World Applied Sciences Journal 33 (6): 1052-1058, 2015
ISSN 1818-4952

DOI: 10.5829/idosi.wasj.2015.33.06.287

Rice Bran Lipase: Partial Purification, Immobilization in Calcium Alginate Beads, Characterization and Application as a Detergent Additive

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Abstract: Lipases from plant sources have interesting applications in different fields. Rice bran, a by-product obtained during the conversion of brown rice into white rice is a potential source of lipase. Only few studies have reported the extraction of lipase from this source for commercial use. In this study, the conditions of extraction such as defatting solvents, buffer pH and number of extraction cycles were optimized. The extracted lipase was purified 4.61-fold, immobilized in calcium alginate beads and comparative characterization of free and immobilized enzymes was performed. The entrapped lipase showed good thermo-stability and retained 77.96% of its activity upon exposure to a high temperature of 70°C when compared to 49.9% for the free enzyme. The immobilized and free enzymes were most stable in the acidic pH range of 5.0-7.0. They showed good stability towards metal ions at 1mM concentration. Polar organic solvents inhibited the enzyme stability to a lesser extent than non-polar solvents. The compatibility of the enzyme with various commercial detergents was checked. The study on oil stain removal from cotton fabric indicated that oil removal was superior in the presence of lipase and detergent, than the detergent alone.

Key words: Rice bran lipase · Calcium alginate · Commercial detergents

INTRODUCTION

Lipase (triacylglycerol acyl hydrolase; EC 3.1.1.3) belongs to the hydrolase class of enzymes and its main biological function is to catalyze the hydrolysis of triacylglycerols to free fatty acids, mono and diacylglycerols by actingat the oil-water interface. In addition to this natural function, it also catalyzesthe esterification, interesterification and transesterification reactions in non-aqueous media. The enzyme possesses certainremarkable properties such as stability in the presence of extreme pH and temperature and in the presence of organic solvents. It also displaysregio, stereo and substrate specificity [1].

Plant lipases have very interesting features for application in different fields. However, the knowledge about plant lipases is still very limited, when compared tomicrobial lipases. Lipase in oilseeds is localized in the aleurone layer of the grain, whereas the fatty materials are dispersed in the sub-aleurone layer and the endosperm. Aqueous extraction of proteins is difficult due to the presence of this fatty material. Therefore defatting is employed as the first step in the extraction process [2]. Lipases from rice bran and sunflower seedshave been studied [3]. Rice bran is the brown layer between the outer husk and inner white grain and is a by-product used in the food and feed industry. The United Nations Industrial Development Organization (UNIDO) has designated rice bran as an under-utilized raw material [4].

In the present study, lipase extracted from rice bran was immobilized in calcium alginate gel beads. The entrapped lipase was characterized and compared with free enzyme. The enzymes' stability at different pH and temperature ranges, as well as in the presence of organic solvents, metal ions and surfactants was studied. The lipase was incorporated in washing systems containing detergent and the degree of oil removal was studied.

MATERIALS AND METHODS

Chemicals and Samples: All chemicals and reagents used were of analytical grade. Hexane, heptane, diethyl ether, petroleum ether, ethanol and phenolphthalein were

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procured from HiMedia, India. Ponni variety of rice bran was collected from a local mill located in the Tirupur District of Tamil Nadu, India.

Defatting of Rice Bran, Lipase Extraction and Activity Assay: Defatting is the first step to remove oil and other lipid substances present in the bran. For this purpose, 10 g of rice bran was stirred for 30 min with 30 ml of *n*-hexane. The defatted bran from the above process was allowed to air-dry for about 1 h so that all solvent was practically removed and then used for lipase extraction [5]. Solvents such as petroleum ether, *n*- heptane and diethyl ether were also tested and their defatting efficiencies compared.

This defatted rice bran was used for the extraction of lipase.The rice bran was incubated with 50mM phosphate buffer of pH 7, at10°C for 30 min. The suspension was then centrifuged for 15 min at 5000 g and the supernatant was used as the crude lipase extract. The extraction conditions were further optimized.Phosphate buffers of pH 5.0-9.0 weretested in order to determine the optimum pH. Six successive extraction cycles were carried out and the lipase activity obtained from each cycle was calculated.

