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Alcoholysis of salicornia oil using free and covalently bound lipase onto chitosan beads

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Abstract

Porcine pancreatic lipase was immobilized on chitosan by covalent binding and retention of its activity was examined. The activities of free and immobilized lipase were determined using olive oil as substrate. The free and immobilized enzymes showed pH 9 as optimum and retained 50% of activity after five cycles. When the substrate concentration was kept constant and enzyme concentration was varied, the K_m and V_{max} were observed to be 4.0×10^{-7} and 0.32, and 3.32×10^{-7} and 0.32, respectively, for free and covalently bound enzyme. This indicates that there is no possibility of conformational change during immobilization. Immobilized enzyme showed improved thermal and storage stability. Alcoholysis of salicornia oil, mediated by free and immobilized lipase, was carried out at 25 °C using methanol in hexane and acetone. Free and immobilized enzyme in hexane produced, respectively, 45% and 55% of fatty acid methyl ester after 12 h.

Keywords: Lipase; Immobilization; Chitosan; Salicornia oil; Alcoholysis

1. Introduction

The immobilization of biologically active species is becoming an increasingly important tool for an efficient production of useful compounds without side reactions. The selection of support material and the method of immobilization are very important for carrying out the desired enzymatic reaction. Naturally-occurring polymers, such as chitin and chitosan, offer certain advantages such as porous structure, low bulk density and little or no toxicity. They are practically insoluble in water, alkalies and organic solvents, but soluble in acidic media. Chitosan can be used more conveniently than chitin owing to the presence of free amino groups. The amino groups of chitosan facilitate the immobilization of enzyme, either by adsorption or chemical reaction (Kasumi, Tsuji, Hayashi, & Tsumura, 1977).

Microbial lipase belongs to a class of *Serine Hydrolase* (EC. 3.1.1.3), which is produced by various microorganisms (Sugiura, 1984). Lipase from *Candida cylindracea* was immobilized on chitosan by a covalent binding method for the hydrolysis of beef tallow by Sakakibara, Okada, Takahashi, and Tokiwa (1993). It is reported that *porcine pancreatic* lipase can be immobilized on a macro porous cross-linked polymer of styrene with divinyl benzene and used for transesterification of methyl butyrins with 1-butanol. It was found that the catalytic activity of immobilized *porcine pancreatic* lipase was twice higher than that of the free one (Xie, He, & Lu, 1994). The immobilization of porcine pancreatic lipase on C_8 HPLC beads was reported by (van Tol, Stevens, Veldhuizen, Jongejan, & Duine, 1995) and

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carried out by the transesterification of hexanol and tributyrin in different organic solvents for a kinetic parameter study. Lipase was also immobilized on modified chitosan by the introduction of hydrophobic groups into porous cross-linked chitosan beads which were further used for the transesterification of racemic 1-phenyl ethyl alcohol and vinyl acetate in organic solvents to investigate the effects of water and hydrophobic groups on the enzyme activities (Kawamura, Okano, & Tanibe, 1998).

The enzymatic alcoholysis of vegetable oils and animal fats is an environmentally friendly process for manufacturing short-chain fatty acid alkyl esters. In industry, alcoholysis is generally carried out by heating vegetable oil and alcohols at relatively high temperatures. Because of the relatively high energy cost of the conventional process and the anticipated lower prices of enzymes, industrial application of lipase in the oleochemical industry has become more beneficial (Shaw & Wang, 1991). Methyl and ethyl esters of long chain fatty acids are also excellent alternatives for biofuel applications (Lawson & Hughes, 1988). Enzymatic alcoholysis of oils with short-chain alcohols has been reported in the literature (Kanasawud, Phutrakul, Bloomer, Adlercreutz, & Mattiasson, 1992). Enzymatic alcoholysis of Black currant oil in ethanol at 30 °C was reported to yield 52% of esters after 8 h (Vacek et al., 2001). Lipases from *Mucor miehei*, *Pseudomonas fluorescens* and *Candida* sp. were examined by Mittelbach (1990) for enzymatic alcoholysis of sunflower oil with ethanol and methanol in petroleum ether solvent. The most active lipase for alcoholysis with ethanol was reported to be from *Pseudomonas fluorescens*, which gave 99% conversion in light petroleum at 45 °C after 5 h. The other two enzymes from *Mucor miehei* and *Candida* sp. were not so active for mediating enzymatic alcoholysis with ethanol. Alcoholysis of palm oil midfraction (PMF) with methanol and propanol in hexane by lipase from *Rhizopus rhizopodiformis* was reported and difference was observed between methanolysis and propanolysis (Basri et al., 1997). The conversion of PMF to propyl palmitate and propyl oleate after 10 h were and 15%, respectively, whereas, for methyl palmitate and methyl oleate, they were 60% and 10%, respectively.

