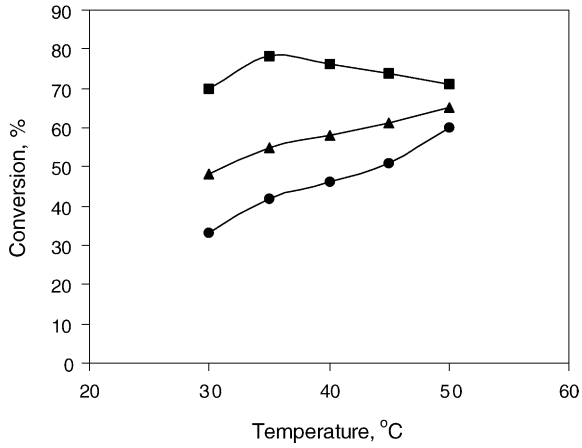


Fig. 8. Effect of temperature on hydrolysis of olive oil (1% olive oil concentration). Flow rate ($cm^3$ $min^{-1}$): (■), 0.50 ml/min (▲), 1.0 ml/min (●).

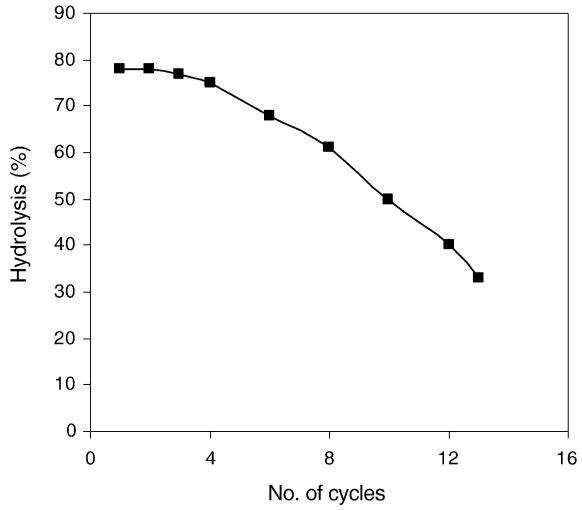


Fig. 9. Reactor stability for the hydrolysis of olive oil in two phase system.

*rugosa* lipase in alginate beads and used for palm oil hydrolysis in lecithin/isooctane system. They reported that the immobilized lipase lost a little activity up to three cycles and retained only 10% of its initial activity after 7 cycles whereas we have observed retention of 30% activity for 12 cycles for the continuous hydrolysis in the present study (Fig. 9).

## 4. Conclusion

*Porcine pancrease* lipase entrapped in K-carrageenan beads is found to be superior to the free enzyme under all conditions tested for the enzyme efficacy and stability. Use of K-carrageenan beads for entrapment of lipase results into 32 mg/g enzyme on dry weight of K-carrageenan. The amount of entrapped lipase and compressive strength of gel cured in PEI-phosphate buffer are observed to be two times higher than the gel cured in PEI-water. Immobilized lipase shows better thermal and storage stability.

The enzyme is found to retain 50% of its activity after repeated use of 5 cycles. The Michaelis constant ($K_m$) and the maximum reaction velocity ($V_{max}$) of the free and immobilized enzyme are almost same indicating that there is no conformational change during immobilization. Hydrolysis of olive oil is carried out using fixed bed reactor and it is observed that maximum hydrolysis takes place at 1.0% olive oil concentration, 0.25 $cm^3\,min^{-1}$ flow rate and 35 °C temperature.

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