J Am Oil Chem Soc (2008) 85:739–748
DOI 10.1007/s11746-008-1261-7

ORIGINAL PAPER

Efficient Immobilization of Lecitase in Gelatin Hydrogel and Degumming of Rice Bran Oil Using a Spinning Basket Reactor

Gurrala Sheelu · G. Kavitha · Nitin W. Fadnavis

Received: 17 October 2007 / Revised: 8 May 2008 / Accepted: 3 June 2008 / Published online: 26 June 2008

Abstract Immobilization of Lecitase (Phospholipase A1) in gelatin hydrogel and its stability is studied with a view to utilizing the immobilized enzyme for degumming rice bran oil. Excellent retention of enzyme activity (>80%) is observed in hydrogel containing 43.5% gelatin crosslinked with glutaraldehyde. Compared to the free enzyme which has a broad pH-activity profile (6.5–8.0), the activity of the immobilized enzyme is strongly dependent on pH and has a pH-optimum of pH 7.5. The optimum temperature of enzyme activity increases from 37 to 50 °C. Compared to the free enzyme which loses all its activity in 72 h at 50 °C, the immobilized enzyme retains its activity in full. The immobilized enzyme has been used efficiently in a spinning basket bioreactor for the degumming of rice bran oil with 6 recycles without loss of enzyme activity. The phosphorus content of the oil decreases from 400 ppm to 50–70 ppm in each cycle. After charcoal treatment and dewaxing, a second enzymatic treatment brings down the phosphorus content to <5 ppm.

Keywords Phospholipase A1 · Immobilization · Gelatin · Rice bran oil · Degumming · Spinning basket reactor

Introduction

Rice bran oil is extensively consumed as an edible oil due to its potential as a neutraceutical food [1]. It is the only known vegetable oil to contain γ-oryzanol, a complex mixture of ferulate esters with sterols and triterpene alcohols [2]. γ-Oryzanol is known to exhibit useful medicinal properties such as hypocholesterolemic activity, decreasing early atherosclerosis and treatment of inflammatory processes. It also contains tocopherols/tocotrienols that are associated with antioxidant and anti-tumor activities [3–7]. Japan began producing rice bran oil 50 years ago, where it is popularly known as 'heart oil' because of its special properties. Rice bran oil also contains a good balance of saturated (20%), monounsaturated (45%) and polyunsaturated fats (35%) [8].

The chemical refining of rice bran oil generally results in considerably higher losses than observed in the refining of other vegetable oils. This is due to the presence of relatively large amounts of free fatty acids and non-triacylglycerol compounds [9]. Refining losses can be substantially reduced by using physical refining with the enzyme Lecitase. The enzyme Phospholipase A1, sold under the brand name Lecitase by Novozymes, Denmark, converts non-hydratable phospholipids into water-soluble lyso-phospholipids, which are then removed by centrifugation. Residual phosphorus levels of 0–5 ppm are obtained after enzymatic degumming, bleaching and dewaxing. This gives a good-quality edible rice bran oil. The enzymatic degumming process minimizes losses of oil, produces consistent degumming results, reduces water consumption, and is more environmentally sound [10]. However, present technology utilizes an enzyme solution for degumming which is lost after a single cycle and it would be advantageous to recycle the enzyme by immobilizing it on a solid support. One should of course consider the cost of immobilization. In many cases, the enzyme cost is getting lower due to advances in genetic and process engineering. A single use of non-immobilized enzyme

G. Sheelu · G. Kavitha · N. W. Fadnavis (✉)
Biotransformations Laboratory,
Indian Institute of Chemical Technology,
Uppal Road, Habsiguda, Hyderabad 500007, India
e-mail: fadnavis@iict.res.in; fadnavisnw@yahoo.com

could be economically more viable than multiple use of the immobilized enzyme. Besides, for a food item, it is also essential to consider the large tonnage (>10 TPD) of the process, biocompatibility of the matrix, its cost, ease and efficiency of immobilization, and recovery of enzyme activity after each recycle.

In the present work, we report immobilization of Lecitase in a gelatin hydrogel matrix crosslinked with glutaraldehyde. The immobilization matrix is quite cheap and biocompatible, the immobilized enzyme is found to be thermally stable and can be used several times for degumming of rice bran oil. A spinning basket reactor is found to be highly effective in overcoming the problems of attrition of the gel and separation of the immobilized enzyme from the oil.

Materials and Methods

Lecitase (a 50% solution of the enzyme in a buffer) was obtained from Novozymes, Denmark. Eupergit C and XAD-7 were obtained from Fluka. These were washed with methanol and dried in a vacuum before use. All other chemicals were of analytical or of reagent grade obtained from Hi Media, India. All experiments were repeated 3 times. The values of enzyme activities reported in the text and figures are the average of 3 determinations and were reproducible within ±5%.

Enzyme Activity Assay

The assay procedure provided by the manufacturers along with the enzyme is based on hydrolysis of the substrate 1-(*S*-decanoyl)-2-decanoyl-1-thio-*sn*-glycero-3-phosphocholine [11]. However, we chose the assay system recommended by the Food and Agriculture Organization of the United Nations (FAO) based on an emulsion containing 2% lecithin, 6.4 mM calcium chloride and 3.2 mM sodium deoxycholate in distilled water [12]. Composition of this assay solution was modified to obtain reproducible results, especially for samples with traces of rice bran oil with free fatty acids. For our experiments, the assay buffer consisted of 135 mM calcium chloride, 8.35 mM sodium deoxycholate and 1.5% soybean lecithin in Tris-HCl buffer (0.01 M, pH 7.5). Thus a solution of calcium chloride (15 g) was prepared in Tris-HCl buffer (0.01 M, pH 7.5), sodium deoxycholate (3.5 g) and soybean lecithin (15 g) were added to the buffer, the mixture was warmed at 60 °C for 10 min and sonicated for 15 min. The emulsion was filtered through a cotton plug to remove any lumps and stored at room temperature. The assay buffer was stable for at least 3 days.