Salicornia is a halophyte (salt-tolerant plant) which belongs to *chenopodiaceae* family, grown in saline soil and water (Weete, Rivers, & Weber, 1970). The seeds of the plant are small, containing 28–30% of oil with an appreciable linoleic acid content (Austenfeld, 1986). The enzymatic alcoholysis of this oil has not been reported.

In the present work, an attempt is made to optimize the conditions for covalent coupling of lipase onto chitosan and subsequent alcoholysis of salicornia oil using free and immobilized lipase.

2. Materials and methods

2.1. Materials

Porcine pancreatic lipase (EC 3.1.1.3) was obtained from Sigma Chemicals Co., USA. Chitosan was received from the Central Institute of Fisheries Technology (CIFT, Cochin, India). Triethanolamine hydrochloride, copper nitrate and gluteraldehyde were obtained from S.D. Fine-Chemicals Ltd., Mumbai, India. All other reagents and chemicals used were of analytical grade.

2.2. Assay of lipase activity

The hydrolytic activities of free and immobilized lipase were determined spectroscopically by a reported method (Schmidt, Stork, & Von Dahl, 1974) using 1 M triethanolamine hydrochloride buffer of pH 8.5, 1% (w/v) olive oil solution in 0.89% (w/v) NaCl and 1% (w/v) gum arabic solution. The absorbance was measured at 440 nm and the activity of the enzyme was determined from the calibration plot considering one unit of hydrolytic activity of lipase as the amount of enzyme that catalyzes the hydrolysis of olive oil to form 1 μmol fatty acid per min at 35 °C. The measurements were taken in triplicate and mean values, along with standard deviation, were presented. The results were observed within less than 3% variation limit.

2.3. Preparation of immobilized lipase

Chitosan beads were prepared according to the procedure given by Sun and Payne (1996). Immobilization of lipase was carried out by covalent coupling. Chitosan beads (100 mg) were incubated with various concentrations (0.01–5.0%) of gluteraldehyde for the activation of free amino groups. The beads were washed with distilled water to remove excess gluteraldehyde. The activated support was further used for coupling of lipase in phosphate buffers of different pH at 5 °C using 0.5–10 mg of enzyme for different time intervals (0.5–20 h). The treated beads were washed with distilled water to remove free enzyme. The protein content in immobilized beads was estimated by determining the protein from the supernatant liquid by Lowry's method (Lowry, Rosenbrough, Farr, & Randall, 1951) and using a differential method.

2.4. Thermal stability

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 (Soni, Desai, & Devi, 2001):

$$\ln A_t = \ln A_0 - K_d(t),$$

where A_0 is the initial activity, and A_t is the activity after heat treatment for t minutes.

2.5. pH activity profile

The activities of free and immobilized lipase were measured by incubating them at 35 °C for 10 min in buffer solutions of pH ranging from 2.0 to 12.0, using olive oil as a substrate.

2.6. Storage stability

The residual activities of the free and immobilized enzymes stored at 35 °C were determined and expressed as the percentage retention of their residual activities at different time intervals.

2.7. Reusability of immobilized lipase

To evaluate the reusability of the immobilized lipase, the beads were washed with water and buffer after use and then suspended again in a fresh reaction mixture to measure the enzymatic activity. The procedure was repeated until the enzyme lost 50% of its total activity.

