IMMOBILIZATION OF LIPASE IN K-CARRAGEENAN BY ENCAPSULATION – AN ENVIRONMENTAL FRIENDLY APPROACH

Kenthorai Raman Jegannathan, Chan Eng Seng and Pogaku Ravindra*
Centre of Materials and Minerals, School of Engineering and Information Technology, Universiti Malaysia Sabah, Sabah, (MALAYSIA)

Received July 15, 2009 *Accepted December 12, 2009*

ABSTRACT

The objective of this work was to immobilize lipase enzyme in a natural polymer matrix forming a liquid core capsule. The immobilization was done by encapsulation method using a coaxial needle with lipase enzyme solution as inner core solution and κ-carrageenan solution as outer core solution. Both the solutions were dropped by extrusion through the coaxial needle in a carrier stream (palm oil) followed by hardening. The microscopic picture of the encapsulated lipase shows the lipase core enzyme confined in the middle surrounded by the polymer membrane. The encapsulated lipase in κ-carrageenan was spherical in shape with an average diameter of 1.6 mm and 5% coefficient of variance. The activity studies revealed that the immobilized enzyme was stable at pH range 5-9 and temperature range of 25-45 ^{o}C. The encapsulated lipase shows good operational and reusable properties. The immobilized lipase enzyme can be a potential heterogeneous catalyst for various hydrolysis and esterification reactions. The encapsulation of lipase in a natural polymer makes the immobilization process environmental friendly.

Key Words : Immobilization, Encapsulation, κ-carrageenan, Lipase, Environmental friendly.

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

* Author for correspondence

control over catalysis and process economics.

Lipase has been immobilized on various supports either by physical adsorption, cross-linkage, entrapment and encapsulation[4-7]. Among the immobilization techniques entrapment and encapsulation of lipase on natural polymers are becoming more familiar due to their low cost, process repeatability, lack of toxicity and environmental friendly nature. 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]. 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[9]. 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. No attempt has been made to encapsulate lipase enzyme in κ-carrageenan forming liquid core capsules. Therefore, in this study lipase from *Burkholderia cepacia* was encapsulated in κ-carrageenan and the physical and stability characteristics of the lipase core capsules are reported.

MATERIAL AND METHODS

Materials

Burkholderia 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 of analytical grade.

Lipase encapsulation

Encapsulation of *Burkholderia 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 ^{o}C for an hour and cooling on to 45 ^{o}C. The inner core *Burkholderia 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 reused fort the next immobilization batch. Encapsulated *Burkholderia cepacia* lipase was filtered using a sieve and washed to remove excess oil and hardened in KCl solution for 24 h at 4 ^{o}C. After 24 h the capsules were filtered, dried in room temperature and used for further studies.

Lipase activity

The activity of encapsulated *Burkholderia cepacia* lipase in κ-carrageenan was measured using *p*-NPP as substrate by modifying the method of Hung et al (2003)[10]. *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 *Burkholderia cepacia* lipase in κ-carrageenan. After 5 min of reaction at 30 ^{o}C the reaction was terminated by adding 2 ml of 0.5 N 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 15000 $M^{-1}cm^{-1}$ for *p*-nitrophenol was used[11]. One unit of activity was defined as the amount of enzyme necessary to hydrolyze 1 µmol per min of *p*-NPP under the conditions of assay. The protein content of the encapsulated *Burkholderia cepacia* lipase in κ-carrageenan was estimated according to the method of Lowry using bovine serum as standard.

Capsule size and coefficient of variance

The size and the membrane thickness of the encapsulated *Burkholderia cepacia* lipase in κ-carrageenan was 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.

Moisture content

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

pH stability

The pH stability of the free lipase and the encapsulated *Burkholderia cepacia* lipase in κ-carrageenan was carried out by incubating the enzyme at 30 ^{o}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 *Burkholderia cepacia* lipase in κ-carrageenan after incubation to the activity at the optimum reaction pH.

Temperature stability

The thermal stability of the soluble lipase and encapsulated *Burkholderia cepacia* lipase in κ-carrageenan was conducted by incubating the immobilized enzyme at various temperatures in the range of 25-45^{o}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.

