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Immobilization of acidic lipase derived from *Pseudomonas gessardii* onto mesoporous activated carbon for the hydrolysis of olive oil

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ABSTRACT

Mesoporous activated carbon (MAC) derived from rice husk is used for the immobilization of acidic lipase (ALIP) produced from *Pseudomonas gessardii*. The purified acidic lipase had the specific activity and molecular weight of 1473 U/mg and 94 kDa respectively. To determine the optimum conditions for the immobilization of lipase onto MAC, the experiments were carried out by varying the time (10–180 min), pH (2–8), temperature (10–50 °C) and the initial lipase activity (49×10^3, 98×10^3, 147×10^3 and 196×10^3 U/l in acetate buffer). The optimum conditions for immobilization of acidic lipase were found to be: time—120 min; pH 3.5; temperature—30 °C, which resulted in achieving a maximum immobilization of 1834 U/g. The thermal stability of the immobilized lipase was comparatively higher than that in its free form. The free and immobilized enzyme kinetic parameters (K_m and V_{max}) were found using Michaelis–Menten enzyme kinetics. The K_m values for free enzyme and immobilized one were 0.655 and 0.243 mM respectively. The immobilization of acidic lipase onto MAC was confirmed using Fourier Transform-Infrared Spectroscopy, X-ray diffraction analysis and scanning electron microscopy.

1. Introduction

Enzymes are biocatalysts with high specificity, high catalytic efficiency and bio-degradability and hence find major applications in industrial sectors and in medical sciences as they increase the rate of chemical reaction by lowering the activation energy [1]. Lipase (triacylglycerol hydrolase; EC 3.1.1.3) is one of the most extensively used enzymes that catalyses the hydrolysis of triacylglycerol to glycerol and fatty acids in oil–water interface systems [2,3]. Depending on the nature of substrate and reaction conditions, lipases can catalyze a wide range of enantio- and regioselective reactions such as hydrolysis, esterifications, transesterifications, aminolysis and ammoniolysis [4–6]. Lipase is an important enzyme with a broad variety of applications in the food industry, fine chemicals, and pharmaceutical industry [5,7]. Also it constitutes the most important group of biocatalysts for biotechnological applications. Novel biotechnological applications have been successfully established, using lipases for the synthesis of biopolymers, agrochemicals, flavour compounds and biodiesel [8]. A variety of environmental conditions inactivates the lipases engaged during a particular reaction and further brings difficulties while separating from the reaction system for its reuse and hence industrial applications of lipase are limited. In most instances enzyme immobilization on solid supports may be an effective tool to improve their pH, thermal and operational stabilities, recoverability, and reduce the cost and thereby finding a way for wide industrial applications [9–11]. Therefore, numerous efforts have been shed on the preparation of lipases in immobilized forms, which involve a variety of support materials as well as immobilization methods [12,13].

Selection of the right support for biocatalysis of enzyme is vital, as enzyme efficiency depends largely on the support and its linkage to it. The selected support should have a well-developed internal structure and a large surface area, provided by high porosity. The support should have high affinity (or capacity) for enzymes and a suitable chemical structure (% hydrophobicity) to provide maximum enzyme activity and enzyme–substrate contact. The support also should be thermally stable, chemically durable, resistant to contamination, and available at a reasonable cost [14].

The physical methods, especially adsorption, have an advantage over the chemical methods for the immobilization of enzymes onto carriers in that it is simple, less expensive and can retain high catalytic activity [15]. The adsorption of lipase onto porous support may be one of the most widely employed ways used in the continuously operated packed beds and the stirred tank reactors, especially in large-scale operations [16]. The mesopores may provide ample space for lipase molecules so that there will be little resistance for

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their immobilization. Based on these factors, mesoporous activated carbon obtained from rice husk was found to be a suitable carrier matrix for enzyme.

Many supports have been studied including polymers and resins [17–19], silica and silica–alumina composites [20–23], clay and bentonite materials [11,24,25]. However, many researchers were reported that porous materials had favouring features for the immobilization of enzymes compared to non-porous materials, owing to their pore size, large surface area, pore-volume and open structure [26–28]. Therefore, in the present investigation, the feasibility of immobilization of acidic lipase onto mesoporous activated carbon matrix has been explored.

The most important application of lipase in oleochemical industry is the production of fatty acids from oils through hydrolysis [29]. Partial hydrolysis of triglycerides yields mono-, di-glycerides and fatty acids. The mono-, di- and triglycerides yield fatty acids and glycerols on the completion of hydrolysis. Thereupon, enzyme-catalyzed hydrolysis has been considered as an energy saving method, especially for producing high value-added products and fatty acids [30,31]. Hence the present study is focused towards the immobilization of acidic lipase onto mesoporous activated carbon and further to be employed for olive oil hydrolysis.

2. Materials and methods

2.1. Mesoporous activated carbon (MAC) preparation

Mesoporous activated carbon (MAC) was prepared from rice husk by two-stage process: precarbonization and chemical activation. The precarbonization of rice husk was carried out at 400 °C in an air tight crucible, followed by chemical activation using phosphoric acid at 800 °C [32] in order to render it as mesoporous in nature. The preparation studies suggested that the MAC heat treated at 800 °C possessed the highest surface area of 412 m^2/g.

2.2. Characterization of the mesoporous activated carbon matrix

The carbon, hydrogen and nitrogen contents of the MAC were determined using CHNS 1108 model Carlo–Erba analyzer. The pH of the point of zero charge, pH_{PZC}, i.e. the pH above which the total surface of carbon particles is negatively charged and it was measured by the pH drift method [33]. Matter soluble in water (%), matter soluble in acid (%), phenol number (mg/g) and ion exchange capacity (m^2/g) was carried out according to the method of BIST (Bureau of Indian Standards) [34]. The surface area and pore size distribution were derived from the N_2 adsorption–desorption isotherms. The N_2 adsorption–desorption isotherms of MAC were measured using Quantachrome Corp. Nova-1000 gas sorption analyzer. Prior to measurement, MAC was degassed at 150 °C overnight. The nitrogen adsorption–desorption data was recorded at liquid nitrogen temperature 77 K. The surface area was calculated using BET equation, which is the most widely used model for determining the specific surface area (m^2/g).