2.8. Determination of kinetic constants

The Michaelis constant (K_m) and the maximum reaction velocity constant (V_{max}) for the free and immobilized lipase were determined by measuring the velocity of the reaction at varying enzyme concentrations ranging form 0.83×10^{-10} to 4.17×10^{-10} M and keeping substrate concentration constant at 4.42×10^{-5} M. Free and immobilized enzymes in the optimum pH buffer were incubated with substrate for 30 min at 35 °C. From the activity of the enzymes K_m and V_{max} were calculated using a Lineweaver–Burk plot of $1/S$ vs $1/V$.

2.9. Properties of salicornia oil

Salicornia oil was obtained through Soxhlet extraction of salicornia brachiata seeds. The oil was analyzed for typical physical and chemical properties. The conversion of fatty oil to ester was carried by the AOCS standard method and the detection of fatty esters was done by using a Waters (LC-4000) HPLC system equipped with a variable refractive index detector (Model R 401) and Millenium 2010 software.

2.10. Enzyme-mediated alcoholysis

Enzymatic alcoholysis of salicornia oil was carried out using 0.50 mM oil and 0.5–3.0 mM methanol, 6 ml solvent and fixed quantities of free or immobilized lipase. The reaction was carried out at 25 °C under constant stirring for different time intervals. The reaction temperature was maintained at 25 °C owing to the instability of free enzyme at higher temperatures (Fig. 2). A control run was carried out without using enzyme. After different time intervals, the reaction mixtures were filtered and separated and washed with hexane. The solvent was evaporated and the mixture was separated by preparative thin-layer chromatography (TLC), yielding the requested fatty acid methyl esters. The separation was done using precoated TLC sheets with a silica gel layer (0.2 mm), and developed in a solvent mixture containing light petroleum/diethyl ether/acetic acid (80:20:1.6 by volume). Spots of different groups were identified using iodine vapour. Fractions corresponding to fatty acid methyl esters were extracted from the plate with acetonitrile. The methyl ester content was analyzed by the HPLC system described earlier. The mobile phase acetonitrile was used at 30 ml min^{-1} flow rate. The yield was calculated, based on the conversion of initial oleic, palmitic and linoleic acid equivalents in salicornia oil to the corresponding oleic, palmitic and linoleic ester present at the time of termination of the reaction.

3. Results and discussion

3.1. Immobilization of lipase

For food, pharmaceutical, medical and agricultural applications, nontoxicity and biocompatibility of materials are also required. To respond to the growing public health and environment awareness, the material should be biodegradable, and prove economical and inexpensive. Chitosan is biocompatible, biodegrading to harmless products and nontoxic; it shows physiological inertness and remarkable affinity for proteins, hemostatic, fingistatic, antitumoral and anticholesteremic. It is also eco-friendly and safe for humans and the natural environment. The process for covalent binding using gluteraldehyde is also very simple. The process is superior to that of other immobilization processes on the synthetic supports.

During the process of covalent binding, the amino groups of chitosan were activated with gluteraldehyde to form Schiff bases (Coughlin, Aizawa, Alexander, & Charles, 1975) followed by covalent coupling of enzyme to the active sites. The maximum coupling of lipase was observed at 0.02% of gluteraldehyde, at pH 7, at 16 h and 5 °C temperature (Table 1). The protein coupled to the support was observed to be 6.67 mg/g of the dry support.

3.2. Thermal stability

The thermal stability of the enzyme is one of the most important factors for its application. Free and

Table 1
Effect of enzyme concentration on immobilization of lipase (Weight of chitosan beads 100 mg, pH 8.5, temperature 35 °C)

Enzyme employed (mg)	Protein content on bead (μg)	Active protein on bead (μg)	Retention of activity on bead (%)
1.0	51.9	16.2	31.2
2.0	131	41.0	31.3
3.0	151	56.1	31.2
4.0	208	82.2	39.6
6.0	213	83.0	39.0