Lipase activity was estimated titrimetrically according tomodified Jenson's method [6]. One unit of enzyme activity was defined as the amount of enzyme that released 1 µM of free fatty acids per minute under the assay conditions. Castor oil, olive oil, palm oil and coconut oil were used as substrates for lipase in the assay.

Partial Purification and Immobilization of Lipase: Crude lipase obtained after extraction was subjected to 50% and 75% ammonium sulfate precipitation, followed bydialysis. The fractions were assayed for lipase activity and protein content. They were also subjected to SDS-PAGE on a 12% separating gel, to check for purity and estimate molecular weight. This partially purified lipase solution was mixed with sodium alginate 3% (w/v) and the lipase-alginate mixture was added using a syringe into 1% calcium chloride solution from a constant distance. The beads were allowed to harden in calcium chloride solution for an hour. The free and immobilized lipases were designated as FL and IL, respectively.

Characterization of Lipase

Stability Studies: The enzymes were characterized by performing the following stability tests. In each case, the percent relative activity was calculated by taking the activity of the unincubated enzyme sample (control) to be 100%.

Thermo-stability of the enzyme was determined by pre-incubating the FL and IL at different temperatures (30-70°C) in 50 mM phosphate buffer, pH 7.0, for 1 h at 30°C. The pH stability was investigated by mixing the FL and IL in equal volumes of various buffers such as sodium phosphate (pH 5.0–8.0) and glycine-NaOH (pH 9.0–11.0) and incubating themat 30°C for 1 h. To check the organic solvent stability of enzyme, FL and IL were treated with various organic solvents viz., ethanol, isopropanol, methanol, hexane, ethyl acetate and petroleum ether for 1h at 30°C. Each solvent was studied at 10 and 20% (v/v) concentration. The effects of various metal salts such as $CaCl_2$, $MgSO_4$, KCl, NaCl and $CuSO_4$ at 1 and 3 mM concentrations were studied. Finally, the FL and IL were pre-incubated with selected surfactants (1% v/v) for 1 h at 35°C prior to the lipase assay. The surfactants Tween 80, Triton X-100, Sodium taurochlo rate and SDS were used.

Reusability: In order to test the reusability of lipase entrapped in calcium alginate beads, the beads were repeatedly used for the hydrolysis reaction. After each trial, the beads were separated and the reaction medium was freshlyreplaced.

Application of the Lipase in Detergent Formulations: The compatibility of the enzyme with commercial detergents was detected by pre-incubating itwith different commercial detergents (0.5% (w/v) for 1 h. The enzyme without detergent was taken as control and relative activity (%) was calculated.

The effect of lipase oncotton fabric spotted with olive oil was determined. The cotton fabric was washed and cut into the size of 2 cm x 2 cm. 0.5 mL of olive oil was spotted on the cotton fabric and then allowed to dry. Theinitial weight of the oil-spotted fabric was determined. Different washing solutions werepreparedusing commercial detergents and lipase. The oil stained fabrics wereintroducedinto conical flasks containing the washing solutions (comprising of 0.5% (w/v) commercial detergent and 1% (v/v) lipase) and incubated for 30 min at 35°C with slight agitation. Then the fabrics were rinsed with distilled water and air dried. The final weight of the fabric was determined and percent oil removal was calculated. Lipase assay was performed withthe spentwashing solutionand the amount of free fatty acids released was determined [7].