Enzyme activity was determined by a subsequent hydrolysis of soybean lecithin in sodium deoxycholate-lecithin emulsion. Upon hydrolysis at a fixed pH (e.g. 7.5), a long chain fatty acid is released which is neutralized by the addition of 0.2 N sodium hydroxide solution to maintain a constant pH. Typically, the assay was performed with 100 µl of free enzyme solution or 2.3 g (wet) gel in 25 ml of assay buffer at 30 °C for 20 min. 1 U of enzyme activity was defined as 1 µmol of NaOH consumed per min (equivalent to 1 µmol of fatty acid released per min under the assay condition). The free enzyme showed an activity of 425–475 units/ml at pH between 6.5 and 7.5.

Immobilization of Lecitase (Phospholipase A1) in Gelatin

Gelatin (1 g) was added to distilled water (2 ml) and the mixture was warmed at 60 °C to dissolve the gelatin. The enzyme stock solution (100 µl) was added with vigorous stirring and then a glutaraldehyde solution (100 µl of 25% aqueous solution) was added with continuous stirring with a glass rod. The thick jelly was cooled in ice and left overnight in refrigerator at 5 °C. The gel was then frozen with liquid nitrogen and crushed into small pieces (1–2 mm) with a pestle and mortar. The immobilized phospholipase enzyme thus obtained was washed once with cold sodium citrate buffer (10 ml, 0.01 M, pH 7.5) and stored in a refrigerator. At pH 7.5, assay of the gel showed an activity of 85 units and assay of the supernatant wash showed an activity of 10–12 units. Since the apparent immobilized enzyme activity is dependent on several factors such as particle size, pH, temperature, nature of the matrix etc. (see "Results and Discussion"), the immobilization efficiency was calculated to be 80–85% based on the activity in supernatant washings. At pH 6.5, the activity of the enzyme in free and immobilized state was similar and hence for comparison of activities of free and immobilized enzymes under different conditions, enzyme assays were mostly performed at pH 6.5.

Enzyme Stability Studies

All the studies were performed in triplicate. Several sets of conical flasks containing the buffer (or rice bran oil) and the enzyme (free or immobilized) were prepared simultaneously and one set was used for each assay.

To study the thermal stability in aqueous solution, the enzyme (100 µl of free enzyme or 2.3 g immobilized enzyme) was incubated in 5 mM sodium citrate buffer (pH 7.5, 5 ml) at different temperatures for varying periods. For the assay, the pH was adjusted to 6.5 with 0.2 N NaOH, the lecithin assay solution (20 ml, pH 6.5) was added and

residual enzyme activity was assayed by continuous titration with 0.2 N NaOH for 20 min. at pH 6.5.

For thermal stability studies in the rice bran oil medium, the immobilized enzyme (2.3 g) was pre-incubated in aqueous sodium citrate buffer of pH 7.5 (5 ml), the aqueous buffer was decanted, excess water was removed with filter paper, the gel was suspended in rice bran oil (10 ml) and incubated for periods of up to 80 h at different temperatures. In parallel experiments, the pH of the commercial enzyme solution was adjusted to 7.5, the enzyme solution (100 µl) was mixed with rice bran oil (1 ml) and incubated at 50 °C. For the determination of residual enzyme activity, the rice bran oil was separated from the gel, the gel was suspended in water (5 ml) and the acid components formed due to enzymatic reaction were neutralized by adjusting the pH to 6.5 with 0.1 N NaOH. The activity of the enzyme was then determined by a titrimetric method after addition of lecithin assay solution (20 ml). In the case of the free enzyme, the whole of the oil along with the enzyme was used for the assay.

Enzyme Recycle

In the recycle studies, 10 sets of conical flasks with the immobilized enzyme (2.3 g gel) suspended either in rice bran oil (20 ml) or aqueous buffer (20 ml, 5 mM sodium citrate, pH 7.5) were shaken on an orbital shaker at 150 rpm and 40 °C for 12 h. From one flask, the oil (or buffer) was decanted and the enzyme activity in the gel was assayed titrimetrically. The oil (or buffer) in the remaining flasks was decanted and replaced with fresh oil (or buffer) and shaking was continued again for 12 h. Thus studies on 10 recycles were performed with same batch of immobilized enzyme.

Degumming of Rice Bran Oil in the Spinning Basket Bioreactor

The spinning basket reactor (Fig. 8) consists of a catalytic basket made of stainless steel with length of 8 cm and diameter of 14 cm. The basket wall has pores of 0.5 mm diameter for using catalytic particles with a size greater than 1 mm. The solid catalyst particles (25 g corresponding to 2 ml of enzyme solution) were packed loosely in the rotating basket moved by a shaft. The basket containing the catalyst was placed in a temperature controlled double walled vessel containing crude rice bran oil (500 ml). The temperature of the vessel was maintained at 30 °C and the basket was rotated at a speed of 400 rpm so that the reactants circulate past the catalyst's active surface avoiding attrition of the catalyst. After the reaction for 2 h, stirring was stopped and the oil was drained out of the vessel from the drain valve provided at the bottom of the vessel.

Recycling of the Immobilized Enzyme in the Stirred Basket Reactor

In the recycle studies, the crude rice bran oil that had been treated with the enzyme in the stirred basket reactor was drained out, fresh oil (500 ml) and sodium citrate buffer (0.1 M, pH 7.5, 5 ml) were then added to the reactor and stirred for 2 h. The treated oil was then heated with 4% (w/v) bleaching earth and 1% (w/v) activated charcoal at 80–100 °C for 30 min with stirring, then cooled slowly to room temperature and centrifuged at 10,000 rpm for 15 min. The bleached and dewaxed oil was then analyzed for its phosphorus content.

Determination of the Phosphorus Content of Rice Bran Oil

The method of phosphorus determination is based on the ignition of the test portion, followed by nitric acid attack of the ashes and formation of a yellow phosphovanadomolybdic complex. The vegetable oil (5 g) is mixed with magnesium oxide (100 mg) in a ceramic crucible and the mixture is ashed in a furnace at 900 °C. The ash is then dissolved in nitric acid (5 ml, 6 N). To this, 20 ml of 1:1 mixture of ammonium molybdate solution (5% in water) and ammonium vanadate (0.25% in 0.2 N nitric acid) was added and absorbance of the solution was measured at 460 nm against a blank after 20 min incubation. A standard curve was also made with solutions of dipotassium hydrogen phosphate containing 5–500 ppm phosphorus.