2.3. Isolation of Pseudomonas gessardii

P. gessardii was isolated from the animal tallow acclimatized black soil. The acclimatization of the animal tallow was carried out to isolate an efficient strain from the soil for the production of high yield lipase using animal tallow as the substrate. Accordingly, 10 g of beef tallow was buried in black soil for a period of 3 weeks. The soil acclimatized with tallow was added to nutrient broth (NB) of composition: peptone (5.0 g/l); yeast extract (1.5 g/l); beef extract (1.5 g/l); NaCl (0.5 g/l) and was incubated at 37 °C for 48 h. Then the cultured broth was plated on the tributyrin agar and incubated at 37 °C for 24 h. The colony showing clear zone in the tributyrin agar plate and higher lipase activity in the presence of tallow was selected and identified as *P. gessardii* by 16S rDNA sequencing and phylogenetical analysis. It was deposited in the Gene bank and assigned the accession number, FJ943496.

2.4. Production of acidic lipase

The acidic lipase was produced from *P. gessardii* in a basal medium containing KH_2PO_4 (1.0 g/l); ammonium sulphate (0.5 g/l); $CaCl_2$ (1.0 g/l); 0.3% (v/v) goat tallow and 1% (v/v) gum arabic. One gram of solid goat tallow was melted and 0.3 ml from this melt was emulsified in distilled water containing 1% gum arabic using ultrasonic sonicator. Culture conditions maintained were: 300 ml of medium in 1 l Erlenmeyar flasks; rotation at 100 rpm for 48 h at pH 3.5 and temperature 37 °C [35]. The maximum lipase activity produced in the optimized culture conditions was 166 U/ml. *P. gessardii* acidic lipase produced in the present study is an extracellular enzyme as it has been separated from the medium without sonication.

2.5. Lipase assay

Lipase activity was measured by titrimetric assay according to an olive oil emulsion [36] with slight modifications. Olive oil 10% (v/v) was emulsified in distilled water containing 2% (w/v) of polyvinyl alcohol in a Bandelin ultrasonic sonicator. Lipase assay was formed with 5 ml of olive oil emulsion, 2 ml of 0.030% Triton X-100, 2 ml of 3 M NaCl, 1 ml of 0.075% $CaCl_2$, and 5 ml of acetate buffer (pH 3.5). The enzyme–substrate mixture was incubated at 30 °C for 5 min. The reaction was terminated by adding 10 ml acetone:ethanol (1:1) to the mixture. The liberated fatty acids were titrated against 0.02 M NaOH using phenolphthalein as an indicator. Unit of lipase activity was defined as the amount of enzyme that released 1 μmole of fatty acid per minute under assay conditions.

2.6. Purification of acidic lipase

Biomass in the broth was removed by centrifugation and the supernatant was subjected to ammonium sulphate precipitation at 4 °C. Precipitation was allowed for overnight, followed by centrifugation at 6500 × *g* in a refrigerated centrifuge for 30 min. The precipitate was dissolved in a minimal amount of 0.1 M acetate buffer (pH 3.5) and dialyzed for 24 h with three changes in the same buffer. This crude enzyme was applied to ion exchange (DEAE-cellulose) chromatography and gel filtration (Sephadex G-100) column chromatography as follows.

2.6.1. Step 1: DEAE-cellulose column chromatography

The crude enzyme was loaded onto a DEAE-cellulose column (1.0 cm × 15 cm) previously equilibrated with the 0.1 M acetate buffer (pH 3.5). After washing with two bed volumes of the initial buffer, the elution was performed with a negative linear gradient of 0–1.0 M NaCl at a flow rate of 30 ml/h. Fractions showing lipase activity were pooled, and subjected to Sephadex G-100 column chromatography for further purification.

2.6.2. Step 2: Sephadex G-100 column chromatography

The pooled enzyme from DEAE-cellulose column was loaded onto the Sephadex G-100 column (1.0 cm × 15 cm) previously equilibrated with the 0.1 M acetate buffer (pH 3.5). After washing the column with two bed volumes of the initial buffer, the elution was performed with a negative linear gradient of 0–1.0 M NaCl at a flow rate of 30 ml/h. Fractions showing lipase activity were collected and lyophilized for further use.

2.7. Protein determination

Protein concentration was determined by Lowry method [37] employing bovine serum albumin as standard.

2.8. Determination of molecular weight

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using the method of Laemmli [38], on a 5% polyacrylamide stacking gel and a 12% polyacrylamide-resolving gel. The protein marker ranging from 14.3 to 94.7 kDa was used as a standard marker for the determination of molecular weight. Protein bands were visualized by silver staining.

2.9. Acidic lipase immobilization onto MAC

The optimum conditions for the immobilization of acidic lipase onto MAC were carried out by varying the parameters such as time (10–180 min), pH (2–8), temperature (10–50 °C) and initial concentration of lipase (49×10^3, 98×10^3, 147×10^3 and 196×10^3 U/l in acetate buffer). The mass of MAC used for the immobilization of acidic lipase is 1 g/15 ml of buffered lipase solution. Lipase immobilization was investigated with hydrolysis activity as objective function. The residual lipase activity was determined using lipase assay. The immobilized lipase activity was calculated by subtracting the final activity of lipase solution from the initial activity.

2.10. Thermal and storage stability

The thermal stability of free lipase (ALIP) and immobilized lipase (MAC–ALIP) was investigated by incubating them in an acetate buffer (pH 3.5, 0.1 M) and exposing to various temperatures (10–50 °C) for 24 h. The lipase activities of heated biocatalysts were measured by taking an unheated control to be 100% active. The storage stability of the ALIP and MAC–ALIP was determined by storing it at 4 °C for 50 days. The remaining percentage of free and immobilized lipase activity was calculated in each determination using the method followed by Naci and Ali [36]. In these experiments, the immobilized lipase activity and residual immobilized lipase activity were determined by adding olive oil emulsion to 1.0 g of MAC–ALIP.