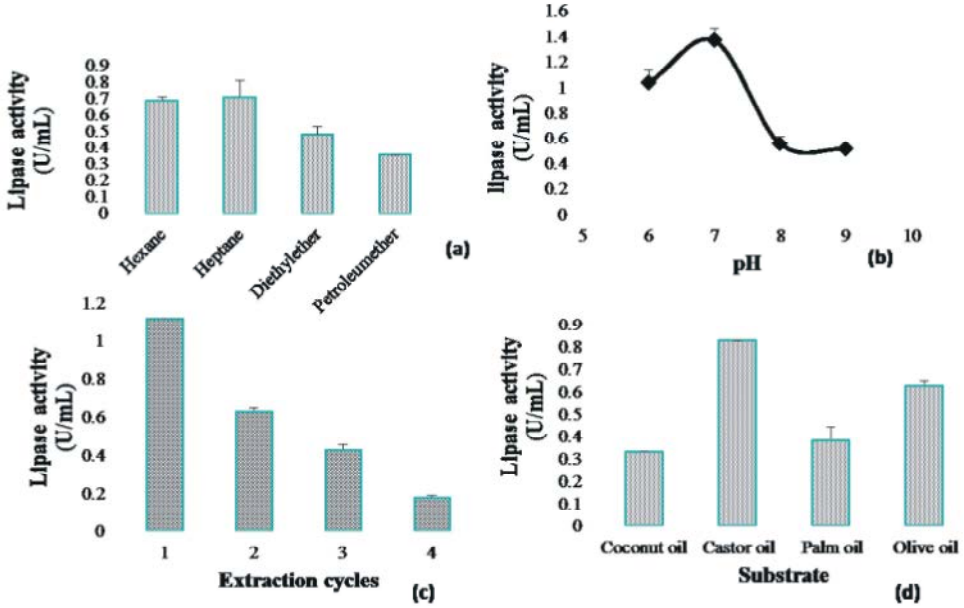

Fig. 1: Effects of various parameters on the obtained lipase activity: (a) organic solvents used for defatting of rice bran, (b) pH of the extraction buffer, (c) number of extraction cycles, (d) substrates used in the assay

RESULTS AND DISCUSSION

Defatting of Rice Bran: Direct aqueous extraction of proteins from rice bran is difficult due to the presence of the fatty material. For this reason, defatting constituted the first step in the extraction process. Non-polar solvents were generally preferred for the defatting process. Among the solvents tested, *n*-heptane appeared to give better results, followed by *n*- hexane (Figure 1a). Lipase assay was carried out in the remaining solvent after the defatting process. This step was incorporated in order to determine enzyme loss during the defatting process. Lipase activity in the solvent extract was found to be negligible (data not shown). It has been previously reported that good defatting efficiency occurs with *n*-hexane [5].

Optimum pH of the Extracting Buffer: Maximum lipase activity of1.16 U/mL was observed with extraction buffer of pH 7.0 (Figure 1b). Similar results have been reported by Ravikumar *et al*. [8] who obtained maximum lipase activity of 1.97 U/mL with pH 7.0 buffer.

Number of Extraction Cycles: Extraction of lipase was carried out for four successive cycles. Maximum lipase activity of 1.12 U/mL was obtained in the first cycle and the activity decreased during successive cycles. In the fourth cycle, negligible activity was obtained. This result clearly shows that maximum lipase present in the bran was extracted in the first cycle itself (Figure 1c).

Lipase Assay Using Different Substrates: Among the vegetable oils tested, the activity was found to be high for castor oil (0.86 U/mL) followed by olive oil (0.60 U/mL) (Figure 1d). This result differs from previously reported literature. It has been reported that olive oil was hydrolyzed better than other oils [9]. The hydrolysis depends on the fitting of chemical structure of the substrate with the enzyme. In castor oil, ricinoleic acid is the main component, which is absent in other oils.

Partial Purification and Immobilization of Lipase: Crude enzyme with specific activity of 2.22 U/mg was precipitated by giving 75% ammonium sulfate cut, resulting in specific activity of 10.24 U/mg. Fold purification and percent yield obtained were 4.61 and 46.01%, respectively (Table 1). Weerasooriya *et al*. [10] have reported that rubber seed lipase on ammonium sulfate precipitation exhibited 0.91 U/mg of specific activity with purification fold of 2.84. In another study, Ismail and Aytan [11] have reported 6.92-fold purification of hazelnut seed lipase. The electrophoretic mobilities of crude and partially purified lipase samples are represented in Figure 2. The lipase-entrapped calcium alginate beads are depicted in Figure 3.