Immobilization of Lecitase (Phospholipase A1) on Eupergit C and XAD-7

Eupergit C or XAD-7 (1 g) was added to a solution of enzyme stock solution (100 µl) in a sodium citrate buffer (10 ml, 0.1 M, pH 7.5). The contents were shaken on an orbital shaker at room temperature overnight. The supernatant was then decanted and the polymer beads were washed with the sodium citrate buffer (3 × 10 ml). In the case of Eupergit C, the activity in the beads at pH 7.5 was found to be 15 units/g of the polymer. In the case of XAD-7, the activity of the immobilized enzyme was found to be 11 units/g of support.

Immobilization of Lecitase in Microemulsion-Based Organo-Gel

Gelatin (5 g) was heated with distilled water (8.5 ml) at 60 °C for 15 min to complete gelation. Dioctyl sodium sulfosuccinate (AOT) solution (35 ml, 0.3 M in isooctane) was added with vigorous stirring. The viscous and turbid gel so obtained was cooled in ice with shaking for 10 min to get a

transparent free flowing liquid. The enzyme solution (500 μl) was slowly added to the cold solution with vigorous stirring to achieve a uniform distribution of the enzyme. The cooling bath was removed and glutaraldehyde (1 ml, 25% solution) was added. The contents were stirred at room temperature with glass rod till the contents start became viscous, then poured into a petri dish and left at room temperature overnight. The dry gel was cut into small pieces and washed with isooctane and diisopropyl ether and vacuum dried at room temperature for 12 h to obtain immobilized enzyme (9 g). The determination of the water content by Karl-Fisher titration showed that the water content of the gel was 7.7% (w/w). Determination of enzyme activity of the gel showed that the enzyme was completely inactive.

Immobilization of Lecitase (Phospholipase A1) on Celite

Celite (1 g) was added in small portions to a solution of Lecitase (100 μl) in a sodium citrate buffer (1 ml, 0.1 M, pH 7.5) while mixing the contents with a glass rod at room temperature. The slightly moist free flowing powder was stored in a refrigerator. Assay of the enzyme adsorbed on celite at pH 7.5 showed activity of 95 units. This was two times higher than observed for the free enzyme (50 units) in aqueous solution.

Results and Discussion

There are several reports of phospholipase immobilization, most of the work being confined to immobilization of Phospholipase A2 from cobra venom for biomedical applications. Supports such as CM-Sephadex [13], Eupergit C [14], alginate-silicate sol-gel matrix [15], chitosan [16], porous glass and silica matrix [17–19] have been shown to immobilize the phospholipases with good efficiencies. Acylation of ε-amino groups of lysines with 4-nitro-3-octanoyl-oxybenzoic acid and subsequent covalent coupling onto carbonyldiimidazole-activated cross-linked agarose beads, has also been shown to produce high retention of activity of PLA2 [20]. Anthonsen and co-workers have described immobilization of Phospholipase C from *Bacillus cereus* on XAD7, Sepabeads FP-DA, Eupergit C, Celite 547, Silica gel 60 by covalent attachment or by adsorption [21]. To the best of our knowledge, the only reported examples of immobilization of PLA1 is immobilization on epoxy resin Eupergit C [22], by adsorbtion on octyl agarose at low ionic strength, by covalent immobilization on supports coated with polyethyleneimine (PEI) and in cyanogen bromide agarose [23]. These reported procedures are however quite expensive and economically unviable for degumming of vegetable oils on a commercial scale.

Generally, lipases are sturdy enzymes and they can be immobilized on a variety of supports with fair efficiency [24–29]. Thus initially we explored the possibilities of immobilizing phospholipase A1 (Lecitase) by simple techniques such as adsorption on celite or XAD-7 resin, entrapment in organo-gels [29] and covalent binding to Eupergit C. In the case of XAD-7 and Eupergit C, the activity of the immobilized enzyme as determined by the titrimetric method was reasonable (10–15 units/g at pH 7.5) but in both cases the activity was completely lost after a second recycle during degumming of rice bran oil. In the case of celite, the apparent activity of the enzyme increased two fold on adsorption (95 units after adsorption compared to 50 units in aqueous solution for the same quantity of enzyme solution at pH 7.5) but after 2 recycles with rice bran oil, the matrix was covered with strongly adsorbed gums and hydrolyzed phosphatidic acids causing inhibition of enzyme activity. Immobilization of Lecitase in microemulsion based gelatin organo-gel resulted in complete loss of enzyme activity. We finally discovered that gelatin hydrogel was the most appropriate matrix for Lecitase immobilization.

Effect of Gelatin Concentration on Efficiency of Immobilization

Gelatin as an immobilization matrix is cheap and biocompatible. It has been used for immobilization of enzymes either as such [24], blended with calcium alginate [25] or in organo-gels with the use of surfactants [29]. Typically, immobilization of an enzyme in gelatin matrix is done by mixing the enzyme and gelatin solutions and crosslinking with glutaraldehyde. The resultant gel contains entrapped enzyme, partially bonded covalently to gelatin through formation of a Schiff's base. The efficiency of immobilization and the stability of the immobilized enzyme depends upon the crosslinking of the matrix which, in turn, is directly related to the concentration of gelatin. Thus the effect of gelatin concentration on enzyme immobilization was studied at a fixed enzyme concentration. The amount of gelatin in the gel was varied and the enzyme activity of the entrapped enzyme was measured. Figure 1 shows the effect of gelatin concentration on the efficiency of enzyme entrapment. It was observed that the amount of entrapped enzyme increases with the amount of gelatin. Due to limitations of viscosity, further increase in gelatin concentration was not possible. All further studies were made on the enzyme immobilized in 43.5% gelatin gels.