2.11. Operational stability of MAC–ALIP

To study the operational stability of immobilized lipase, it was repeatedly used in the olive oil hydrolysis to determine the lipase activity. When one activity measurement was over, the MAC–ALIP was thoroughly rinsed with acetate buffer (pH 3.5, 0.1 M). Then, it was added into a fresh reaction mixture to catalyze the hydrolysis reaction as before.

2.12. Determination of kinetic parameters

Lipase activity measurement was made for different substrate concentrations at constant temperature and pH to determine kinetic parameters, maximum reaction rate (V_{max}) and Michaelis–Menten constant (K_m) for free and immobilized lipase. Olive oil was used as the substrate and the resulting product (liberated fatty acid) concentration was determined according to the method followed by Naci and Ali [36]. It is assumed that, polyvinyl alcohol (PVA) mixed with the substrate (olive oil) in the lipase assay produces emulsion of olive oil and acts only as a stabiliser of olive oil [39], but it does not take part in the hydrolysis reaction. The kinetic parameters were estimated from the Michaelis–Menten equation in Eq. (1).

$$V = \frac{[S]V_{max}}{[S] + K_m} \quad (1)$$

where [S] is the substrate concentration (mM) and V is the initial reaction rate of the enzyme (mM/min).

Table 1
Characteristics of MAC.

S. No.	Parameters	Values
1	Carbon (%)	45.4
2	Hydrogen (%)	0.9
3	Nitrogen (%)	0.1
4	Moisture content (%)	5.2
5	Ash content (%)	36.4
6	Bulk density (g/ml)	0.52
7	Apparent density (g/ml)	0.106
8	Average pore diameter (Å)	11.43
9	Matter soluble in water (%)	4.206
10	Matter soluble in acid (%)	4.35
11	Point of zero charge (PZC)	6.4
12	Decolorizing capacity (mg/g)	44
13	Phenol number (mg/g)	4.31
14	Ion exchange capacity (mg/g)	0.062
15	Surface area (m^2/g)	412

2.13. FT-IR studies

A Perkin-Elmer infrared spectrophotometer was used for the investigation of the surface functional groups. The samples with KBr (spectroscopic grade) pellets were prepared in the size of about 10–13 mm in diameter and 1 mm in thickness. The samples were scanned in the spectral range of 4000–400 cm^{-1}.

2.14. XRD analysis

The XRD pattern of MAC, ALIP, and MAC–ALIP was determined using XRD analysis with high resolution GUINER powder X-ray diffractometer (SEIFERT, Germany).

2.15. Scanning electron microscopy

The surface morphology of mesoporous carrier and lipase immobilized mesoporous carrier was determined using Leo-Jeol Scanning Electron Microscope at the magnification of 2500–10,000×. The carbon samples were coated with gold by a gold sputtering device for the clear visibility of the surface morphology.

3. Results and discussion

3.1. Characterization of MAC

The characterization of the MAC such as surface area, pore size, elemental composition was given in Table 1.

3.2. Purification and molecular weight determination of acidic lipase

The acidic lipase produced from *P. gessardii* by aerobic fermentation was extracted and purified using ammonium sulphate precipitation, ion exchange (DEAE-cellulose) and gel filtration (Sephadex G-100) column chromatography. The specific activity and purification fold of the purified lipase were found to be 1473 U/mg and 8.1 respectively (Table 2). As shown in Fig. 1, the purified lipase showed single protein band in the SDS-PAGE (Fig. 1, lane 2) indicating the protein content with high level purity. The molecular mass of the protein was found to be around 94 kDa based on SDS-PAGE.

Table 2
Summary of the purification procedure of ALIP derived from *Pseudomonas gessardii*.

Purification steps	Total activity (U)	Protein content (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude enzyme	166,000	912.6	181.9	100	1.0
Ammonium sulphate precipitate	45,800	223.6	204.8	27.6	1.13
Dialysis	34,400	116.4	295.53	20.72	1.625
DEAE-cellulose	29,220	35.6	568	17.6	3.12
Sephadex G-100	22,680	15.4	1473	13.7	8.1

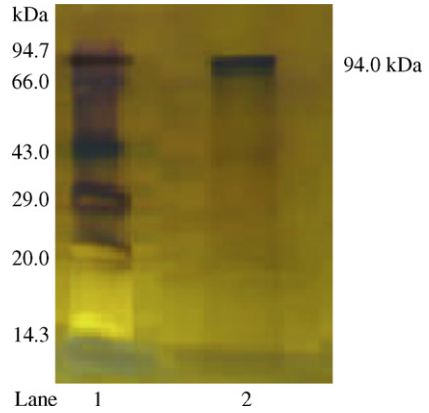

Fig. 1. Molecular mass determination by SDS-PAGE—lane 1: molecular mass standards (14.3–94.7 kDa), lane 2: lipase (10 μg), purified by ammonium sulphate precipitation in combination with ion exchange chromatography and gel filtration chromatography.

3.3. Immobilization of acidic lipase onto MAC

3.3.1. Effect of immobilizing time

The effect of time for the immobilization of acidic lipase was carried out in order to determine the equilibrium points. The activity of lipase was measured at different time intervals (10–180 min) during immobilization (Fig. 2). The immobilization was performed for different time periods (10–180 min) at pH 3.5 and 30 °C. It was found that in all experiments, the immobilization was rapid up to 20 min and then there was a slight increase in immobilization until the equilibrium (120 min). Initially the number of adsorption sites available is higher and the driving force for the mass transfer is greater. As the immobilization time was increased, the number of bare active sites became less and the lipase molecules may become clustered inside the carbon particles, thus impairing the diffusion of lipase.