Characterization of Free and Immobilized Lipases

Effect of Temperature on Lipase Stability: TheFL was stable in a temperature range of 30-40°C, while IL was stable in a broader range of 30-50°C. At an elevated

Table 1: Purification chart for rice bran lipase

Sample	Total volume (mL)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude supernatant	100	12.35	27.38	2.22	1.00	100
75% $(NH_4)_2SO_4$	5	1.23	12.6	10.24	4.61	46.01

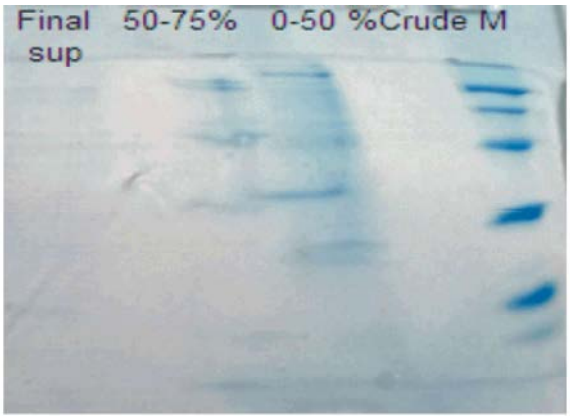

Fig. 2: SDS PAGE of crude and partially purified lipase preparations

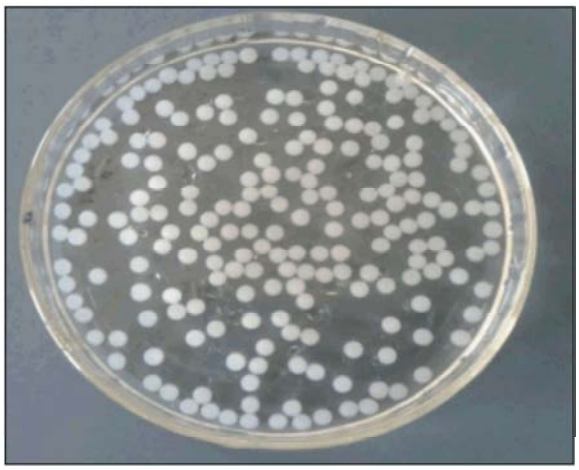

Fig. 3: Entrapment of lipase in calcium alginate beads

temperature of 70°C, the relative activity of the IL was 77.96% compared to 49.9% for the FL (Figure 4a). Thermo-stability of lipase entrapped in alginate matrices is thus far better than that of the free enzyme. Shafei and Allam [12] have also observed that the immobilized lipase remained active up to 50°C while the free enzyme activity decreased from 40°Conwards.

Effect of pH on Lipase Stability: The FL and IL were stable in the pH range of 5.0-7.0.Then gradual fall in the activity was noticed in both cases. The immobilized lipase beads did not show any activity at pH 10.0 while free enzyme lost its activity completely at pH11.0 (Figure 4b). Shafei and Allam [12] have reported that the IL was stable in the pH range of 6.5-8.0, while the FL was stable in the range of 6.5-7.5. The surface of the beads in which the enzyme is localized has a cationic or anionic nature. This produces a charged microenvironment that affects the nature of the enzyme and altersits pH stability [13].

Effect of Organic Solvents on Lipase Stability: The results obtained from this study indicate that the water-miscible organic solvents such as propanol and ethanol affect lipase activity to a lesser extent. Relative activity of FL and IL in the presence of 10% (v/v) propanol was 69.71% and 57.57%, respectively (Table 2). Non polar solvents such as petroleum ether and *n*-hexane inhibited the activity even at 10% concentration. In general, the FL had better organic