Effect of pH on Enzyme Activity

The protein matrix of gelatin is complex. It possesses amino acid residues with polar non-ionic groups such as hydroxy, ionic groups such as carboxy and amino, and also

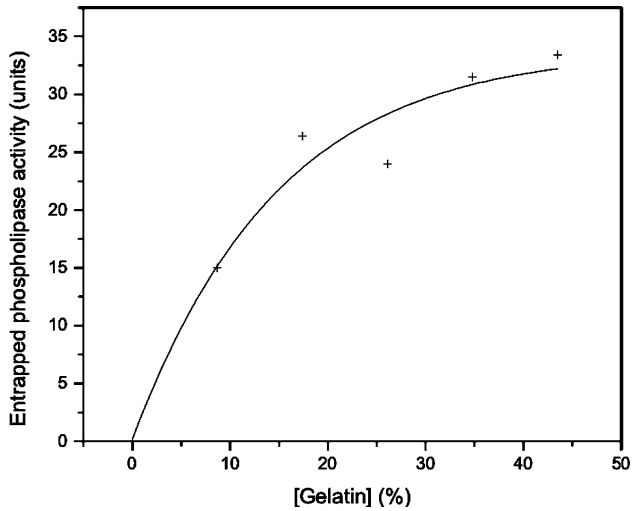

Fig. 1 Effect of gelatin concentration on efficiency of enzyme immobilization. Enzyme activity measured in 25 ml of assay buffer (pH 6.5) at 30 °C with total gel (2.3 g) obtained after immobilization of 100 µl of commercial Lecitase solution

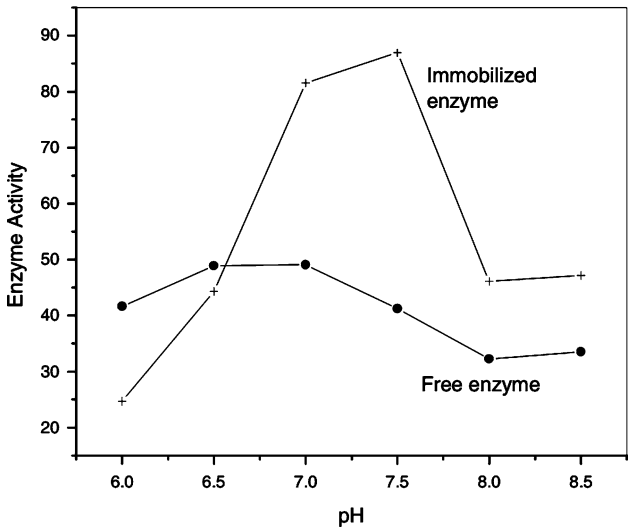

Fig. 2 Effect of pH on (*filled circles*) free and (*plus*) immobilized enzyme. Enzyme activity measured in 25 ml of assay buffer (pH 6.5–8.5) at 30 °C with total gel (2.3 g) obtained after immobilization of 100 µl of Lecitase solution

hydrophobic domains. Close interactions of these residues with those on Lecitase can cause shifts in the dissociation constants of various amino acid residues, and hence the pH-optimum. Thus the effect of pH on the activity of the immobilized enzyme was studied. Figure 2 shows the effect of pH on the activity of free and immobilized enzyme. In these experiments the gel (2.3 g) was equilibrated with citrate buffer (5 mM) of appropriate pH for 15 min and then assayed at a constant pH. The pH of assay buffer was varied from 6.0 to 8.5. It is observed that the free enzyme has a broad pH-optimum between pH 6.5 and 8.5 (400–500 units/ml) while the pH-activity profile for immobilized enzyme is much narrower with a pH-optimum between pH 7.0 and 7.5. Interestingly, the apparent activity of the enzyme increases when immobilized in a gelatin matrix and on adsorption on celite. For example, 1 ml of free enzyme solution shows an activity of 450 units. When the same amount of enzyme is immobilized in gelatin or adsorbed on celite, the apparent activity increases to 850–900 units. Most probably, immobilization causes a small conformational change in the enzyme resulting in its increased activity. It is well known that proteins undergo conformational changes upon adsorption on solid-liquid interfaces [30, 31] and when confined in water-pools of reverse micelles where many enzymes have been found to be 'superactive' [32].

For a comparison of the behavior of immobilized Lecitase in rice bran oil, the gelatin gel was pre-incubated in 0.5 M sodium citrate buffers of different pH (pH 6.0–8.5) and degumming of rice bran oil was performed using a fixed amount of gel to oil ratio (20 g gel for 200 ml oil). The enzyme activity was estimated from the residual phosphorus content of the oil after dewaxing and charcoal treatment (see "Materials and methods"). The enzyme activity was found to be higher at pH 8.5 than at pH 6, but the overall effect of pre-incubation was not very significant. Apparently, the amount of aqueous buffer present in the gelatin matrix is too low to change the overall pH of the medium (Fig. 3).

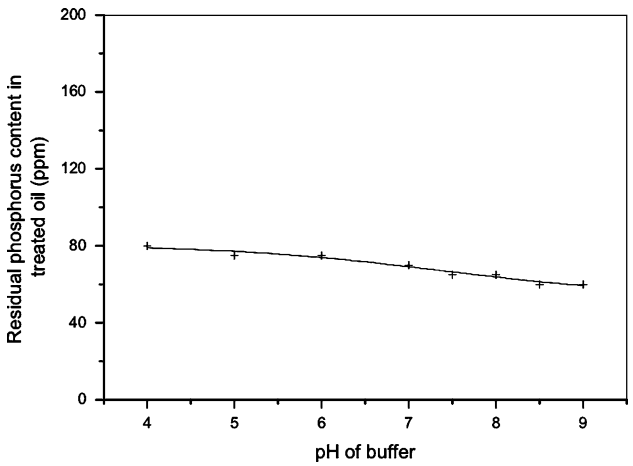

Fig. 3 Effect of pre-incubation at different pH values on the degumming efficiency of immobilized Lecitase. Gelatin gel (20 g) was pre-incubated in 0.5 M citrate buffers of different pH (pH 6.0–8.5) and degumming of rice bran oil (200 ml) was performed at 40 °C for 2 h. The residual phosphorus content of the oil was determined after dewaxing and charcoal treatment

Effect of Temperature on Phospholipase Activity in Aqueous Medium

Temperature causes changes in enzyme conformation and stability. Since stability of the enzyme is of prime importance in industry, the effect of temperature on the enzyme activity in an aqueous assay solution was studied. Figure 4 shows that the optimum temperature for the free enzyme in an aqueous medium is between 37 and 40 °C while on immobilization it increases to 50 °C. Apparently, binding of the enzyme in gelatin gel restricts enzyme movement, leading to a shift in the temperature optimum. A similar shift in optimum temperature has been observed for enzymes entrapped in gel matrices and the phenomenon has been attributed mainly to enzyme stabilization due to restricted enzyme movement. [33–35].