3.3.2. Effect of pH on the immobilization of lipase

In order to determine the optimum pH for the immobilization of the lipase onto MAC, the experiments were carried out at varying pH values between pH 2 and 8 at an initial lipase activity 49×10^3 U/l and temperature 30 °C. A bell shaped curve was obtained for free lipase, in the studied pH range with a maximum lipase activity at pH 3.5 (figure not shown). When the immobilization conditions were also studied in the same pH range, a bell shaped curve was also obtained with a maxima reaching at pH 3.5 as shown in Fig. 3. At this pH the maximum lipase loading was achieved to be 684 U/g. However at its closest pH of 3 and 4, the lipase loading was 589 and 522 U/g respectively. Hence the optimum pH for immobilization was fixed to be 3.5.

3.3.3. Effect of temperature on the immobilization of lipase

The optimum temperature for the immobilization of the lipase onto MAC was carried out by varying temperature from 10 to 50 °C at an initial lipase activity 49×10^3 U/l and pH 3.5. A bell shaped curve was obtained for free lipase, in the studied temperature range with a maximum lipase activity at temperature 30 °C (figure not shown). The immobilization conditions also were studied in the same temperature range and initial lipase activity, a bell shaped curve was also obtained with a maxima reaching at 30 °C as shown in Fig. 4. At this temperature the maximum lipase loading was achieved to be 688 U/g. However at its closest temperature of 20 and 40 °C, the lipase loading was 512 and 607 U/g respectively. Hence the optimum temperature for immobilization was fixed to be 30 °C.

3.3.4. Effect of initial enzyme activity

The enzyme load of MAC increased with increasing lipase activity. The enzyme load at initial lipase activities 49×10^3, 98×10^3, 147×10^3 and 196×10^3 U/l was found to be 684, 1150, 1497, 1834 U/g at an operating conditions of pH 3.5 and temperature 30 °C. The enzyme load onto MAC increased with increase in initial lipase activity but however, the percentage immobilization decreased with increase in lipase activity. Hence the results sug-

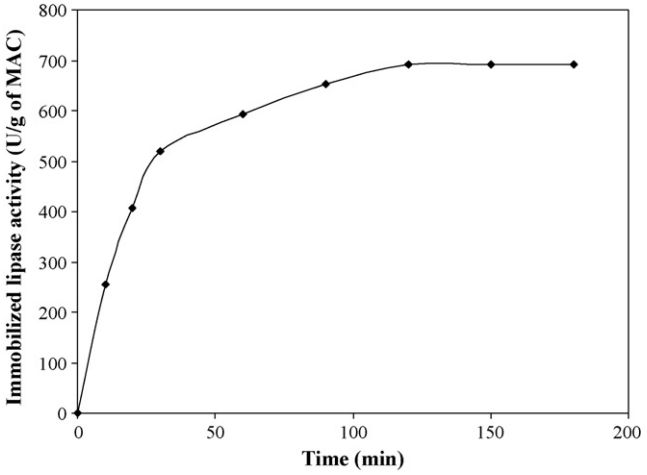

Fig. 2. Effect of immobilization time (conditions: pH 3.5; temperature 30 °C; initial lipase activity 49×10^3 U/l in acetate buffer; MAC 1 g).

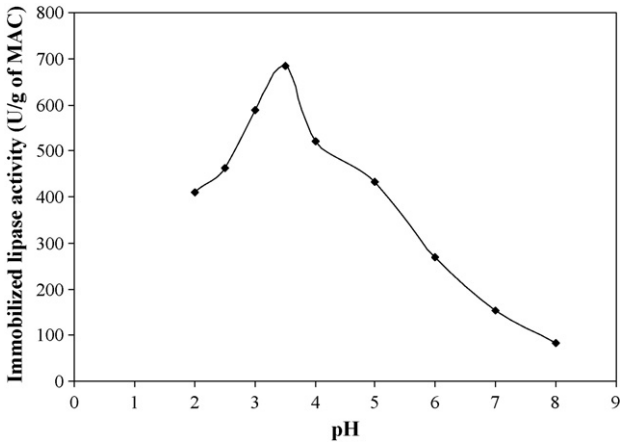

Fig. 3. Effect of pH on the immobilization of lipase (conditions: time 120 min; temperature 30 °C; initial lipase activity 49×10^3 U/l in acetate buffer; MAC 1 g).

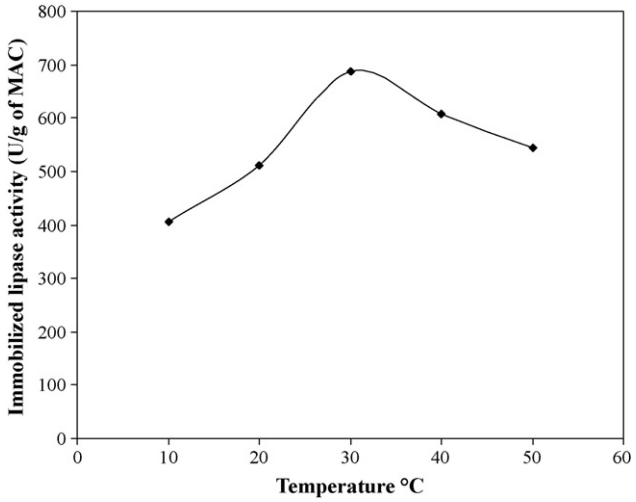

Fig. 4. Effect of temperature on the immobilization of lipase (conditions: time 120 min; pH 3.5; initial lipase activity 49×10^3 U/l in acetate buffer; MAC 1 g).

gest that initial lipase activity of 49×10^3 U/l is sufficient enough for achieving a maximum loading (93%) of lipase onto the mass of MAC taken. The lower immobilization percentage at higher lipase activities could be due to the crowding or agglomeration of enzyme molecules near the pore entrances and at the active sites of the support.

