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Immobilisation and Applications of a Thermostable Lipase from the Seeds of Macrotylomauniflorum

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ABSTRACT

Lipases (triacylglycerol acylhydrolases EC 3.1.1.3) have proved to be one of the most important industrial enzymes as they are able to perform in both aqueous and water-restricted environments catalyzing stereospecific hydrolysis and transesterification reactions respectively. Lipases from plant sources have interesting features for application in different fields. In the present study, a lipase from seeds of Macrotylomauniflorum was isolated and purified using hydrophobic interaction chromatography. The enzyme was found to be thermoactive, having maximum hydrolytic activity at 60° C and stability over the range of 20 to 70° C. The optimum pH for the enzyme is 8.6 and is stable in alkaline range upto pH 10. The enzyme was inhibited by Cd^{2+}, Hg^{2+}, tween 20, tween 40 as well as pesticides malathion, methyl parathion and chlorpyrifos. Immobilization of enzyme on calcium alginate beads has been done and its application in carrying out transesterification reactions in non-polar solvents has been investigated.

Keywords: Lipase, Macrotylomauniflorum, Thermostable, Immobilization.

I. INTRODUCTION

Enzymes are extensively being used in many industries for biotechnological, pharmaceutical and food applications on account of their unique characteristics. The limiting factors in widespread applicability of enzymes are their substantially unstable nature and requirement of stringent conditions of pH and temperature. Also, the enzyme once used in a reaction, cannot be recovered for repeated use. Immobilisation of enzyme by different methods is the key to improve enzyme stability and reusability[1,2].

Lipases (EC 3.1.1.3)are the biocatalysts that have inherent ability to hydrolysetriacylglycerols into free fatty acids and glycerol. The unique competence of lipases to catalyse stereospecific transesterification reactionsin water-restricted environments has made them one of the most favourable candidates in organic biosynthesis [3].To date, a large number of lipases have been profoundly studied from the bacterial and fungal sources whereas;the knowledge about plant lipases isstill very limited regardless of their biocompatibility and low production cost[4].

Macrotylomauniflorum (Lam.) Verdc. commonly known as horse gram or Kulithis one of the lesser known legumes. It is mainly used as cattle fodder though it is also consumed as cooked food in some parts of India. It has been reported to have hepatoprotective, hypercholesteramic and antioxidant activities [5].

In the present study, a lipase enzyme was isolated and partially purified from the seeds of Macrotylomauniflorum. The partially purified lipase was immobilised by entrapment in calcium alginate beads [6] in order tocatalyse the transesterfication reaction to form Ethyl propanoate.

II. METHODS AND MATERIAL

Materials

The seeds of Macrotylomauniflorum were purchased from local market. P-nitrophenylpalmitate was purchased from Sigma-Aldrich while sodium alginate and other chemicals were procured from Sisco Research Laboratories Pvt. Ltd. (India).

Protein estimation

Protein in samples was estimated by Lowry's Method [7] using Bovine Serum albumin as a standard.

Lipase Assay

Lipase activity was determined with slight modifications to Winkler and Stuckmann method [8] using p-Nitrophenyl Palmitate as a substrate. p-Nitrophenyl Palmitate was dissolved (3mg/ml) in isopropanol and then 1 part of it was added dropwise to 9 parts of Tris-HCl buffer, pH 8.6, 50mM solution containing 0.4% Triton x-100. The assay was performed by adding 0.7 ml of the substrate solution to 0.3 ml enzyme in Tris-HCl buffer. After incubating the reaction mixture for 20 minutes, absorbance was measured at 410nm. The µmoles of p-nitrophenol released was determined by using molar extinction coefficient of it (0.012750 $\mu m^{-1}cm^{-1}$). One unit of enzyme activity is defined as the amount of enzyme liberating one micromole of p-Nitrophenol in one minute [9].

Partial Purification of Lipase from Macrotylomauniflorum

M. uniflorum seeds were allowed to germinate for 36 hours, extracted in four volumes of physiological saline followed by 30-80% ammonium sulphate precipitation. The precipitated protein was collected after centrifugation, dissolved in minimum amount of distilled water and dialysed against distilled water and finally against Tris buffer.The dialysed protein was subjected to Hydrophobic Interaction Chromatography using a column packed with phenyl sepharose matrix equilibrated in phosphate buffer pH 7.0, 50mM containing 1M ammonium Sulphate. The column was washed with discontinuous gradient of ammonium sulphate in buffer and finally with distilled water. Sephadex G-100 gel filtration column was used to further purify HIC eluted lipase.