Thermostability of Phospholipase in Aqueous Buffer

The thermostability of the enzyme in free and immobilized states was studied in an aqueous buffer by incubating at a fixed temperature for 72 h. It was observed that the free enzyme was fairly stable up to 40 °C and retained 30% activity after 72 h. Enzyme stability decreased at higher temperatures. At 50 °C the free enzyme lost >95% activity in 72 h. Enzyme deactivation followed a typical first order kinetics ($t_{1/2} = 27$ h). In comparison, the immobilized enzyme lost 60% of its initial activity after 72 h under similar conditions (Fig. 5). The loss of activity in the case of the immobilized enzyme was mostly due to deactivation of the enzyme that had leached out into the surrounding aqueous buffer in which the immobilized enzyme was suspended. This was further confirmed from studies in a rice bran oil medium where the enzyme cannot leach out.

Thermostability of Phospholipase in Rice Bran Oil

The thermal stability of the free and immobilized enzyme in a rice bran oil medium was studied after incubating the enzyme suspension in rice bran oil. At regular intervals, the residual enzyme activity was determined by the titrimetric method. In rice bran oil, the optimum temperature for both free and immobilized enzyme was found to be 40 °C. Compared to aqueous solution, the free enzyme was far more stable in rice bran oil. For example, after 72 h incubation at 40 °C, the enzyme lost only 10% of its activity. It retained 50% of its activity after 72 h incubation at 50 °C. The immobilized enzyme was even more stable. It did not lose any activity at 40 °C and lost only 20% of its activity at 50 °C after incubation for 72 h (Fig. 6).

Recycling of the Immobilized Enzyme

For an immobilized enzyme to be cost effective, it is important to study if it can be recycled efficiently. Thus recycle studies were made with the immobilized enzyme in aqueous buffer as well as in a rice bran oil medium. Figure 7 shows that the activity of the immobilized enzyme remains unchanged within ±5% for 10 recycles in rice bran oil in contrast to aqueous solution where the enzyme activity is slowly lost due to leaching.

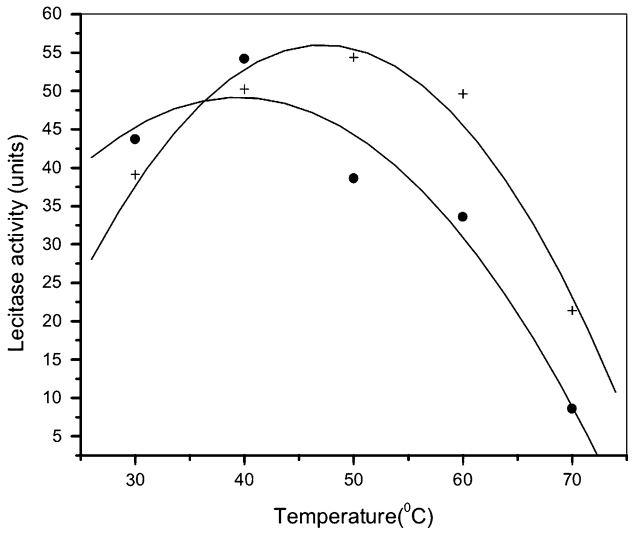

Fig. 4 Effect of temperature on Lecitase activity. (*filled circles*) free enzyme solution (100 µl); (*plus*) immobilized enzyme (gel obtained after immobilization of 100 µl of commercial Lecitase solution). Enzyme activity was measured in 25 ml of assay buffer (pH 6.5) at a constant temperature

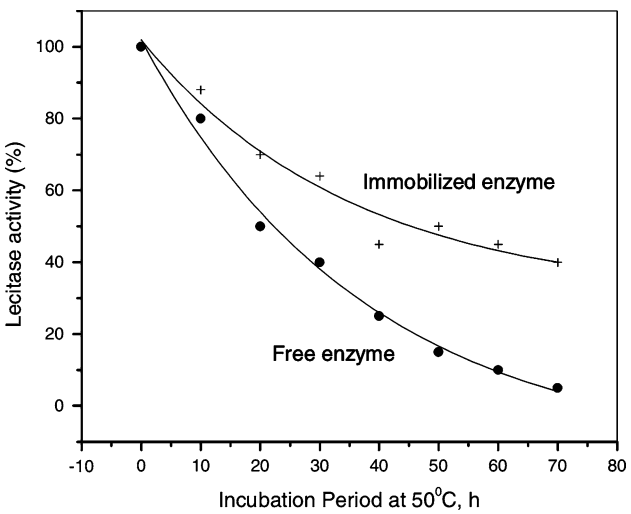

Fig. 5 Stability of Lecitase at 50 °C in 5 mM citrate buffer, pH 7.5. (*filled circles*) free enzyme (100% = 37 units); (*plus*) immobilized enzyme (100% = 55 units). Enzyme assay conditions as in Fig. 1

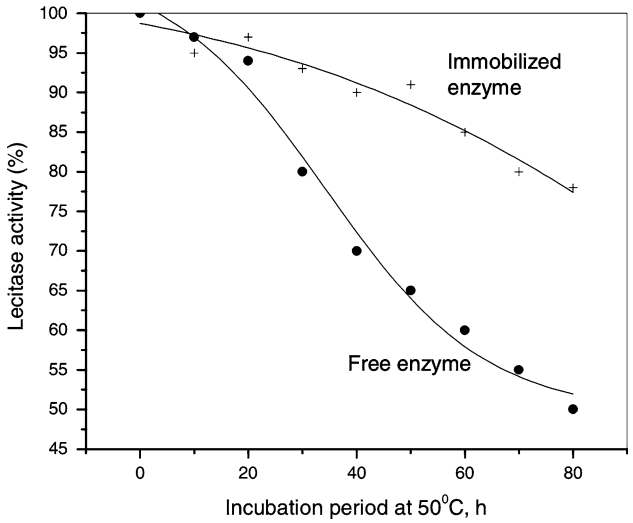

Fig. 6 Stability of Lecitase at 50 °C in rice bran oil. (*filled circles*) free enzyme, pH 7.5, 100 µl suspended in 1 ml rice bran oil (100% = 37 units); (*plus*) immobilized enzyme pre-incubated at pH 7.5 suspended in 10 ml rice bran oil (100% = 55 units). Enzyme assay conditions as in Fig. 1