3.4. Thermal stability

Thermal stability of enzyme is one of the most important criteria for long term and commercial application. The immobilized enzyme is known to be more resistant against heat than that native state. Free and immobilized lipases were incubated at various temperatures (10–50 °C) for 24 h and the residual activity was measured. When compared to the free lipase, the immobilized lipase exhibited a better thermal stability at all temperatures tested (Fig. 5). We observed that upon incubation for 24 h at 40 °C, the free lipase retained the activity about 65% while the immobilized lipase displayed a residual activity of about 88%. At 50 °C after 24 h of incubation, the immobilized lipase retained 69% of its original activity while the free enzyme showed 38%. These data indicated that the thermal stability of lipase could be enhanced by the immobilization process. Ozturk et al. [40] have reported that after 240 min of incubation at 40 °C, the *Candida*

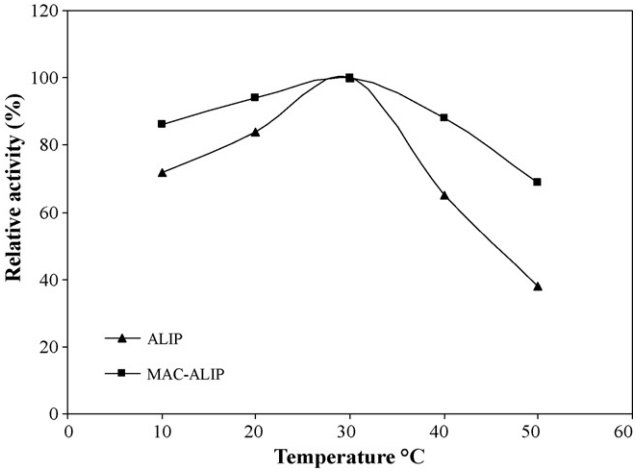

Fig. 5. Thermal stability of ALIP and MAC–ALIP (conditions: time 24 h; pH 3.5).

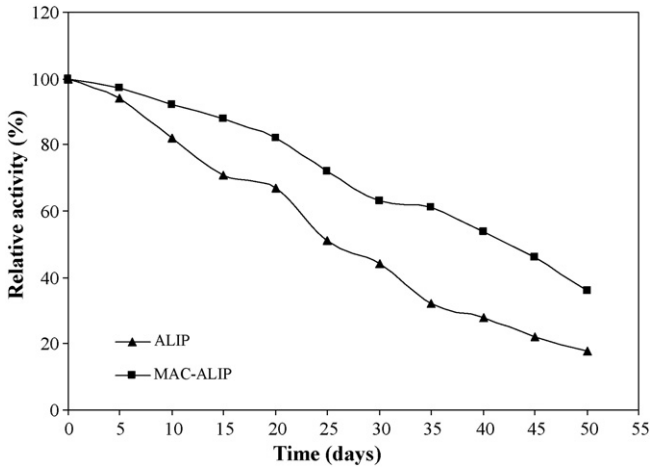

Fig. 6. Storage stability of ALIP and MAC–ALIP.

rugosa lipase immobilized on poly-2-hydroxyethyl methacrylate and N-methacryloyl-(L)-phenylalanine methyl ester retained 66% of its initial activity, while the free lipase retained 26.5%. At 50 °C, the immobilized lipase showed 62.5% of its initial activity, while the free lipase showed 19.2% of its initial activity. Ghamgui et al. [41] also reported that, the *Rhizopus oryzae* lipase immobilized onto $CaCO_3$ heat treated for 24 h at 50 °C, retained 67% of the residual activity. Under the same conditions, the free lipase lost 94% of its activity [41]. On the basis of these observations, lipase immobilized MAC showed significant thermal stability compared to that of its free state.

3.5. Storage stability

Storage stability for the immobilized lipase is one of the significant indexes to evaluate the properties of enzyme, which can make the immobilized lipase more advantageous than that of the free one. Enzymes are not stable during storage in solutions and their activities decrease gradually with time. Hence, for the determination of storage stability and application of the immobilized enzyme systems in the physiological environment, it is important to examine the storage stability. The storage stability was investigated for 50 days, by measuring the enzyme activities at certain time intervals and the results are given in Fig. 6. After 15 days, there was a slight decrease in the activity by about 12% for the immobilized lipase and 29% for the free lipase. Moreover, the immobilized enzyme provided a prominent advantage in stability over the free enzyme, especially at a longer duration.

3.6. Operational stability of immobilized enzymes

Operational stability of the immobilized lipase in batch hydrolysis of olive oil was tested by repetitive use of MAC–ALIP. As shown

Table 3
Operational stability of MAC–ALIP (conditions: pH 3.5; temperature 30 °C).

No of cycles	Relative activity (%)
1	100
3	100
5	100
7	98
10	94
15	82
20	61
25	43

in Table 3, the activity of the immobilized lipase retained 98% up to seven cycles and thereafter reduced to 43% at 25th cycle of operation. The maximum operational stability in the initial runs may be attributed to the strong covalent attachment of the enzymes on the MAC surfaces. The reduction in the operational stability at higher cycles may be due to the strains induced in the immobilized enzyme by the periodic run of the substrate leading to conformational changes affecting the native active sites. Although the maximum stability is observed till seven cycles in the present study, further increase in stability for higher number of cycles can be obtained by modifying the surface properties of the MAC used.

3.7. Determination of kinetic parameters

Kinetic parameters for the activity of free and immobilized lipase, i.e. V_{max}, and K_m (Fig. 7), were determined using the Michaelis–Menten plot by the emulsified olive oil as the substrate. The lipase activity/mass of ALIP and MAC–ALIP used for the determination of kinetic parameters were 684 U (0.47 mg of free lipase) and 1.0 g (MAC–ALIP) respectively. Unit of lipase activity (U) is defined as the amount of enzyme that hydrolyzed olive oil to release 1.0 μmol of fatty acid per minute under the assay conditions. The K_m values signify the extent to which the enzymes have access to the substrates [42]. The lower value of K_m represents higher affinity between enzymes and substrates. It is observed that lipase immobilized in MAC has lower K_m (0.243 mM) value than the free enzyme (0.655 mM), indicating a higher affinity of the MAC–ALIP towards substrate olive oil (enzyme–substrate complex formation). The V_{max} value for MAC–ALIP was 0.221 mM min^{-1} and for the free lipase was 0.285 mM min^{-1}. The observed findings corroborate with values reported by Kennedy et al. [43] during the immobilization of polyphenol oxidase onto mesoporous activated carbon. On the other hand, some researchers reported the higher K_m values for immobilized lipase, attributed to conformational changes or lower accessibility of the substrate to the active sites of the immobilized enzyme [9,44,45].

