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Physical and stability characteristics of *Burkholderia cepacia* lipase encapsulated in κ-carrageenan

Kenthorai Raman Jegannathan, Eng-Seng Chan, Pogaku Ravindra*

Centre of Materials and Minerals, School of Engineering and Information Technology, Universiti Malaysia Sabah, 88999 Kota Kinabalu, Sabah, Malaysia

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ABSTRACT

In this work, a novel approach for lipase immobilization was exploited. Lipase from *Burkholderia cepacia* was encapsulated into κ-carrageenan by co-extrusion method to form a liquid core capsule. The diameter of the encapsulated lipase was found to be in the range of 1.3–1.8 mm with an average membrane thickness of 200 μm and 5% coefficient of variance. The encapsulation efficiency was 42.6% and 97% moisture content respectively. The encapsulated lipase was stable between pH 6 and 9 and temperature until 50 °C. The encapsulated lipase was stable until disintegration of the carrier when stored at 27 °C and retained 72.3% of its original activity after 6 cycles of hydrolysis of *p*-NPP. The encapsulated lipase was stable in various organic solvents including methanol, ethanol, *iso*-propanol, *n*-hexane and *n*-heptane. Kinetic parameters K_m and V_{max} were found to be 0.22 mM and 0.06 μmol/min for free lipase and 0.25 mM and 0.05 μmol/min for encapsulated lipase respectively.

1. Introduction

Lipases constitute the most important group of biocatalysts for biotechnological applications. Novel biotechnological applications have been successfully established using lipases for the synthesis of biopolymers, enantiopure pharmaceuticals, agrochemicals, flavour compounds and biodiesel [1]. The use of enzymes and other proteins has been limited due to their considerably unstable nature and the resulting requirement of stringent conditions, such as particular pH and temperature [2]. Many attempts have been made over the years to improve the catalytic activity and operational stability of the industrial enzymes through the use of genetic engineering, immobilization and /or process alterations. Enzyme immobilization is the most commonly used strategy to impart the desirable features of conventional heterogeneous catalysts onto biological catalysts [3]. Besides enhanced stability, immobilization is also known to offer several advantages such as reusability, continuous operation, ease of product separation, greater control over catalysis and process economics.

Lipase has been immobilized on various supports either by physical adsorption, covalent binding, entrapment and encapsulation [4–7]. Among the immobilization techniques entrapment and encapsulation on natural polymers are becoming more familiar due to their low cost, process repeatability, lack of toxicity and environmental friendly. Enzyme encapsulation is the physical confinement of enzymes inside a polymeric membrane by the phase transition of polymer solution containing the enzyme. Encapsulation can be carried out by various processes, such as coacervation phase separation, interfacial polymerization, solvent evaporation, spray coating, multiorifice centrifugation and air suspension [8]. Method of producing microcapsules is the subject of several review articles [8–11]. Although, numerous methods are described in these articles, the majority are not suitable for producing large (>50 μm diameter) mononuclear microcapsules which show true shell–core morphology and are capable of containing an aqueous-based solution as a core. Such capsules can be prepared by co-extrusion [12]. The process of co-extrusion involves ejecting two liquid streams through concentric nozzles under a force. In this manner, the centre nozzle carries liquid solution to be encapsulated while the outer nozzle carries the polymer.

κ-Carrageenan is a naturally occurring polysaccharide isolated from a marine red algae. 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. κ-Carrageenan can be easily converted into gel in the presence of metal ions, amines, amino acids derivative and water soluble organic solvents [13]. *Burkholderia cepacia* lipase is tolerant towards methanol which favors its use in both hydrolysis and esterification reactions. No attempt has been made to encapsulate lipase enzyme

* Corresponding author at: Department of Chemical Engineering, School of Engineering and IT, Universiti Malaysia Sabah, 88999 Kota Kinabalu, Sabah, Malaysia.
E-mail address: dr_ravindra@hotmail.com (P. Ravindra).

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in κ-carrageenan forming liquid core capsules. Therefore, in this study lipase from *B. cepacia* was encapsulated in κ-carrageenan and the physical and stability characteristics of the lipase core capsules are reported.