Solvent stability

The stability of encapsulated *Burkholderia 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 *Burkholderia cepacia* lipase in κ-carrageenan suspended in phosphate buffer was taken as the reference for calculating relative activity.

Storage stability

The relative activities of the free and encapsulated *Burkholderia 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.

Reusability of immobilized lipase

To evaluate the reusability of the encapsulated *Burkholderia 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.

RESULTS AND DISCUSSION

Encapsulation of *Burkholderia cepacia* lipase

The encapsulation of *Burkholderia 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.1)** and the coefficient of variance accounting 60 capsules was found to be 5%.

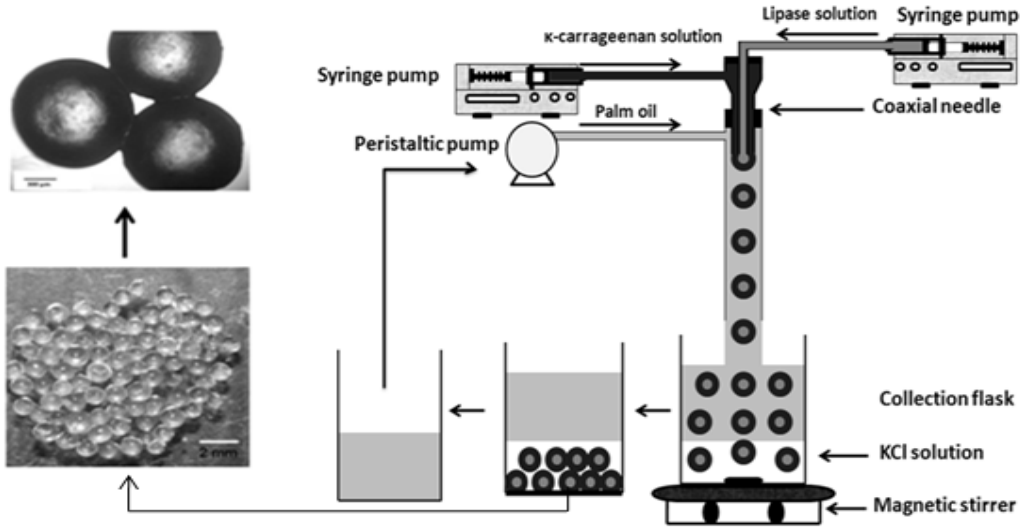

Fig. 1 : Schematic diagram of encapsulation procedure using natural polymer with oil recycle.

In the micrographic picture of encapsulated lipase 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 ^{o}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.

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 ^{o}C. Relative activity as a function of pH is shown in **(Fig. 2)**. 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 ^{o}C to 50 ^{o}C at optimum pH 7. The study of temperature could not be beyond 50 ^{o}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.