3.8. FT-IR spectra

The infrared spectra of mesoporous activated carbon have a wide band at about 3401.75 cm^{-1} due to O–H stretching mode of hexagonal groups and adsorbed water. The position and symmetry of this band at lower wave numbers indicate the presence of strong hydrogen bonds. The phosphate bond, which arises due to the phosphorous acid activation, exhibited at 1103.25 cm^{-1} along

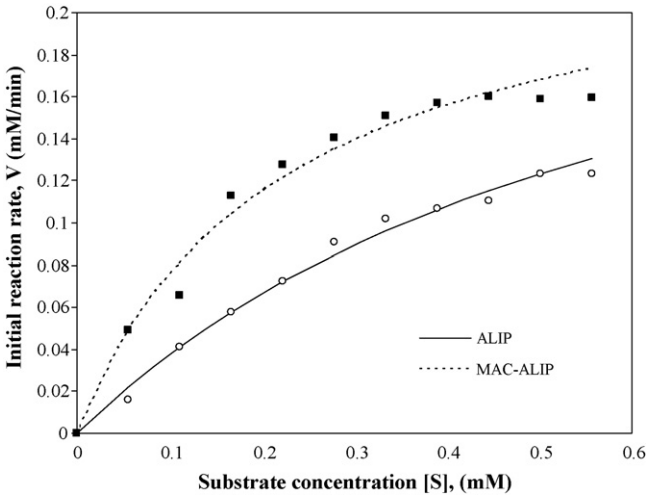

Fig. 7. Michaelis–Menten plots of ALIP and MAC–ALIP.

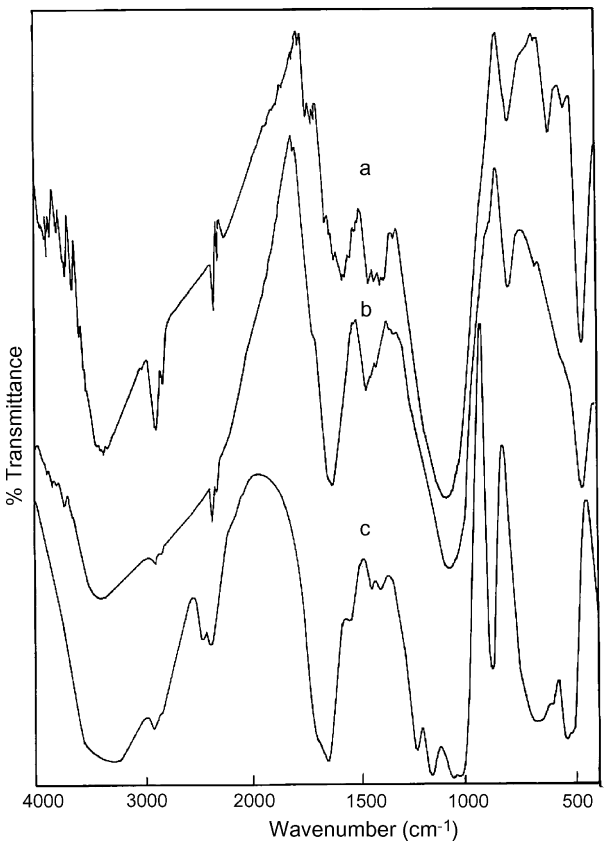

Fig. 8. FT-IR spectra of (a) MAC, (b) MAC–ALIP and (c) ALIP.

with P–O–P stretch at 795.28 cm^{-1}. Asymmetrical stretching of carboxylate group in MAC is indicated at 1579.08 and 1459.07 cm^{-1} respectively (Fig. 8(a)).

Natalello et al. [46] reported that the FT-IR absorption spectrum of pure lipase contained three major bands in the 1800–1300 cm^{-1} spectral region, assigned to peptide group stretching vibrations. The FT-IR spectrum of free acidic lipase in the present study shows a broad band at 3431.35 cm^{-1} due to the overlap of –NH stretching of amide group of proteins. The (C=O) wagging and N–H stretching of amide-I are shown at 1629.32 and 672.81 cm^{-1} respectively. A moderate peak at 1458.98 cm^{-1} can be attributed to the C–N stretching of amide (Fig. 8(c)).

The FT-IR of MAC–ALIP shows the N–H stretching frequency in the region of 3431.35 cm^{-1}. Complete shift of the asymmetrical stretching of carboxylate group around 1583.95 cm^{-1} showed that there is a strong interaction between functional groups of MAC and the enzyme. This can be further confirmed by the shift of amide-I band from 1653.54 to 1629.32 cm^{-1} (sharp band). C–N stretching of amide is at 1458.98 cm^{-1} (Fig. 8(b)).

3.9. XRD analysis

X-ray diffraction patterns of the free lipase (ALIP), free MAC and lipase immobilized MAC are shown in Fig. 9. The XRD pattern of MAC (Fig. 9(a)) shows amorphous structure, with a broad peak around 2θ value of 22°. This corresponds to the amorphous silica that is present as an impurity in the raw material of the MAC [32]. ALIP (Fig. 9(b)) shows the crystalline structure of lipase, which is also observed in the MAC–ALIP (Fig. 9(c)), owing to the immobilized acidic lipase. The peaks observed in MAC–ALIP at 2θ region of 32° and 35° are reported to be crystalline phases of lipase. This is in agreement with the values reported by Gupta et al. [47].