2. Materials and methods

2.1. Materials

B. cepacia lipase was purchased from Amano enzymes Inc (Japan). κ-Carrageenan was procured from FMC Biopolymer (USA). *p*-NPP from Sigma Chemicals Co. (USA), refined palm oil was obtained from Lam soon edible oil Sdn Bhd (Malaysia). All other chemicals and solvents used were analytical grade.

2.2. Lipase encapsulation

Encapsulation of *B. cepacia* lipase using κ-carrageenan was carried out using coaxial needle by co-extrusion method as shown in Fig. 1. The outer shell κ-carrageenan 3% (w/v) solution in water was prepared by heating up to 80 °C for an hour and cooling on to 45 °C. The inner core *B. cepacia* lipase enzyme solution was prepared by adding lipase enzyme powder (1 g/ml) in phosphate buffer (pH 7) and dissolved completely by swirling. Refined palm oil was used as the continuous phase. The inner and outer diameter of the coaxial needle was 0.8 and 1.6 mm in diameter. The enzyme solution in the inner needle and κ-carrageenen solution in the outer needle were coextruded at a flow rate of 5 ml/h and 50 ml/h using a syringe pump (Model TE-331) Thermo (Japan). Refined palm oil was used as the continuous phase and the flow rate of continuous phase was maintained at 60 ml/h using a peristaltic pump, Watson and Marlow (England). The continuous phase stream flowed into a stirred beaker containing 2% KCl solution. After the lipase solution in the syringe pump was extruded out, the beaker containing the KCl solution and encapsulated lipase was removed out of the magnetic stirrer and allowed to settle the capsules in the aqueous phase. Then, the oil was removed and the encapsulated *B. cepacia* lipase was filtered using a sieve and washed to remove excess oil and hardened in KCl solution for 24 h at 4 °C. After 24 h the capsules were filtered, dried in room temperature and used for further studies.

2.3. Lipase activity

The activity of encapsulated *B. cepacia* lipase in κ-carrageenan was measured using *p*-NPP as substrate by modifying the method of [14]. *p*-NPP was dissolved at a concentration of 0.1% (w/v) in reagent grade (95%) ethanol. The reaction mixture consisted of 100 μl substrate, 1 ml of 0.05 M phosphate buffer (pH 7) and 50 μl of free lipase solution (stock solution 1 mg/ml) or 100 mg encapsulated *B. cepacia* lipase in κ-carrageenan. After 5 min of reaction at 30 °C the reaction was terminated by adding 2 ml of 0.5N Na_2CO_3. The mixture was centrifuged at 10,000 rpm for 10 min, and the absorbance of the supernatant was measured at 410 nm using a spectrophotometer, Spectronic 4001 (USA). A molar extinction coefficient of 15,000 M^{-1} cm^{-1} for *p*-nitrophenol was used [15]. One unit of activity was defined as the amount of enzyme necessary to hydrolyze 1 μmol/min of *p*-NPP under the conditions of assay. The protein content of the encapsulated *B. cepacia* lipase in κ-carrageenan was estimated according to the method of Lowry using bovine serum as standard.

2.4. Immobilization efficiency

The encapsulation efficiency was evaluated in terms of protein coupling

$$\text{Protein coupling yield}\,(\%) = \frac{\text{amount of protein coupled}}{\text{amount of protein introduced}} \times 100$$

2.5. Capsule size and coefficient of variance

The size and the membrane thickness of the encapsulated *B. cepacia* lipase in κ-carrageenan were measured by the Motic software, using the microscopic picture taken by an optical microscope attached with a camera (Motic, China). The coefficient of variance of the capsule size produced was calculated accounting 60 capsules.

2.6. Moisture content

Moisture content was determined for a known weight of encapsulated *B. cepacia* lipase in κ-carrageenan using a hot air oven by heating the capsules at 102 °C until a constant weight.