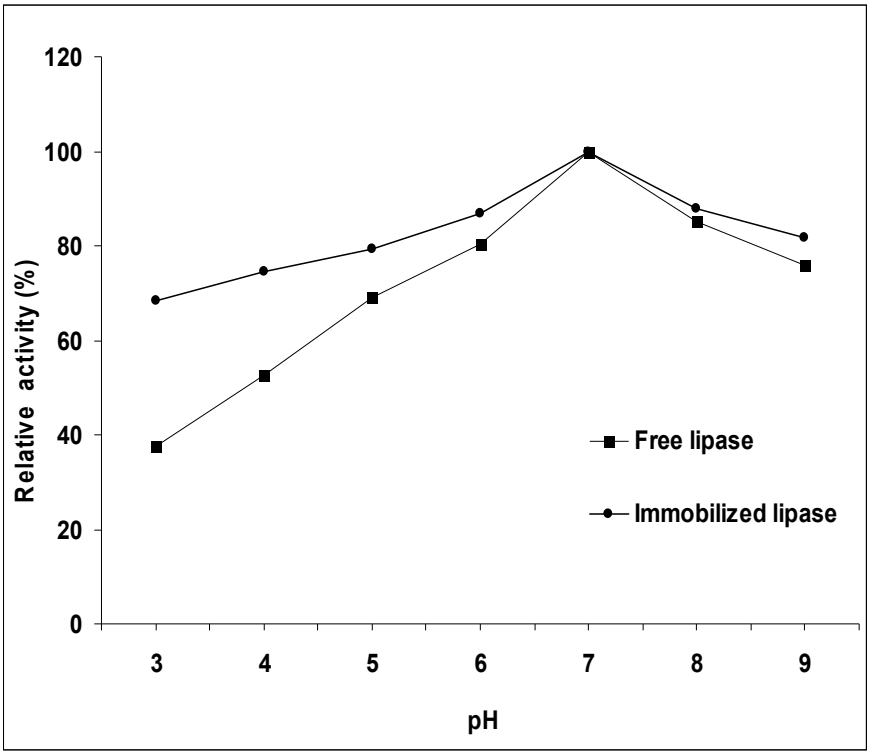

Fig. 2 : pH stability of free and immobilized lipase at 30ºC.

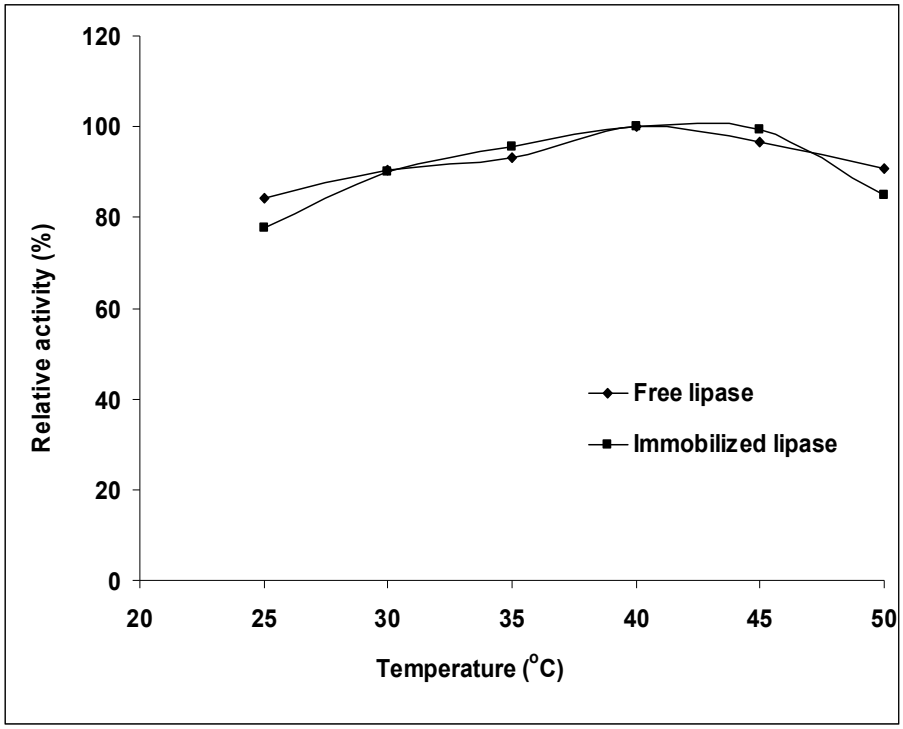

Fig. 3 : Temperature stability of free and immobilized lipase at pH 7.

However, most of the enzymatic reactions do not require high temperature. Relative activity as a function of temperature is shown in **(Fig.3)**. Optimum temperature for free and encapsulated lipase in κ-carrageenan was found to be 30 °C. Encapsulation is the physical enclosure of enzyme within a polymeric membrane. In this method, enzymes do not make chemical 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[12].

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[13]. 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[14]. The stability of encapsulated lipase in κ-carrageenan in various solvents is shown in (Table 1). There was no collapse of the gel structure when the encapsulated lipase was suspended in solvents. The activity studies showed that the encapsulated *Burkholderia cepacia* lipase was stable in alcohol and hexane compared to acetone. *Burkholderia cepacia* lipase showed good stability towards methanol.