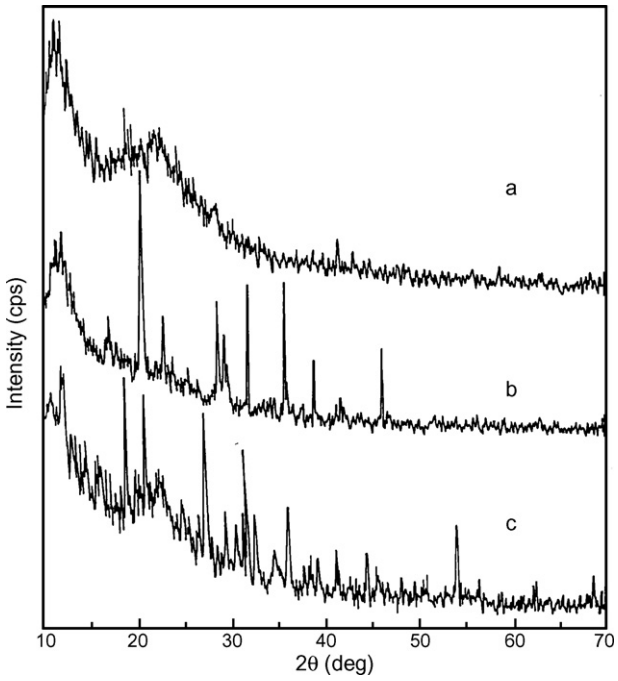

Fig. 9. XRD pattern of (a) MAC, (b) ALIP and (c) MAC–ALIP.

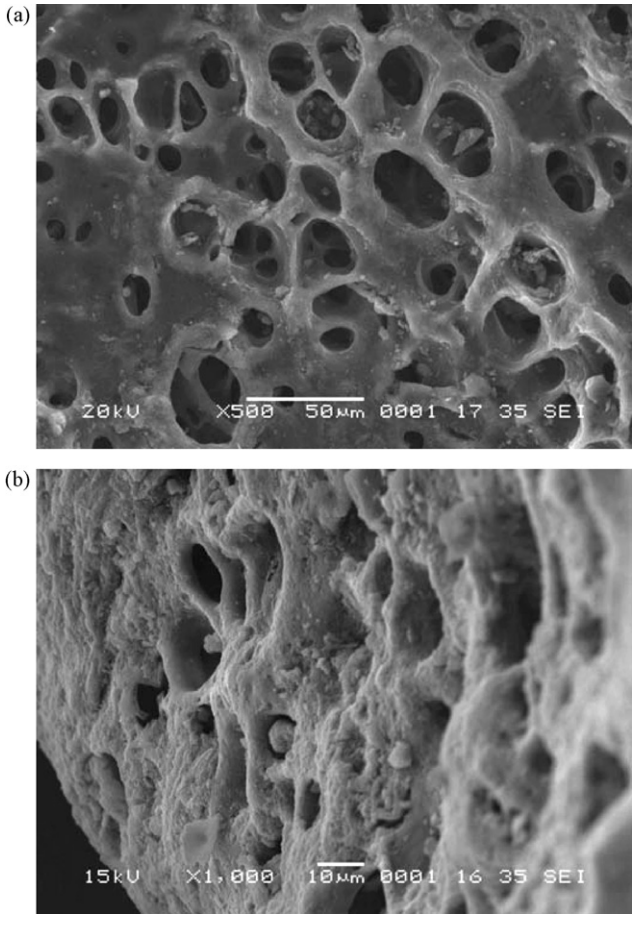

Fig. 10. Surface morphology of (a) MAC and (b) MAC–ALIP by SEM analysis.

3.10. Scanning electron microscopy (SEM)

Surface morphology of MAC is shown in Fig. 10(a), the micrograph clearly shows the presence of highly porous nature and differences in pore sizes due to chemical activation by phosphoric acid. The opening of the pores in the surface of the rice husk should be due to the extraction of some materials so as to create, upon activation, micro- and mesopores in the carbon matrix. As a result of the creation of pores, there was an increase in both the surface area and the pore-volume in the activated carbon. The surface morphology of the MAC–ALIP is shown in Fig. 10(b). The micrograph presents that the enzyme molecules are well bound to the inner walls of the pores in the carbon matrix. On account of this, the pore wall surfaces are fully modified. The enzyme molecules enter into the deep pores of the carbon matrix and after filling the pores they reside at the entrance of the mouth of the pores and thereafter it spreads to the outer pore surface area.

4. Conclusion

The acidic lipase produced from *P. gessardii* was used for the immobilization onto mesoporous activated carbon matrix. The optimum conditions for the immobilization of acidic lipase onto MAC were found to be: time 120 min, pH 3.5, temperature 30 °C and initial lipase activity 49×10^3 U/l. The maximum enzyme load of the MAC was 1834 U/g at the optimum conditions. The thermal stability of the immobilized lipase was higher than that in its free form. Kinetic parameters for free and immobilized enzymes, determined according to the Michaelis–Menten equation, showed a lower K_m value for MAC–ALIP than the free enzyme, indicating a higher affinity towards substrate. The results obtained from the instrumental studies such as FT-IR, X-ray diffraction and scanning electron microscopy evidenced the immobilization of acidic lipase onto MAC. The SEM pictures of MAC–ALIP revealed the immobilization occurred on both, the surface and the pores of the MAC leading to high immobilization intensity. Therefore, the results obtained in the present study concluded that the MAC can be considered as the best carrier matrix for enzyme molecules and this can find wide application as biocatalysts in various organic syntheses, through a simple and inexpensive method.