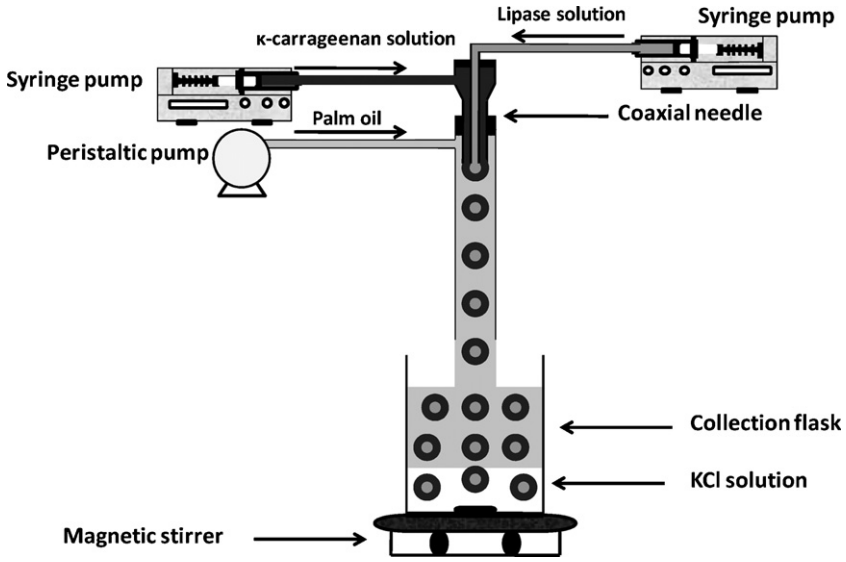

Fig. 1. Schematic diagram of encapsulation procedure.

2.7. Stability studies

2.7.1. pH stability

The pH stability of the free lipase and the encapsulated *B. cepacia* lipase in κ-carrageenan was carried out by incubating the enzyme at 30 °C in the buffers of varying pH in the range of 3–9 for 1 h. After 1 h the capsules were separated from the buffer solution using a filter, dried in room temperature and its catalytic activity was determined as described in Section 2.3. Relative activities were calculated as the ratio of the activity of the encapsulated *B. cepacia* lipase in κ-carrageenan after incubation to the activity at the optimum reaction pH.

2.7.2. Temperature stability

The thermal stability of the soluble lipase and encapsulated *B. cepacia* lipase in κ-carrageenan was conducted by incubating the immobilized enzyme at various temperatures in the range of 25–45 °C for 1 h at pH 7 and the capsules were separated from the buffer solution using a filter, dried in room temperature and its catalytic activity was determined as described in Section 2.3. Relative activities were calculated as mentioned above and plotted against temperature.

2.7.3. Solvent stability

The stability of encapsulated *B. cepacia* lipase in κ-carrageenan in various solvents including phosphate buffer pH 7, methanol, ethanol, *iso*-propanol, acetone, *n*-heptane and *n*-hexane was studied by incubating the immobilized enzyme at various solvents at 30 °C for 1 h. Thereafter, the capsules were separated from the solvents using a filter, dried in room temperature and the immobilized enzyme was assayed for relative lipase activity. The activity of *B. cepacia* lipase in κ-carrageenan suspended in phosphate buffer was taken as the reference for calculating relative activity.

2.7.4. Storage stability

The relative activities of the free and encapsulated *B. cepacia* lipase on κ-carrageenan stored at room temperature 27 °C were determined and the activities were expressed as the percentage retention of their activities at various time intervals.

2.8. Kinetic parameters

The Michaelis constant K_m and the maximum reaction velocity V_{max} for the free and encapsulated *B. cepacia* lipase on κ-carrageenan were determined by measuring the initial velocity of the reaction by varying the substrate concentration at a constant enzyme concentration. The initial rate was determined at 1 min. However, the final conversion was at 5 min for both free and immobilized lipase. The free and encapsulated *B. cepacia* lipase in κ-carrageenan at pH 7 was incubated with *p*-NPP substrate for 1 min at 30 °C. From the measured activity of the free and encapsulated enzymes, K_m and V_{max} was calculated using Lineweaver–Burk plot of $1/S$ versus $1/V$.

2.9. Reusability of immobilized lipase

To evaluate the reusability of the encapsulated *B. cepacia* lipase in κ-carrageenan, the capsules were washed with buffer solution after use in hydrolysis of *p*-NPP and suspended again in fresh aliquot of the substrate to measure the enzymatic activity.