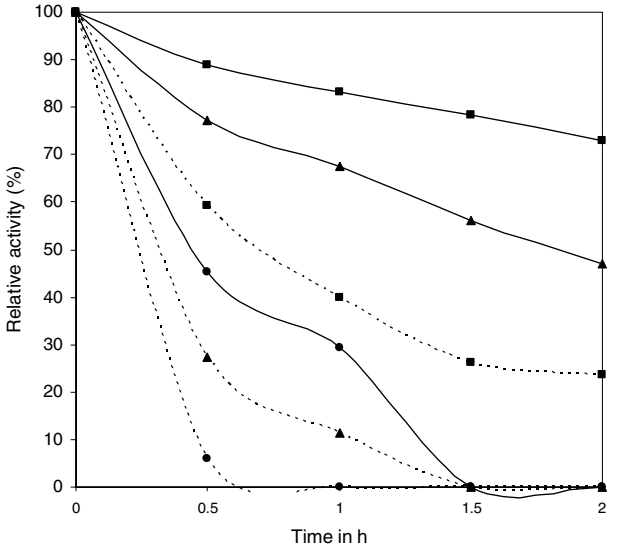

Fig. 1. Effect of temperature on the activities of free and immobilized enzymes at pH 8.5, 40 °C (●), 50 °C (♦), 60 °C (▲), free enzyme (----), immobilized enzyme (—).

immobilized enzymes were incubated for 2 h at various temperatures and the enzyme activity was measured at various time intervals after cooling the enzyme to 35 °C and following the procedure described earlier. Results obtained are illustrated in Fig. 1. Covalently bound enzyme showed better thermal stability at all temperatures and time periods. However, marked reduction in the activity for both free and covalently bound enzyme was observed at 60 °C. Thermodeactivation constants calculated are given in Table 2. A higher value of thermodeactivation constant indicates a higher rate of thermal degradation at the respective time and temperature.

3.3. Optimum pH

pH is one of the most influential parameters altering enzymatic activity in an aqueous medium. Immobilization of enzyme is likely to result in conformational changes of enzyme resulting in a variation of optimum pH. However, Fig. 2 shows that maximum enzyme activity is exhibited at pH 9.0 by free as well as immobilized enzyme, indicating absence of conformational changes in the enzyme during covalent binding.

3.4. Storage stability

Enzymes, being sensitive to higher temperatures, generally need low-temperature storage. Immobilization of enzyme may overcome this constraint and material could be stored, in some cases, at room temperature without much loss in enzyme activity. This is very important for the application of an enzyme on the commercial scale. Hence, storage study of free and immobi-

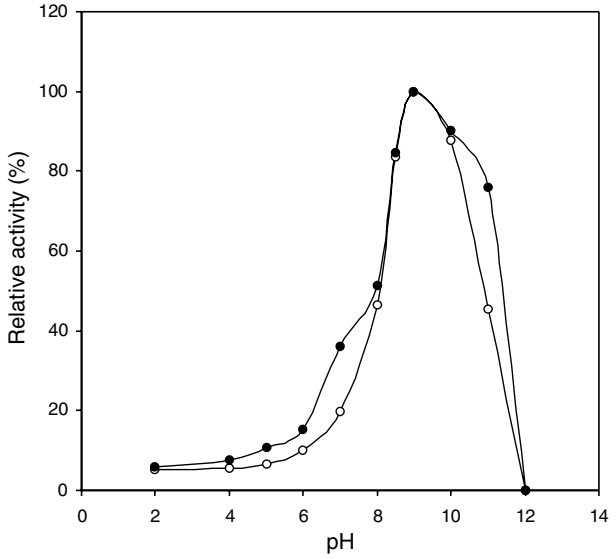

Fig. 2. Effect of pH on the activities of free and immobilized lipase at 35 °C; free enzyme (○), immobilized enzyme (●).