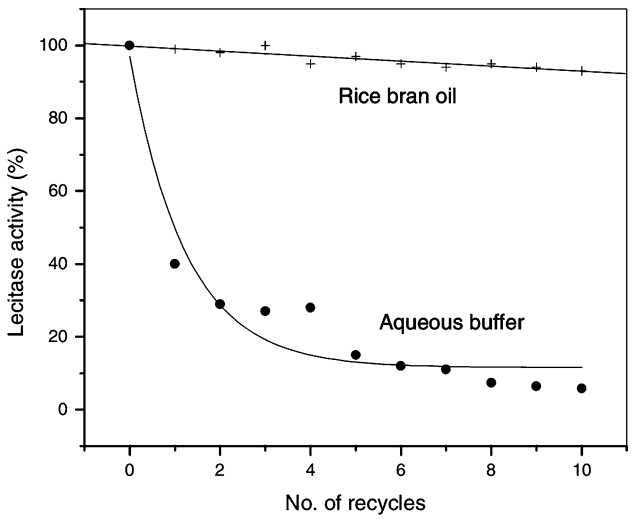

Fig. 7 Recycling of the immobilized enzyme in (*filled circles*) aqueous buffer and in (*plus*) rice bran oil at 40 °C. Recycle after 12 h. Enzyme assay conditions as in Fig. 1

Degumming of Rice Bran Oil in the Spinning Basket Bioreactor

Usually, a stirred tank reactor or a packed bed reactor is used in industry/laboratory for reactions in solid-liquid biphasic systems. However, the fragile nature of the gel prevented us from using a stirred tank reactor where the gel particles are quickly broken up into very small pieces and dispersed in the oil phase. This caused a complete loss of enzyme within a short time. The use of a packed bed reactor was impractical as the gel particles get packed tightly causing a complete stoppage of oil flow and pressure build-up.

An alternative to conventional stirred tank reactor (STR) or a packed bed reactor is to combine the mixing and catalytic function by fixing the catalyst to a stirrer. Although Carberry introduced the stirred basket reactor as a laboratory reactor in 1964 [36], the use of catalytic stirrer reactors for the chemical industry has not received much attention. Some examples from the literature include various hydrogenation reactions [37, 38] and the oligomerization of propylene [39]. The use of a spinning basket reactor type filled with rather large catalyst pellets for hydrodechlorination in the industrial cleaning of liquid waste streams was proposed by Schioppa and coworkers in 2001 [40]. In another configuration, a stirred tank reactor with stationary catalytic basket was designed by Robinson and Mahoney in 1981 [41]. These are known as Robinson–Mahoney or RM reactors and are commercially available from the Autoclave Engineers Company, USA.

Both the Carberry and RM reactors are specially designed for 3 phase systems consisting of solid, liquid and gas. Although the usefulness of such reactors in the chemical industry has been well demonstrated, their utilization as bioreactors has not been exploited. For a relatively simple solid–liquid two-phase system it was not necessary to use a complicated design. Applying the principle of a stirred basket reactor, degumming of rice bran oil has been carried out efficiently in a simplified version as shown in Fig. 8. To the best of our knowledge, ours is the first example of the application of a basket reactor in bioprocessing.

Figure 8 represents a schematic diagram of the spinning basket reactor used in our studies. In consists of a porous catalytic basket made of stainless steel. The solid catalyst particles (dia 1–2 mm) are packed loosely in the basket which is rotated in the jacketed vessel containing crude rice bran oil. Samples are collected at 30-min intervals, treated with bleaching earth and activated charcoal, dewaxed and analyzed for their phosphorus content. In a single operation under optimum conditions, the phosphorus content of the rice bran oil decreases from 400 ppm to 50–70 ppm in 2 h.

Effect of Impeller Speed on the Efficiency of Degumming

One of the major problems with an enzyme immobilized in a hydrophilic matrix is the diffusion of the substrate from the hydrophobic surroundings to the enzyme active site. Decreasing the particle size of the immobilization support and increasing the speed of impeller are some of the strategies for overcoming the diffusion problems. We have maintained the particle size of the gel at a 1–2 mm diameter. A gel of smaller particle size is difficult to handle.

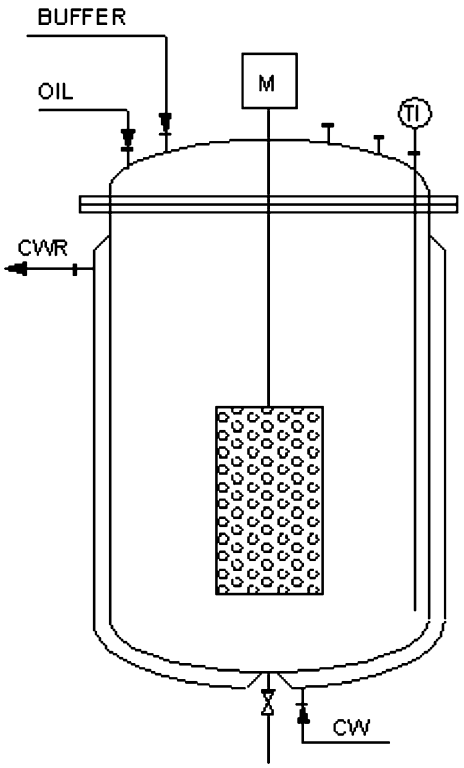

Fig. 8 A schematic representation of spinning basket bioreactor for degumming of rice bran oil