3. Results and discussion

3.1. Encapsulation of B. cepacia lipase

The encapsulation of *B. cepacia* lipase in κ-carrageenan was carried out by co-extrusion through coaxial needle fabricated in our laboratory. Lipase capsules were spherical in shape with a diameter in the range of 1.3–1.8 mm and an average membrane thickness of 200 μm (Fig. 2) and the coefficient of variance accounting 60 capsules was found to be 5%. In the micrographic picture of encapsulated lipase (Fig. 2) a distinct bilayer could be observed showing the inner core of lipase solution and the outer core of κ-carrageenan gel. The moisture content of the encapsulated lipase was found to be 97%. The concentration of the κ-carrageenan solution 3% (w/v) could not be increased further due to clogging of κ-carrageenan in the needle at higher concentration. Increasing the temperature will favor to use κ-carrageenan solution beyond 3% but the rise in temperature beyond 45 °C may inactivate the enzyme. Hence the concentration of κ-carrageenan was fixed to 3% (w/v). All the parameters of the encapsulation process were fixed to produce an encapsulated lipase of diameter in the range of 1.3–1.8 mm. Capsule diameter below this range could be produced by adjusting the encapsulation process parameters but handling those smaller capsules for conducting experiments was tedious.

3.2. Immobilization efficiency

The immobilization efficiency was determined in terms of protein coupling. A protein-coupling yield of 42.6% was observed (Table 1). The loss of enzyme in the manufacturing process has been reported by several other workers and it varies from 33% to 70%. Where as, the encapsulation of *B. cepacia* lipase using the coaxial needle showed a protein loss of 57.4%. The possible reason for loss of lipase in encapsulation procedure could be due to the agitation of the collection flask containing KCl solution. This was confirmed by the protein assay performed on the aqueous phase (KCl solution). The agitation of the collection flask was necessary to keep the capsules separated in the oil until it was settled in the aqueous phase. If not agitated the capsules tend to diffuse each other in the organic phase leading to rupture.

3.3. pH and temperature stability

The pH is one of the important parameter capable of altering enzyme stability in aqueous solution. The effect of pH on the stability of the free and encapsulated lipase in κ-carrageenan was investigated within pH 2.0 and 9.0 at 30 °C. Relative activity as a function of pH is shown in Fig. 3. Optimum pH for free and encapsulated lipase was found to be 7. The effect of temperature on the stability of the free and encapsulated lipase in κ-carrageenan was investigated at temperature 25–50 °C at optimum pH 7. The study of temperature could not be beyond 50 °C for the reason that κ-carrageenan dissolves beyond this temperature. This could be one of the drawback of using κ-carrageenan as a matrix. However, most of the enzymatic reactions do not require high temperature. Relative activity as a function of temperature is shown in Fig. 4. Optimum temperature for free and encapsulated lipase in κ-carrageenan was found to be 30 °C. Encapsulation is the physical enclosure of enzyme

Table 1
Protein coupling yield of encapsulated lipase.

Amount of protein introduced (mg/g-κ-carrageenan)	Amount of protein coupled (mg/g-κ-carrageenan)	Protein coupling yield (%)
3.52	1.50	42.6