Table 2
Thermal stability of enzymes

Time (min)	Thermodeactivation constant × 10^2					
	40 °C		50 °C		60 °C	
	Free	Immobilized	Free	Immobilized	Free	Immobilized
30	1.75	0.69	4.32	0.85	9.32	2.62
60	1.52	0.38	3.62	0.66	–	2.04
90	1.49	0.27	–	0.64	–	–
120	1.20	0.26	–	0.63	–	–

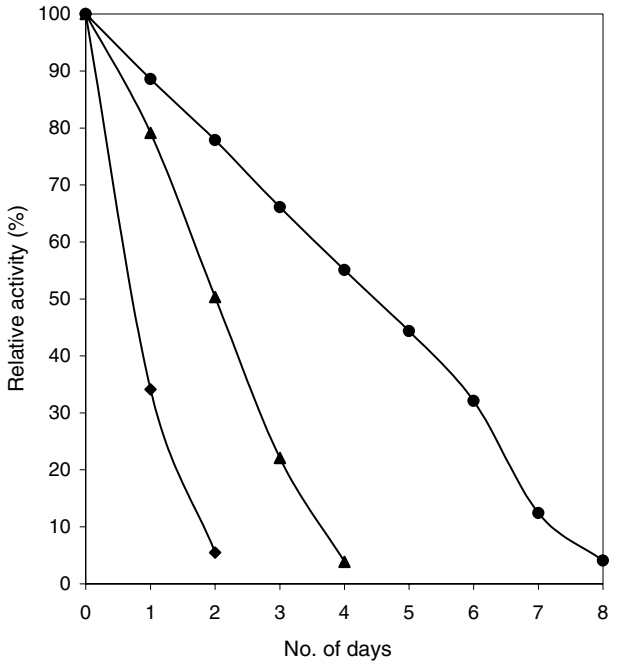

Fig. 3. Storage stability of free and immobilized enzymes at 35 °C; free enzyme (♦), immobilized enzyme (in water) (▲), immobilized enzyme (dry) (●).

lized enzyme was carried out at 35 °C for various time intervals. The immobilized and free enzyme were stored in distilled water; also, immobilized enzyme was stored, without any medium, in a separate container. The residual activity of the enzyme at different time intervals was estimated using olive oil emulsion as substrate and results are given in Fig. 3. At 35 °C, free enzyme lost its activity rapidly, whereas covalently bound enzyme lost its activity slowly. The immobilized enzyme, stored without any medium of suspension, showed higher stability than when stored in distilled water, which indicates that, in aqueous medium, enzyme slowly leaches from the support.

3.5. Reusability of immobilized lipase

A major drawback of free enzymes is that they can not be reused and this problem could be addressed by immobilization. The reusability of enzyme was examined by using the same enzyme repeatedly, with a fresh amount of olive oil emulsion as the substrate. Covalently bound enzyme showed gradual decrease in activity with increasing number of cycles. 50% of activity could be retained, even after five repeated cycles. The K cat value of immobilized enzyme was observed to be 1.6×10^6.

3.6. Determination of kinetic constant

The effect of the substrate concentration on the reaction rate, catalyzed by free and immobilized lipase, was studied using olive oil as substrate. From the Lineweaver Burk plot of $1/V$ vs $1/S$. Michaelis constant (K_m) and the maximum reaction velocity (V_{max}) of the free and immobilized enzyme were calculated. No significant changes in K_m and V_{max} values for free and immobilized enzyme were observed when substrate concentration was kept constant which indicates that there is little possibility of conformational change during immobilization.

3.7. Properties of salicornia oil

The oil content in seed was about 33% (w/w). The physical and chemical properties of salicornia oil are given in Table 3. Salicornia oil contained about 87–88% of unsaturated fatty acid and 12–13% of saturated fatty acid.

3.8. Alcoholysis of salicornia oil

3.8.1. General

Alcoholysis is a transesterification reaction, in which the ester bond of triglyceride is broken by lipase to produce fatty acids which further react with the alcohols to form alkyl esters. A chromatogram of the product of the alcoholysis reaction of salicornia oil and methanol is shown in Fig. 4. Table 4 shows that free and immobilized lipase convert salicornia oil into 45% and 55% of fatty acid methyl ester in 12 h, respectively, when a 1:3 molar ratio of oil and methanol is used in hexane. Zuyi and Ward (1993) have reported only 20% formation of fatty acid isopropyl ester by the enzymatic alcoholysis of cod liver oil with 2-propanol.