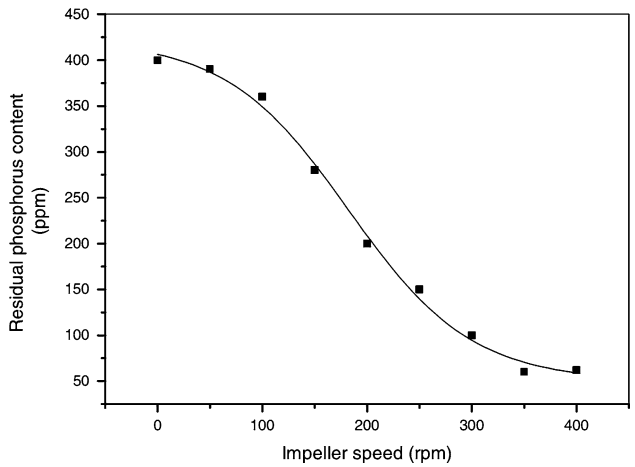

Fig. 9 Effect of impeller speed on the degumming of rice bran oil in the spinning basket bioreactor. *Y*-axis: residual phosphorus content of oil after treatment with enzyme for 2 h, dewaxing and bleaching. Reactions were carried out with 25 g gel corresponding to 2 ml of enzyme solution and 500 ml crude rice bran oil at 40 °C for 2 h

Increasing the speed of the impeller from 50 to 400 rpm shows that the reaction rate increases with impeller speed and between 350 and 400 rpm reaches a constant value (Fig. 9) as estimated from the decrease in phosphorus content of the oil after 2 h reaction at constant temperature and enzyme to oil ratio.

Enzyme Recycle

Degumming of rice bran oil is optimally carried out at 30 °C with catalyst particles of 1–2 mm diameter in the basket reactor spinning at 350–400 rpm for 2 h (see "Materials and methods" for details). The enzyme-treated oil is drained out, fresh oil is added along with sodium citrate buffer, and the enzyme treatment is continued. The immobilized enzyme was recycled 6 times without significant loss of enzyme activity as observed from the assay for enzyme activity. However, the efficiency of phosphorus removal appears to decrease (Fig. 10). This is most probably due to a fall in the local pH in the gel causing a decrease in enzyme activity. This is supported by the observation that prolonging the reaction period for the seventh cycle by several hours did not result in improved phosphorus removal. Treatment of the enzyme-treated oil after bleaching and dewaxing with a fresh batch of enzyme brings down the residual phosphorus content to <5 ppm.

Economic Considerations

According to the information disclosed in the patent application [10], 1 kg of rice bran oil is treated with approx. 300 µl of enzyme for a period of 1–2 h to achieve degumming. In our experiments, we have used immobilized enzyme equivalent to 2 ml of enzyme solution for degumming of 3 kg oil in 6 h. The immobilized enzyme is found to be stable in rice bran oil for at least 10 days. Thus, theoretically, it is possible to process at least 60 kg of oil with 1 ml enzyme. In comparison, 18 ml of enzyme solution is required to process 60 kg oil. The combined cost of gelatin and glutaraldehyde (100 ml for 1 kg gelatin) is

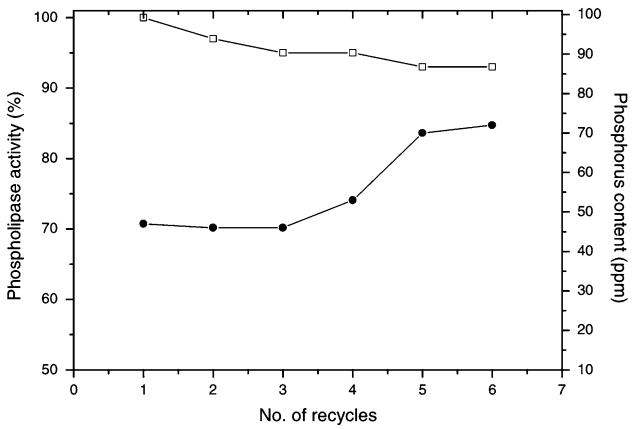

Fig. 10 Changes in phospholipase activity of the immobilized enzyme (*squares*) and the phosphorus content of rice bran oil (*filled circles*) in the spinning basket bioreactor as a function of enzyme recycle. Reactions were carried out with 25 g gel corresponding to 2 ml of enzyme solution and 500 ml crude rice bran oil at 40 °C for 2 h

approximately US $15/kg and one can load up to 100 ml enzyme in 1 kg gelatin. Thus a reusability factor of 18 effectively compensates for the cost of immobilization. With appropriate modifications of the reactor design and process control, the overall processing period can be reduced and the methodology of using gelatin-immobilized Lecitase can become economically feasible.

Acknowledgments We thank Dr. R. B. N. Prasad and Dr P. P. Chakrabarti, Lipid Science and Technology Division, IICT, Hyderabad, for helpful discussions; and CSIR, New Delhi, for financial support.