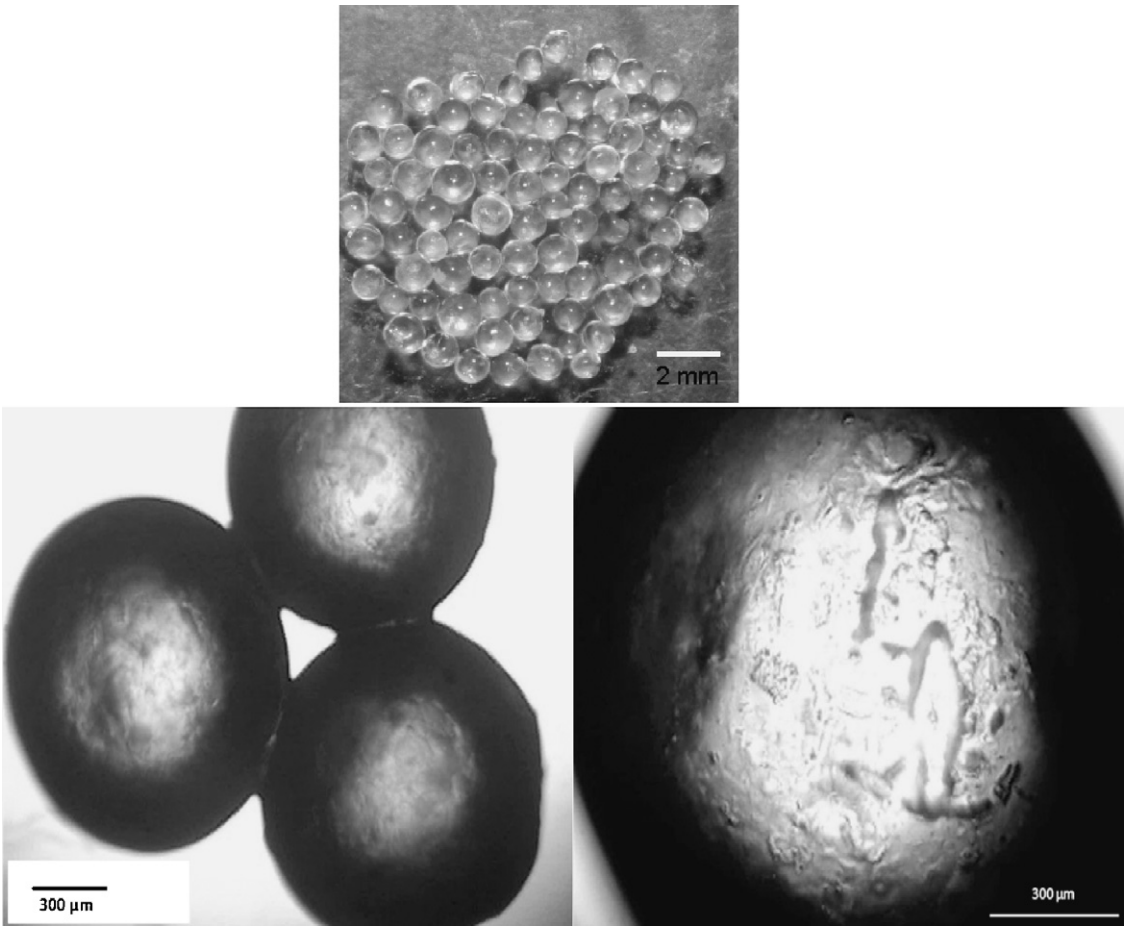

Fig. 2. Microscopic pictures of encapsulated lipase showing the liquid core lipase enzyme in the middle surrounded by κ-carrageenan matrix.

within a polymeric membrane. In this method, enzymes do no chemically bond to polymeric matrices. So, the three-dimensional structure of the enzymes may not be affected by the immobilization procedure and thus the optimum pH and temperature stability of the immobilized enzyme was observed same as the free enzyme [16].

3.4. Stability in organic solvents

The stability in organic solvents is an important characteristic of lipases. It can determine whether the enzyme can be used to catalyze synthetic reactions and also to predict which solvent would be better to perform the reaction. When performing lipase-catalyzed reactions in organic media, the ability to do an esterification reaction instead of hydrolysis is one advantage. The effect of the organic solvent depends on the nature of both enzyme and solvent [17]. It is believed that water-miscible organic solvents strip water from the enzymes, leading to the unfolding of the molecule with exposure of the inner hydrophobic residues and that this denaturation occurs at a much faster rate than in a pure aqueous system [18]. The stability of encapsulated lipase in κ-carrageenan in various solvents is shown in Table 2. There was no collapse of

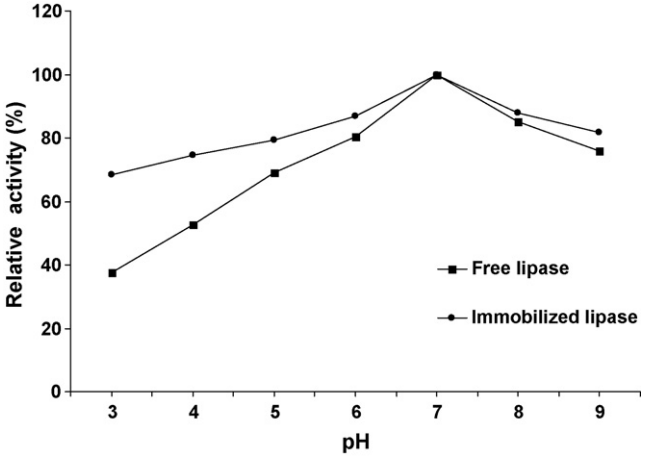

Fig. 3. pH stability of free and immobilized lipase at 30 °C.