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References

[1] T. Palmer, Understanding Enzymes, Prentice Hall, Inc., 1991.
[2] F.M. Gomes, E.B. Pereira, H.F. de Castro, Biomacromolecules 5 (2004) 17.
[3] G. Pugazhemthi, A. Kumar, J. Membr. Sci. 228 (2004) 187.
[4] B.K. Vaidya, G.C. Ingavle, S. Ponrathna, B.D. Kulkarni, S.N. Nene, Bioresour. Technol. 99 (2008) 3623.
[5] S.W. Chang, J.F. Shaw, K.H. Yang, S.F. Chang, C.J. Shieh, Bioresour. Technol. 99 (2008) 2800–2805.
[6] O. Yemul, T. Imae, Biomacromolecules 6 (2005) 2809.
[7] O. Kose, M. Tuter, H.A. Aksoy, Bioresour. Technol. 83 (2002) 125–129.
[8] K.E. Jaeger, T. Eggert, Curr. Opin. Biotechnol. 13 (2002) 390–397.
[9] H. Tutar, E. Yilmaz, E. Pehlivan, M. Yilmaz, Int. J. Biol. Macromol. 45 (2009) 315–320.
[10] H. Zeng, K. Liao, X. Deng, H. Jiang, F. Zhang, Process Biochem. 44 (2009) 791–798.
[11] K.T. Lee, C.C. Akoh, Biotechnol. Tech. 12 (1998) 381–384.
[12] P. Yang, W.K. Teo, Y.P. Ting, Bioresour. Technol. 97 (2006) 39–46.
[13] H. Noureddini, X. Gao, R.S. Philkana, Bioresour. Technol. 96 (2005) 769–777.
[14] P. Gemeiner (Ed.), Enzyme Engineering: Immobilized Biosystems, Ellis Horwood, New York/London, 1992.
[15] X.J. Huang, A.G. Yu, Z.K. Xu, Bioresour. Technol. 99 (2008) 5459–5465.
[16] S. Gao, Y. Wang, G. Luo, Y. Dai, Bioresour. Technol. 100 (2009) 996–999.
[17] M.L. Foresti, M.L. Ferreira, Catal. Today 107/108 (2005) 23–30.
[18] Z.G. Wang, J.Q. Wang, Z.K. Xu, J. Mol. Catal. B: Enzym. 42 (2006) 45–51.

[19] N.N. Gandhi, V. Vijayalakshmi, S.B. Sawant, J.B. Joshi, Chem. Eng. J. 61 (1996) 149–156.
[20] R.M. Blanco, P. Terreros, M. Fernandez-Perez, C. Otero, G.D. Gonzalez, J. Mol. Catal. B: Enzym. 30 (2004) 83–93.
[21] M.D. Serio, C. Maturo, E. De Alteriis, P. Parascandola, R. Tesser, E. Santacesaria, Catal. Today 79/80 (2003) 333–339.
[22] Y.X. Bai, Y.F. Li, Y. Yang, L.X. Yi, J. Biotechnol. 125 (2006) 574–582.
[23] Q.Z.K. Zhou, X.D. Chen, Biochem. Eng. J. 9 (2001) 33–40.
[24] Y. Yesiloglu, Process Biochem. 40 (2005) 2155–2159.
[25] M. Ghiaci, H. Aghaei, S. Soleimanian, M.E. Sedaghat, Appl. Clay Sci. 43 (2009) 289–295.
[26] X.S. Zhao, X.Y. Bao, W.P. Guo, F.Y. Lee, Mater. Today 9 (2006) 32–39.
[27] K.L. Lie, H.L.C. Lina, T.W. Keng, Clin. Biochem. 35 (2002) 181.
[28] J.S. Macedo, L. Otubo, O.P. Ferreira, I.F. Gimenez, I.O. Mazali, L.S. Barreto, Micropor. Mesopor. Mater. 107 (2008) 276.
[29] M.M. Shamel, K.B. Ramachandran, M. Hasan, S. Al-Zuhair, Biochem. Eng. J. 34 (2007) 228–235.
[30] S. Al-Zuhair, M. Hasan, K.B. Ramachandran, Process Biochem. 38 (2003) 1155–1163.
[31] S.W. Tsai, C.S. Chang, J. Chem. Technol. Biotechnol. 57 (1993) 147–154.
[32] L.J. Kennedy, J.J. Vijaya, G. Sekaran, Ind. Eng. Chem. Res. 43 (2004) 1832–1838.
[33] M.V. Lopez-Ramani, F. Stoeckli, C. Moreno-Castilla, M.F. Carraxo, Carbon 37 (1999) 1215–1221.
[34] Activated Carbon Powdered and Granular: Methods of Sampling and Tests, Bureau of Indian Standards, New Delhi, India ISI, 1989, p. 877.
[35] K. Ramani, G. Sekaran, Symposium on Leather Industries Get-together, Chennai, India, 2007, p. 88.
[36] A.M. Naci, D.A.M. Ali, Turk. J. Biol. 26 (2002) 133–143.
[37] O.H. Lowry, N.J. Rosebrough, A.L. Farr, J. Randal, J. Biol. Chem. 193 (1951) 265–275.
[38] U.K. Laemmli, Nature 227 (1970) 680–685.
[39] K. Hayakawa, M. Kawaguchi, T. Kato, Langmuir 13 (1997) 6069–6073.
[40] N. Ozturk, S. Akgol, M. Arisoy, A. Denizli, Sep. Purif. Technol. 58 (2007) 83–90.
[41] H. Ghamgui, N. Miled, M. Karra-chaabouni, Y. Gargouri, Biochem. Eng. J. 37 (2007) 34–41.
[42] L.M. Shuler, F. Kargi, Bioprocess Engineering, 2nd ed., Prentice Hall Inc., 2002.
[43] L.J. Kennedy, P.K. Selvi, P. Aruna, K.N. Hema, G. Sekaran, Chemosphere 69 (2007) 262–270.
[44] X.J. Huang, D. Ge, Z.K. Xu, Eur. Polym. J. 43 (2007) 3710.
[45] G. Pencreah, M. Leullier, J.C. Baratti, Biotechnol. Bioeng. 56 (1997) 181.
[46] A. Natalello, D. Ami, S. Brocca, M. Lotti, S.M. Doglia, Biochem. J. 385 (2005) 511–517.
[47] S. Gupta, S. Yogesha, M. Javiya, C.S. Bhambi, K. Pundir, A. Singh, Bhattacharya, Int. Biol. Macromol. 42 (2008) 145–151.