Table 1 : 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

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 ^{o}C and the activities were measured periodically over duration of 10 days. As can be seen from **Fig.4**, the encapsulated lipase stored at 27 ^{o}C was stable until the disintegration of the carrier. One of the problem 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. 5)** 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.

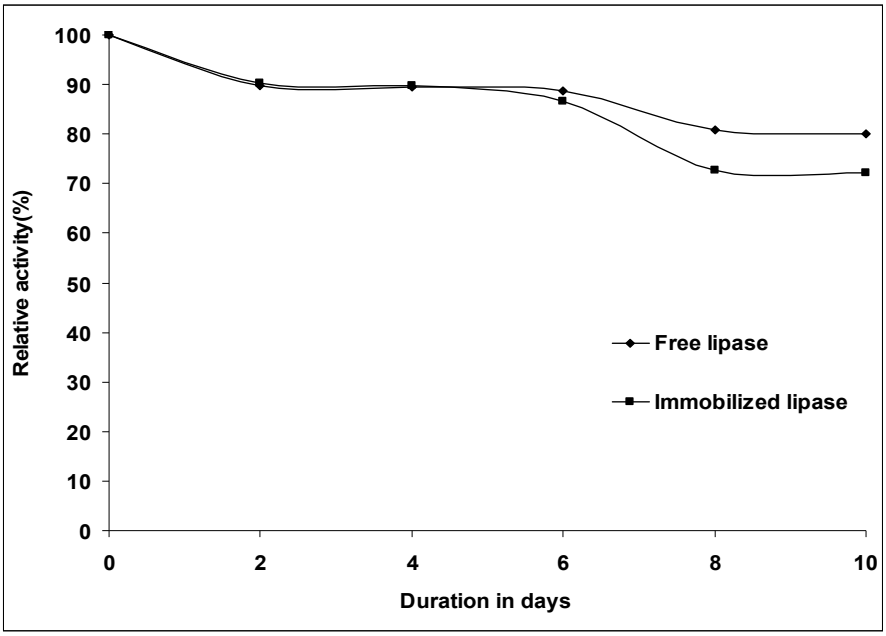

Fig. 4 : Storage stability of free and immobilized lipase at 27ºC.

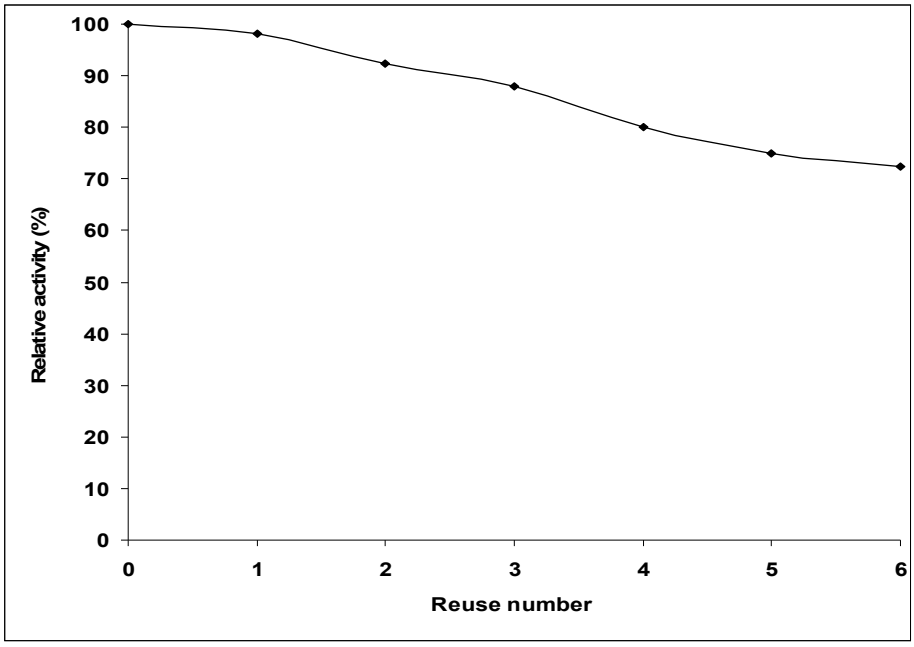

Fig. 5 : Reuse stability of the immobilized lipase in *p*- NPP hydrolysis.