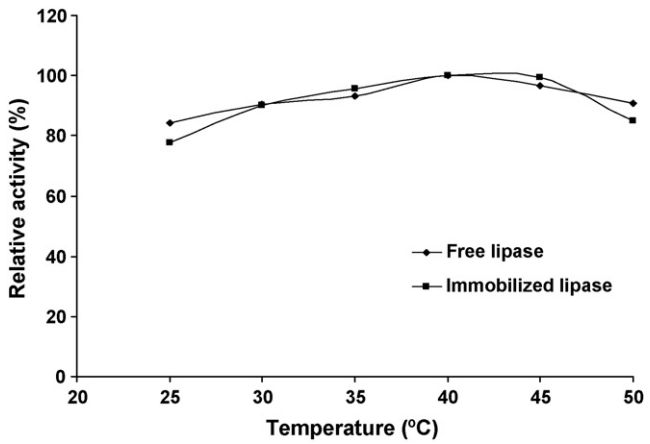

Fig. 4. Temperature stability of free and immobilized lipase at pH 7.

Table 2
Solvent stability of encapsulated lipase.

Solvent	Activity (U/100 mg)	Relative activity (%)
Phosphate buffer pH 7	45.1	100
Methanol	42.2	93.5
n-Hexane	39.2	86.9
Ethanol	39.2	86.9
iso-Propanol	36.5	80.9
n-Heptane	31.2	69.1
Acetone	19.7	43.7

the gel structure when the encapsulated lipase was suspended in solvents. The activity studies showed that the encapsulated *B. cepacia* lipase was stable in alcohol and hexane compared to acetone. *B. cepacia* lipase showed good stability towards methanol.

3.5. Storage stability and reusable studies

The storage stability of an enzyme is of significant importance for scheduling its application in a particular reaction. The encapsulated lipase in κ-carrageenan was stored in 27 °C and the activities were measured periodically over duration of 10 days. As can been seen from Fig. 5, the encapsulated lipase stored at 27 °C was stable until the disintegration of the carrier. One of the problems incorporated using natural polymer is the polymer degradation within a short period at room temperature. Hence the encapsulated lipase in κ-carrageenan is not suitable for long time storage. However, in industries the long-term storage problem could be eliminated by having an in situ encapsulation unit and using the encapsulated enzyme within a short span of time. The main purpose of immobilization is to use the enzyme several times in batch or continuous reactor. The reusable studies of the encapsulated lipase (Fig. 6) showed that the immobilized enzyme retains 72.3% of its original activity after 6th reuse in the hydrolysis of *p*-NPP. The leakage of enzyme could be the reason for lower activity of the encapsulated lipase upon reuse.

3.6. Kinetic parameters

The effect of the substrate concentration on the kinetics of the reaction catalyzed by free and encapsulated lipase was studied using *p*-NPP as substrate at optimum temperature and pH. From the Lineweaver–Burk plot of $1/V$ versus $1/S$ (Fig. 7) Michaelis constant K_m and the initial maximum reaction velocity V_{max} of the free and encapsulated were calculated and the results are given in Table 3. The increase in K_m value of immobilized lipase might be due to the lower accessibility of the substrate to the active site by diffusion limitation [19].