Determination of Optimum pH and temperature

The pH optimum of the semi-purified lipasewas determined by performing standard lipase assay at different pH values ranging from 5.0 to 10.0 in appropriate buffers. For determination of optimum temperature, semi-purified lipase and substrate solution were incubated at different temperatures ranging from 20 to 90 ^{0}C under standard assay conditions.

Thermostability and pH stability of the enzyme

Thermostability of the enzyme was assessed by pre incubating the enzyme at various temperatures (20-90 ^{0}C) for 1 hour and then determining residual enzyme activity at standard assay conditions. The pH stability of the enzyme was determined by measuring residual enzyme activity after incubating the enzyme at different pH ranging from 3.0 to 10.0 for 1 hour at room temperature.

Effect of Metal ions on lipase activity

The semi-purified lipase was preincubated with 5mM solutions of metal ions (K^+, Na^+, Ag^+, Cu^{2+}, Ni^{2+}, Ca^{2+}, Zn^{2+}, Mn^{2+}, Mg^{2+}, Cd^{2+}, Hg^{2+}, Fe^{3+}, Pb^{2+}, Co^{2+}, Sn^{2+}) for 1 hour at room temperature and then residual enzyme activity of each enzyme solution was determined at standard assay conditions.

Effect of detergents on activity of Lipase

The effect of detergents on lipase activity was assessed by incubating the lipase in the presence of surfactants viz. Triton X-100, Tween 20, Tween 40, Sodium taurocholate and four commercial detergents (Tide, Surf excel, Wheel and Rin) at 1% W/V or 1% V/V for 1 hour at room temperature.The residual enzyme activity was determined at pH 8.6 and temperature 60 ^{0}C for 20 minutes.

Effect of Pesticides on Lipase activity

The commonly used organophosphorous pesticides, Malathion, Methyl Parathion and Chlorpyrifos were incubated at 10 mM concentration with the M. uniflorum.Lipase for 1 hour at room Temperature. The lipase activity after incubation was determined at standard assay conditions.

Immobilisation of Lipase

For immobilisation of lipase, equal volumes of lipase were mixed with sodium alginate solutions so as to keep effective concentration of alginate in beads ranging from 6- 12 %. The lipase - Na alginate mixed solutions were added with syringe; dropwise into 50 ml of Calcium Chloride solutions (0.2M) while stirring on a magnetic stirrer to form enzyme entrapped beads. Corresponding control beads were also prepared without adding lipase. After 30 minutes of hardening, beads were separated from $CaCl_2$ solutions and washed three times with Tris-Cl buffer (pH 8.6). The protein content and lipase activity in filtered $CaCl_2$ solutions and three washings was determined so as to determine Loading efficiency [6].

$$\text{Loading efficiency (\%)} = \frac{\text{CiVi} - \text{CfVf}}{\text{CiVi}} \text{ X } 100$$

Where, Ci- Initial protein Concentration, Vi- initial volume of the enzyme solution, Cf- Protein concentration in total filtrate and washings and Vf- Total volume of the filtrate and washings.

Hydrolytic activity of lipase entrapped beads was determined by standard lipase assay using 20 mg beads instead of 0.3 ml enzyme.Immobilisation Yield was calculated as follows:

$$\text{Immobilisation yield (\%)} = \frac{\text{A immb}}{\text{A free}}$$

Where Aimmb- Specific activity of immobilised enzyme and A free- Specific activity of free enzyme [6]

Synthesis of ethyl propionate by immobilised enzyme

Immobilised lipase was used for synthesis of ethyl propionate through transesterification reaction [10]. Reaction mixture (10 ml) contained 50 mg of lipase entrapped beads, 100 mM each of ethanol and propionic acid in n-octane in a glass stoppered bottle. This reaction mixture was incubated at 60 ^{0}C in a shaking incubator for 12 hours. Synthesis of ethyl propionate was confirmed by TLC.

III. RESULTS AND DISCUSSION

Isolation and Partial purification Lipase from the seeds of *Macrotyloma uniflorum*

It is well kown that the activity of lipase is high during germination [11, 12]. The lipase isolated after 36 hours germination was partially purified by salting out, dialysis and column chromatography. As shown in elution profile of hydrophobic interaction chromatography (Fig. 1), lipase was eluted out of phenyl sepharose column with distilled water. Gel filtration further purified the enzyme to near homogeneity.