The kinetics of alcoholysis reaction, with respect to free and immobilized lipase, are illustrated in Fig. 5. Though maximum % conversion was achieved in a

Table 3
Physical and chemical properties of salicornia oil

Physical properties	Chemical properties	
Specific gravity: 0.910 g cm^{-3}at 25 °C	Saponification value: 192	Fatty acid composition (%)
Refractive index: 1.473 (n_D40 °C)	Iodine value: 127	Palmitic acid (16:0): 9.6
Colour index: 11.19		Linoleic acid (18:2): 61.0
		Oleic acid (18:1): 25.6

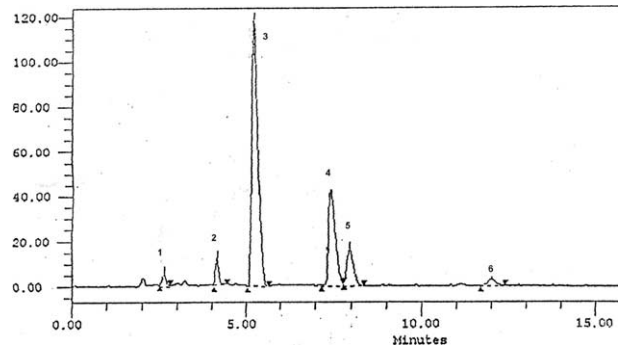

Fig. 4. A typical HPLC chromatogram of alcoholysis products of salicornia oil with methanol; symbols: solvent (1), methyl linolenate (2), methyl linoleate (3), methyl oleate (4), methyl palmitate (5), methyl stearate (6).

Table 4
Effect of molar ratios of oil to methanol and solvents on the alcoholysis of salicornia oil using lipase

Mole ratio of oil/methanol	Solvent	Conversion (%)			
		Linoleic ester	Oleic ester	Palmitic ester	Total ester
1:1	Hexane	19.2 (23.4)	23.4 (29.3)	62.5 (68.8)	23.7 (28.3)
1:2	Hexane	29.3 (45.0)	47.5 (65.4)	89.0 (89.2)	38.6 (52.7)
1:2	Acetone	26.3 (23.1)	49.8 (31.8)	89.5 (52.3)	37.3 (27.2)
1:3	Hexane	39.8 (48.3)	51.3 (67.3)	76.5 (89.1)	44.7 (55.3)
1:3	Acetone	24.3 (27.5)	32.0 (33.9)	56.3 (55.1)	28.4 (30.8)
1:6	Hexane	33.9 (41.2)	37.3 (41.3)	51.2 (48.2)	35.1 (40.4)
1:6	Acetone	29.2 (18.3)	37.0 (25.8)	51.6 (27.8)	32.2 (20.4)

Alcoholysis of salicornia was carried out at 25 °C for 0.50 mM of oil, 1.0 ml water, 0.125 g of free lipase, 12 h.
Figures in brackets are for alcoholysis carried out using 20 g of immobilized system.
% conversion was calculated based on the initial fatty acid present in the oil calculated, i.e., 61% linoleic acid, 25.6% oleic acid, 9.6% palmitic acid.

relatively shorter time by free enzyme, % conversion was higher when immobilized enzyme was used.

3.8.2. Effect of molar ratio of substrate on alcoholysis

The optimum molar ratio of oil to methanol in the alcoholysis reaction required was observed to be 1:3 for free, as well as immobilized enzyme, which indicates that porcine pancreatic lipase is not regiospecific, as with the 1,3-specificity for lipase from *Rhizopodiformis* reported by Basri et al. (1997) (Fig. 6). Increasing the mole ratio of methanol to oil decreased the extent of alcoholysis. This can be attributed to the possible distortion of the essential water layer for the enzyme activity by the excess methanol, as suggested by Laane, Boeren, Vos, and Veeger (1986).