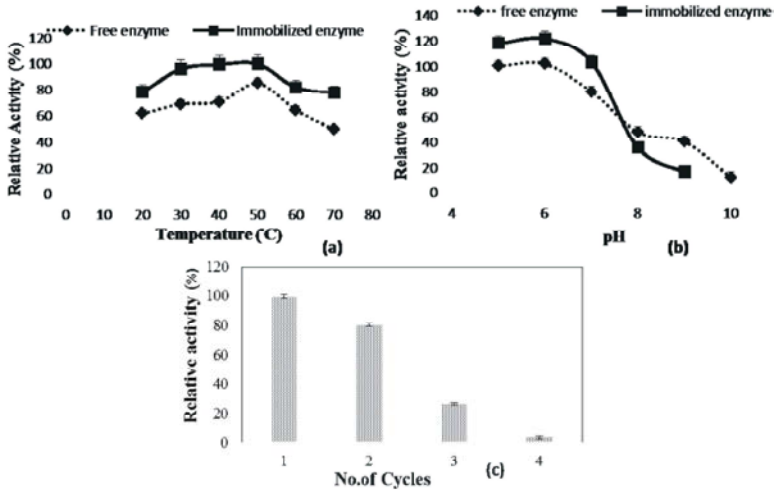

Fig. 4: pH (a) and temperature (b) stabilities of free and immobilized lipase, reusability of immobilized lipase (c)

Table 2: Organic solvent stability of free and immobilized lipase

	10% (v/v) concentration		20% (v/v) concentration	
Organic solvents	FL	IL	FL	IL
Propanol	69.71±2.14	58.66±7.58	61.56±8.40	57.57±2.61
Ethanol	78.88±4.19	62.81±2.58	73.10±9.20	53.84±0.98
Methanol	27.08±3.18	32.32±7.24	42.32±2.72	19.55±1.40
n-Hexane	45.02±2.14	27.38±0.60	42.32±2.45	26.81±3.11
Ethyl acetate	69.52±2.25	74.10±4.85	61.22±2.90	42.17±2.61
Petroleum ether	24.17±1.21	30.78±1.21	57.57±1.55	13.80±0.30

Table 3: Stability of free and immobilized lipase towards metal ions

	1mM concentration		3mM concentration	
Metal ions	FL	IL	FL	IL
$CaCl_2$	97.63±1.12	78.18±0.45	64.51±0.58	26.56±0.61
$MgSO_4$	82.72±2.13	69.82±0.07	68.26±2.60	46.43±0.30
KCl	88.83±2.30	89.18±2.43	49.50±2.65	39.90±6.80
NaCl	92.24±1.54	61.20±5.99	68.08±2.78	46.50±1.91
$CuSO_4$	85.34±0.33	57.61±2.13	62.03±1.49	28.66±2.03

Table 4: Effect of surfactants on lipase stability

	Relative activity (%)	
Surfactants (1% (v/v)	FL	IL
Triton X-100	99.20±1.41	79.81±0.52
Tween 80	168.3±9.62	124.5±3.35
SDS	66.71±0.12	93.52±9.17
Sodium taurochlorate	73.37±9.42	107.9±9.80

solvent stability than the IL. These results are in contrast with the statement that water-miscible solvents tend to strip off water from the enzyme, leading to the unfolding of the molecule and loss of activity [14]. However, Kambourova *et al*. [15] have reported that lipase activity was enhanced by sorbitol and methanol but completely inhibited by butanol, chloroform and diethyl ether.

Effects of Metal Ions on Lipasestability: Metal ions at a lower concentration did not affect the activity of free lipase significantly, but inhibited the activity of immobilized enzyme. In the presence of 1mM $CaCl_2$, relative activities of FL and IL were 97.63% and 78.18%, respectively, but at 3mM they were reduced to 64.51% and 26.56%, respectively (Table 3). 3mM concentration of metal ions severely inhibited the activity of immobilized enzyme as well as the free enzyme Madhikar *et al*. [16] have reported that Ca^{2+} and Mg^{2+}at lower concentration enhanced the activity, whereas at higher concentration they inhibited the activity of sunflower seed lipase. Hence, the metal ions appear to have stimulant effect at lower concentration, but at higher concentrations the enzyme activity decreases because they cover the lipase surface and prevent substrate binding.