References

1. Orthoefer FT (1996) Rice bran oil: healthy lipid source. Food Tech 50:62–64
2. Xu Z, Godber JS (1999) Purification and identification of components of γ-oryzanol in rice bran oil. J Agr Food Chem 47:2724–2728
3. Kim J, Godber J, King J, Prinyawiwatkul W (2001) Inhibition of cholesterol autoxidation by the nonsaponifiable fraction in rice bran in an aqueous model system. J Am Oil Chem Soc 78:685–689
4. Shin TS, Godber JS, Martin DE, Wells JH (1997) Hydrolytic stability and changes in E vitamers and oryzanol of extruded rice bran during storage. J Food Sci 62:704–708
5. Deckere EAM, Korver O (1996) Minor constituents of rice bran oil as functional foods. Nutr Rev 54:20S–126S
6. Eitenmiller RR (1997) Vitamin E content of fats and oils: nutritional implications. Food Tech 51:78–81
7. Qureshi AA, Bradlow BA, Salser WA, Brace LD (1997) Novel tocotrienols of rice bran modulate cardiovascular disease risk parameters of hypercholesterolemic humans. Nutr Biochem 8:290–298
8. Rodrigues CEC, Antoniassi R, Meirelles AJA (2003) Equilibrium data for the system rice bran oil + fatty acids + ethanol + water at 298.2 K. J Chem Eng Data 48:367–373
9. Cvengros J (1995) Physical refining of edible oils. J Am Oil Chem Soc 72:1193–1196
10. Chakrabarti PP, Rao BVSK, Roy SK, Prabhavati Devi BLA, Narayana K, Rani P, Vandana V, Kalyani C, Gadam K, Kale V, Prasad RBN (2004) Process for the pre-treatment of vegetable oils for physical refining. United States Patent Application 20040005399
11. Clausen K (2001) Enzymatic oil-degumming by a novel microbial phospholipase. Eur J Lipid Sci Technol 103:333–340
12. Compendium of food additive specifications—Addendum 13, FAO Corporate Document Repository (2005). Available at: http://www.fao.org/docrep/008/a0044e/a0044e05.htm
13. Madoery RR, González GC, Fidelio GD (1995) Bioconversion of phospholipids by immobilized phospholipase A_2. J Biotechnol 40:145–153
14. Madoery RR, Fidelio GD (2001) A simple method to obtain a covalent immobilized phospholipase A_2. Bioorg Med Chem Lett 11:1663–1664
15. Kim J, Lee CS, Oh J, Kim BG (2001) Production of egg yolk lysolecithin with immobilized phospholipase A_2. Enzyme Microb Technol 29:587–592
16. Chen JP, Chen JY (1998) Preparation and characterization of immobilized phospholipase A_2 on chitosan beads for lowering serum cholesterol concentration. J Mol Catal B Enzym 5:483–490
17. Teke M, Onal S, Kilinc A, Telefoncu A (2003) Immobilization of phospholipase A2 on porous glass and its application for lowering serum cholesterol concentration. Artif Cells Blood Substit Immobil Biotechnol 31:467–478
18. Yaqoob M, Nabi A, Masoom-Yasinzai M (2001) Bioconversion of phosphatidylcholine to phosphatidylserine using immobilized enzyme mini-columns. Process Biochem 36:1181–1185
19. Pantazi D, Drougas E, Loppinet B, Tellis C, Kosmas AM, Lekka ME (2006) Hydrolysis by phospholipase D of phospholipids in solution state or adsorbed on a silica matrix. Chem Phys Lipids 139:20–31
20. Shen Z, Cho W (1995) Highly efficient immobilization of phospholipase A2 and its biomedical applications. J Lipid Res 36:1147–1151
21. Anthonsen T, D'Arrigo P, Pedrocchi-Fantoni G, Secundo F, Servi S, Sundby E (1999) Phospholipids hydrolysis in organic solvents catalysed by immobilised phospholipase C. J Mol Catal B Enzym 6:125–132
22. Basheer S, Kaiyal M, Boltanski A (2006) Immobilization of compounds on polymer matrix. World patent application WO/2004/035773
23. Fernandez-Lorente G, Palomo JM, Guisan JM, Fernandez-Lafuente R (2007) Effect of the immobilization protocol in the activity, stability, and enantioslectivity of Lecitase®. Ultra J Mol Catal B Enzym 47:99–104
24. D'Souza SF (2002) Trends in immobilized enzyme and cell technology. Ind J Biotechnol 1:321–338
25. Fadnavis NW, Sheelu G, Mani Kumar B, Bhalerao MU, Deshpande AA (2003) Gelatin blends with alginate: gels for lipase immobilization and purification. Biotechnol Progr 19:557–564
26. Othman SS, Basri M, Hussein MZ, Basyaruddin M, Rahman A, Rahman RNZA, Salleh AB, Jasmani H (2007) Production of highly enantioselective (−)-methyl butyrate using *Candida rugosa* lipase immobilized on epoxy-activated supports. Food Chem 106:437–443
27. Fernández-Lorente G, Palomo JM, Cabrera Z, Guisán JM, Fernández-Lafuente R (2007) Specificity enhancement towards hydrophobic substrates by immobilization of lipases by interfacial activation on hydrophobic supports. Enz Microb Technol 41:565–569
28. Yu D, Wang Z, Zhao L, Cheng Y, Cao S (2007) Resolution of 2-octanol by SBA-15 immobilized Pseudomonas sp. Lipase. J Mol Catal B Enzym 48:64–69
29. Fadnavis NW, Koteshwar K (1999) An unusual reversible sol-gel transition phenomenon in organogels and its application for enzyme immobilization in gelatin membranes. Biotechnol Prog 15:98–104
30. Haynes CA, Norde W (1995) Structures and stabilities of adsorbed polymers. J Colloid Interface Sci 169:313–328
31. Andrade JD, Hlady V (1986) Protein adsorption and materials biocompatibility: a tutorial review and suggested hypothesis. Adv Polym Sci 79:1–63
32. Luisi PL, Magid L (1993) Solubilization of enzymes and nucleic acids in hydrocarbon micellar solutions. CRC Crit Rev Biochem 20:409–474
33. Altun GD, Cetinus SK (2007) Immobilization of pepsin on chitosan beads. Food Chem 100:964–971
34. Jiang B, Zhang Y (1993) Immobilization of catalase on cross-linked polymeric hydrogels: effect of anion on the activity of immobilized enzyme. Eur Polym J 29:1251–1254
35. Abdel-Naby MA (1993) Immobilization of *Aspergillus niger* NRC 107 xylanase and β-xylosidase, and properties of the immobilized enzymes. Appl Biochem Biotechnol 38:69–81

36. Carberry JJ (1964) Designing laboratory catalytic reactors. Ind Eng Chem 56:39–46
37. Kenney CN, Sedriks W (1972) Effectiveness factors in a three-phase slurry reactor. Reduction of crotonaldehyde over a palladium catalyst. Chem Eng Sci 27:2029–2040
38. Turek F, Winter H (1990) Effectiveness factor in a three-phase spinning basket reactor: hydrogenation of butyndiol. Ind Eng Chem Res 29:1546–1549
39. Peratello S, Molinari M, Bellussi G, Perego C (1999) Olefins oligomerization: thermodynamics an kinetics over a mesoporous silica-alumina. Catal Today 52:271–277
40. Schioppa E, Murena F, Gioia F (2001) Mass transfer resistance in the catalytic hydrodechlorination of polychlorobiphenyls. Experimental results of 2-chlorobiphenyl hydrodechlorination in a slurry reactor and in a rotating basket reactor. Ind Eng Chem Res 40:2011–2016
41. Mahoney JA (1981) Laboratory reactors for mixed phase catalytic studies. NATO Adv Stud Inst Ser E 52:487–513