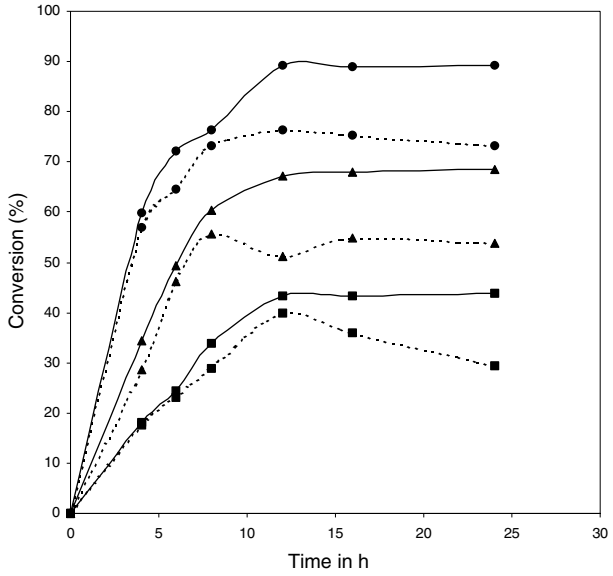

Fig. 5. Effect of time on alcoholysis using free (----) and immobilized (—) lipase; methyl linoleate (■), methyl oleate (▲), methyl palmitate (●).

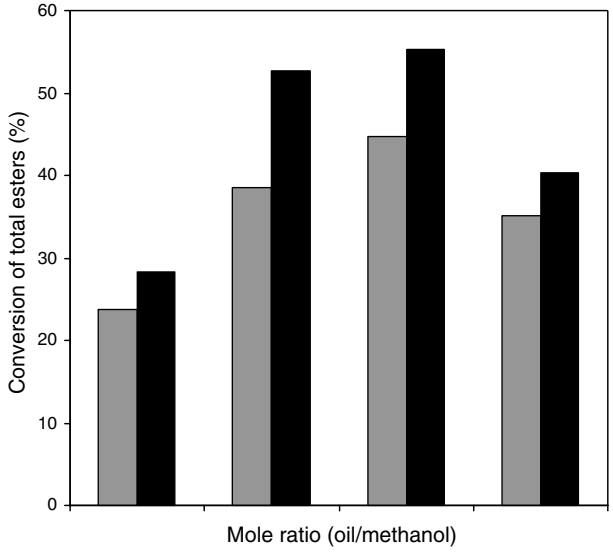

Fig. 6. Effect of the molar ratio on the conversion of alcoholysis products using free (▩) and immobilized lipase (■).

3.8.3. Effect of solvent on alcoholysis

The lipase exhibits higher alcoholysis activities in a non-polar solvent (hexane) than polar solvent (acetone) (Table 4). This finding is in agreement with that of Laane et al. (1986) who reported variation in activities of lipase with change in the reaction medium. The higher enzymatic activity in the non-polar solvent may be attributed to the minimum distortion of the hydration layer around the enzyme by the solvent, whereas the polar solvent, due to its high affinity for water, might remove the essential hydration layer around the enzyme, thus in decreasing enzyme activity.

3.8.4. Role of water in alcoholysis

A quantity of water equivalent to 15.2% of total reaction volume for the normal alcoholysis reaction was employed with free enzyme which led to 45% conversion of fatty acid methyl ester. However, only 13.4% conversion was observed in the alcoholysis reaction when using free enzyme and no water. Mittelbach (1990) have reported increased reaction rate in the presence of 17% water for alcoholysis of sunflower oil with short chain alcohols.

4. Conclusions

Natural polymer chitosan, in the form of beads, was successfully used for covalent coupling of lipase. Immobilized enzyme showed superior properties when compared to the free one. The extent of covalently coupled lipase to chitosan was observed to be 6.67 mg/g dry weight of chitosan. Immobilized lipase showed better thermal and storage stabilities. The enzyme could retain 50% of its activity after five repetition cycles. The Michaelis constant (K_m) and the maximum reaction velocity (V_{max}) of the free and immobilized enzymes were almost the same, indicating no conformational change taking place during immobilization. The immobilized lipase showed better activity towards the alcoholysis reaction of salicornia oil. The optimum molar ratio of oil to alcohol was observed to be 1:3. The free and immobilized lipase exhibited higher alcoholysis activities in non-polar solvents.

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