Effect of pH on Enzyme activity

Optimum pH of the enzyme was found to be 8.6. The enzyme was stable in the alkaline range up to pH 10. The effect of pH is as per shown in Fig. 2 a) and c). Optimum pH for almond seed lipase and rice bran Lipase were found to be 8.5 and 11.0 [13,14]

Effect of temperature on Enzyme activity

The effect of temperature on enzyme activity is shown in Fig 2 b) and d). It is a thermoactiveenzyme showing maximum activity at 60^0 C. The enzyme is stable over the temperature range 20-70^0 C. similar results were obtained for almond seed lipase (65^0 C) and rice bran lipase (80^0 C) [13,14]

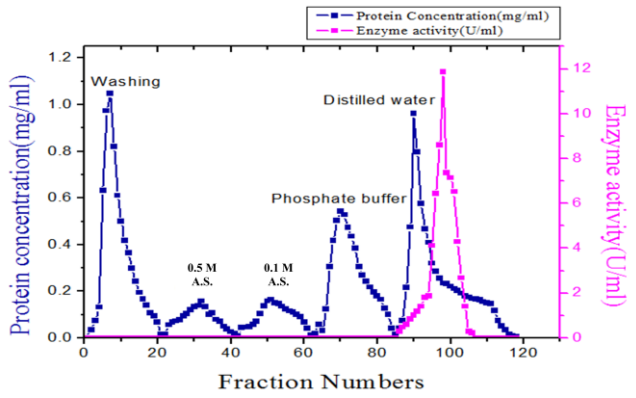

Figure 1. Elution profile of Lipase on Phenyl Sepharosehydrophobic interaction chromatography (A.S.= Ammonium Sulphate in Phosphate Buffer)

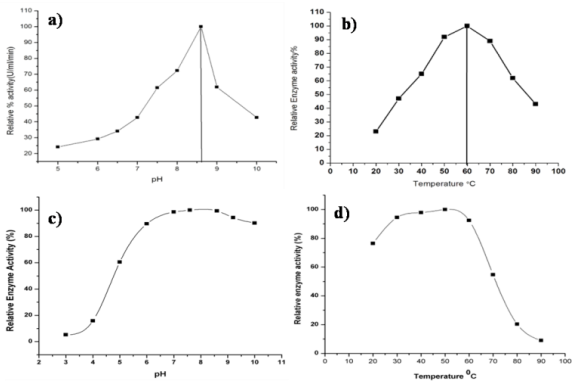

Figure 2. a)Effect of pH on enzyme activity. Optimum pH was found to be 8.6. b) Effect of temperature on enzyme activity. Maximum enzyme activity was obtained at 60 °C. c) pH stability of enzyme d) Temperature stability of enzyme.

Effect of Metal ionson lipase activity:

It is well known fact that metal ions alter the enzyme activity [15]. The effect of monovalent, divalent and trivalent metal ions on lipase activity is as shown in Fig. 3. A notably lower activity of the enzyme was observed in presence of Ag^{+}, Hg^{2+}, Cd^{2+} and Zn^{2+}. whereas Ca^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, and Na^{+} were found to activate enzyme. Bhardwaj et al. have reported an inhibition effect of divalent cations like Mg^{2+}, Zn^{2+}, Cu^{2+}, and Cd^{2+} on rice bran lipase activity [14].Nishio et al. have reported a stimulatory effect of Ca^{2+} and Mg^{2+} on *Pseudomonas fragi* lipase activity [16].

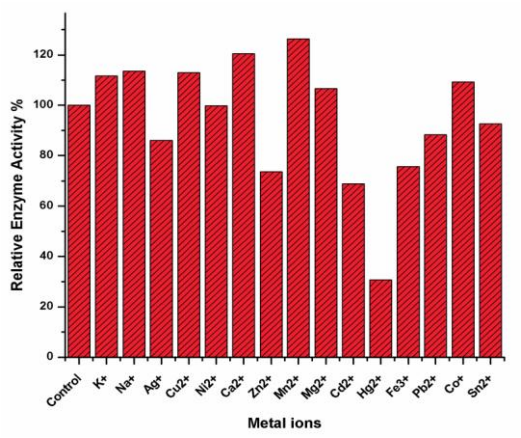

Figure 3. Effect of metal ions on Lipase activity

Effect of detergents on activity of Lipase

A relative lipase activity was observed in presence of surfactants and commercial detergents. The enzyme was strongly inhibited by Tween 20 and Tween 40 while it retains more than 80% of its lipolytic activity with commercial detergents.The results of commercial detergents are in accordance with Polizeli et al. and Weerasooriya et al.[17, 18]