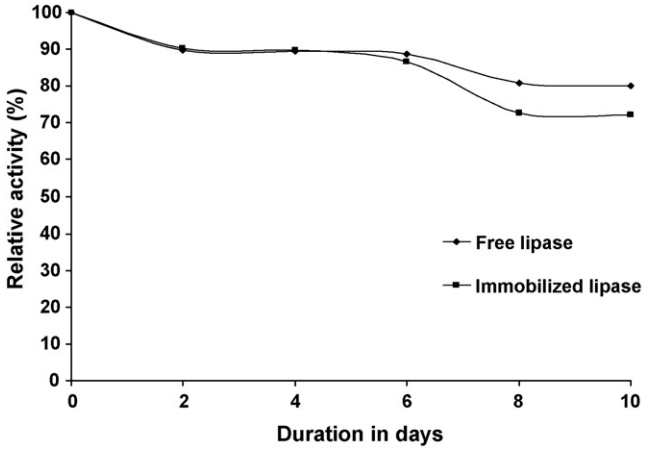

Fig. 5. Storage stability of free and immobilized lipase at 27 °C.

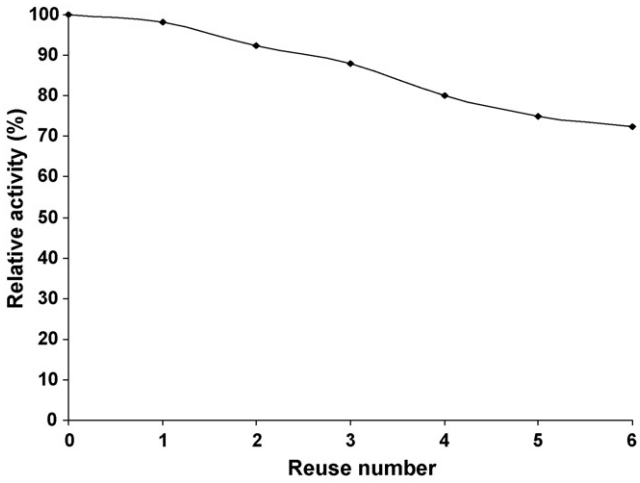

Fig. 6. Reuse stability of the immobilized lipase in *p*-NPP hydrolysis.

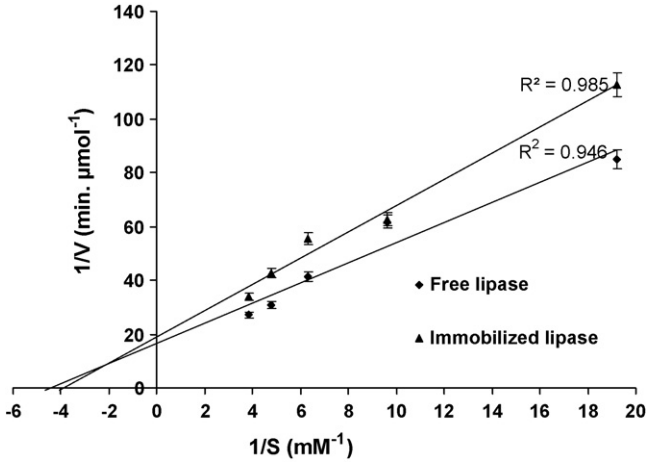

Fig. 7. Lineweaver–Burk plot for *p*-NPP hydrolysis of free and immobilized lipase. Initial reaction rate at 1 min, free lipase 50 μl (1 mg protein) and immobilized lipase 660 mg (1 mg protein).

Table 3
K_m and V_{max} values of free and encapsulated lipase from the Lineweaver–Burk plot of the Michaelis–Menten equation.

	K_m (mM)	V_{max} (μmol/min)
Free lipase	0.22	0.06
Immobilized lipase	0.25	0.05

4. Conclusion

In this work, lipase from *B. cepacia* was encapsulated in κ-carrageenan by co-extrusion method forming a liquid core capsule with an encapsulation efficiency of 42.6%. The liquid core capsules were spherical in shape with a diameter ranging 1.3–1.8 mm and an average membrane thickness of 200 μm. The encapsulated lipase showed good pH, temperature, and storage stability similar to free lipase and retains 72.3% of its original activity after using for 6 cycles. The K_m and V_{max} values were similar to that of free lipase. *B. cepacia* lipase encapsulated in κ-carrageenan showed good stability in various alcohols and alkenes and the encapsulated lipase could be stored at room temperature for 10 days without significant change to its activity. *B. cepacia* lipase encapsulated in

κ-carrageenan could be a potential heterogeneous catalyst for non-conventional media.

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