Effect of Surfactants on Enzyme Stability: From Table 4, it is clear thatthe non-ionic surfactanttween 80 enhanced the activity of FL and IL. The activity of FL was unaltered in the presence of triton X-100, while the IL was somewhat inhibited. The anionic surfactant SDS inhibited the activity of FL to a greater extent. Sodium taurocholate had mixed effects on the FL and IL. Cherif *et al*. [17] found that lipase was highly stable in the presence of non-ionic surfactants triton X-100 and tween. Khoo and Ibrahim [18] stated that the inhibitory effect of SDS was due to the possibility that negatively charged detergents obstructed lipase from the interface, combined with the enzyme, or coated it so that the site for substrate adhesion was being inaccessible.

Reusability of Immobilized Lipase: The reusability of the entrapped enzyme was checked. The immobilized enzyme showed 80.72% of its original activity during the second cycle, after which there was sharp decline in the activity to 28% and complete loss of activity was observed during the fourth cycle (Figure 4c). This might be due to the leakage of enzyme from the beads, which occurred due to the washing of beads at the end of each cycle.

Table 5: Compatibility of lipase with commercial detergents, wash performance analysis of lipase with detergents

Commercial detergent(0.5% w/v)	Relative activity (%)	Washing solution	Free fatty acid released (µ moles/mL)
d_1	80.74±10.88	d_1	0.56±0.15
		d1 + L	1.53±0.21
d_2	94.71±3.40	d_2	0.75±0.11
		d_2 + L	2.54±0.15
d_3	84.51±10.9	d_3	0.85±0.09
		d_3 + L	2.90±0.09
d_4	65.37±5.43	d_4	0.45±0.12
		d_4 + L	1.92±0.38

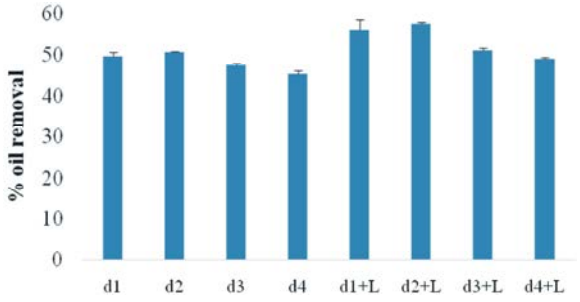

Fig. 5: Effects of different washing solutions on oil removal

Application in Detergent Formulations: The compatibility of the enzyme with commercial detergents (d_1 - Power, d_2 - Tide, d_3 – Ariel,d_4 - Surf) was checked.The enzyme exhibited high stability towards d_2, retaining 94.7% of its activity after 1 h of contact (Table 5). Weerasooriya *et al*. [10] found highrelative activities of 80 and 78% in the presence of Surf and Rin, respectively. Based on thiscompatibility, different washing solutions containing lipase were prepared.

The pre weighed, oil stained cotton fabric was incubated with different washing solutions for 30 min. Then the fabric was removed, rinsed and dried. The final weight of the cloth was measured and percent oil removal was calculated. Oil removal was always better when lipase was used along with the detergents. Percentage oil removal was calculated for different washing solutions. On comparing d_1+L and d_1, the percent oil removals were 56.02 and 49.08%, respectively. Figure 5 shows that the degree of oil removal was slightly higher in detergent with lipase combination than detergent alone.

The spent washing solution was also titrated against NaOHand the micromoles of free fatty acids released were calculated (Table 5). When lipase was used along with the detergent, substantial amounts of free fatty acid were released, which show hydrolysis of the oil present in the fabric. Visual observation of fabrics also yielded agreeable results.

CONCLUSION

In the present study, lipase was obtained from the cheap alternative source of rice bran. Various conditions of extraction have been optimized such as de-fatting solvents, buffer pH and extraction cycles. The enzyme has been partially purified by ammonium sulfate precipitation and 4.61-fold purification has been achieved. The enzyme hasbeen immobilized in calcium alginate beads and comparative characterization of free and immobilized enzymes has been performed. The entrapped lipase has shown good thermo-stability and pH stability. The enzyme has displayedmoderate stability towards isopropanol and ethanol. Both the FL and IL haveexhibited good stability towards the metal ions at 1mM. The IL has retained 80.72% of its activity and could be reused up to 2 cycles. The enzyme has shown compatibility with commercial detergents and possessed ability to remove oil stains. Thus, therice bran lipase could be used as a detergent additive to improve its washing performance.

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