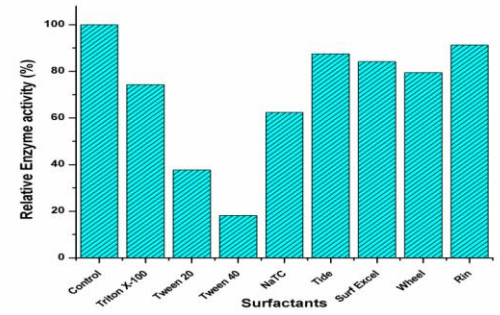

Figure 4. Effect of Surfactants and detergents

Effect of Pesticides on Lipase activity

All the three pesticides inhibited the enzyme profoundly. The results are shown in Fig. 5.Kartalet al. andGangadharaReddy et al. have done similar work in order to design a biosensor for pesticide detection.

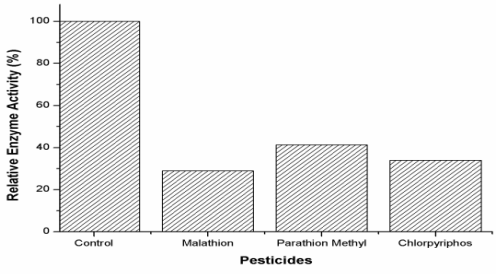

Figure 5. Effect of pesticides on enzyme activity

Immobilisation of Enzyme

M. uniflorum lipase was immobilised by entrapment in Calcium alginate gel beads. The beads retained lipolytic activityeven after repeated usage proving its reusability.

Gelation is the effect of crosslinking between alginate and calcium and thus have substantial effects on immobilisation efficiency. Therefore, effect of alginate concentration on loading efficiency and immobilisation yield was investigated. Alginate concentration was increased from 6% to 12 % keeping other parameters constant. Loading efficiency increased with increasing alginate concentration while immobilisation yield decreased as shown in Fig. 6. As crosslinking is expected to increase with alginate concentration, lipase will not leak from the beads. While higher crosslinking may affect conformation of enzyme leading to decrease in immobilisation yield. Similar results were observed by Keehoon et al. with *Candida rugosa* lipase [6]. Lipase entrapped beads were used for synthesis of ethyl propionate by transesterification which are confirmed by TLC.

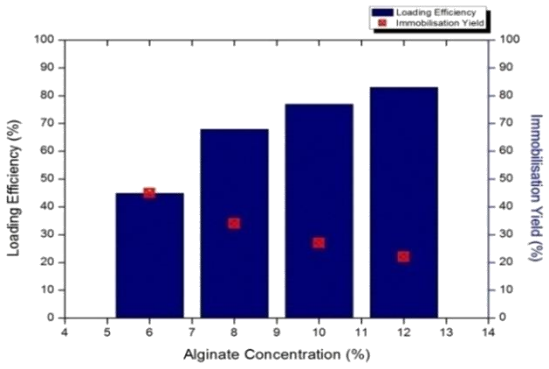

Figure 6. Effect of alginate Concentrationon Immobilization

IV. CONCLUSION

Lipase was isolated and partially purified from *Macrotylomauniflorum* seeds. The enzyme was found to be thermoactive showing maximum activity at pH 8.6 and 60^{0}C temperature. Stability of the lipase enzyme at high temperature and alkaline pH makes it suitable for industrial applications. The lipase was strongly inhibited by heavy metals ions like mercury and cadmium as well as surfactants tween 20 and tween 40. Lipase retains more than 80% activity in the presence of commercial detergents andhydrolytic activity was enhanced in presence of Ca^{2+} and Mn^{2+}. Immobilisation of lipase was done by entrapment in Ca alginate beads and effect of alginate concentration on loading efficiency and immobilisation yield was analysed. At higher concentration of alginate immobilisation yield decreased suggesting need to determine optimum alginate concentration for entrapment. The immobilised lipase can be used to catalyse transesterification reactions in organic solvents so as to synthesize industrially important biocompatible compounds at cheaper cost.

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