CONCLUSION

In this work, lipase from *Burkholderia cepacia* was encapsulated in κ-carrageenan by co-extrusion method forming a liquid core capsule. 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. *Burkholderia 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. Burkholderia cepacia lipase encapsulated in κ-carrageenan could be a potential heterogeneous catalyst for non-conventional media.

ACKNOWLEDGEMENT

The authors wishes to thank the esciencefund, Ministry of Science, Technology and Innovation (MOSTI), Malaysia for funding this project (Grant no: SCF0012-IND-2006).

REFERENCES

1. Jaeger K.E. and Eggert T., Lipases for biotechnology, *Curr. Opin. Biotechnol*, **13**, 390-397, **(2002)**
2. Seema S.B. and Steven H.N., Immobilization of lipase using hydrophilic polymers in the form of hydrogel beads, *Biomaterials,* **23**, 3627-3636, **(2002)**
3. Won K., Kim S., Ki K.J., Park H.W. and Moon S.J., Optimization of lipase entrapment in Ca-aliginate gel beads, *Process Biochem*, **40**, 2149-2154, **(2005)**

4. Rahman M.B.A., Tajudin S.M., Hussein M.Z., Rahman R.A., Salleh A.B. and Basri M., Application of natural kaolin as support for the immobilization of lipase from *Candida rugosa* as biocatalyst for effective esterification, *Appl. Clay Sci.,* **29**, 111-116, **(2005)**

5. Yujun W., Jian X., Guangsheng L. and Youyuan D., Immobilization of lipase by ultrafiltration and cross-linking onto the polysulfone membrane surface, *Bioresource Technol.,* **99**, 2299-2303, **(2007)**

6. Noureddini H., Gao X. and Philkana R.S., Immobilized Pseudomonas cepacia lipase for biodiesel fuel production from soybean oil, *Bioresource Technol*., **96**, 769-777, **(2005)**

7. Orcaire O., Buisson P. and Pierre A.C Application of silica aerogel encapsulated lipases in the synthesis of biodiesel by transesterification reactions, *J. Mol. Catal. B: Enzym*., **42**,106-113, **(2006)**

8. Benita S., Microencapsulation methods and industrial applications, Marcel Dekker, New York, **(1996)**.

9. Jang K.H., Jung S.J., Chang H.S. and Chun U.H., Enzymatic sorbitol production with *Zymomonas mobilis* immobilized in cappa-carageenan, *J. Microbiol. Biotechnol*, **6**, 36-42, **(1996)**

10. Hung T.C., Giridhar R, Chiou S.H ,and Wu W.T., Binary immobilization of Candida rugosa lipase on chitosan, *J. Mol. Catal-B Enzym.,* **26**, 69-78, **(2003)**

11. Okahata Y., Hatano A., and Ijiro K., Enhancing enantioselectivity of a lipid-coated lipase *via* imprinting methods for esterification in organic solvents, *Tetrahedron: asymmetry*., **6**, 1311-1322, **(1995)**

12. Tümtürk H., Karaca N., Demirel G. and Şahin F., Preparation and application of poly(*N*,*N*-dimethylacrylamide-*co*-acrylamide) and poly(*N*-isopropylacrylamide-*co*-acrylamide)/κ-Carrageenan hydrogels for immobilization of lipase, *Int. J. Biol. Macromol.,* **40**, 281-285, **(2006)**

13. Hazarika S., Goswami P., Dutta N.N. and Hazarika A.K., Ethyl oleate synthesis by *Porcine pancreatic* lipase in organic solvents, *Chem. Eng. J.,* **85**,61-68, **(2002)**

14. Azevedo A.M., Prazeres D.M.F., Cabral J.M.S. and Fonseca L.P., Stability of free and immobilised peroxidase in aqueous–organic solvents mixtures, *J. Mol. Catal. B: Enzym.,* **5**, 147-153, **(2001)**

● ● ● ● ●