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Biochemical Characterization of Lipase from Fagopyrum Esculentum Seeds and Its Application

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ABSTRACT

Enzymes are biocatalysts, providing opportunity for economically sustainable industrial applications. Immobilization of enzyme further enhances its reusability. Lipase a hydrolytic enzyme, owing to its enantioselectivity and regioselectivity finds varied industrial applications. Lipases are used in detergent, food and various industries. In the present study, lipase was purified from the seeds of *Fagopyrum esculentum* (buckwheat). The enzyme was immobilized on chitosan beads. Optimization of different parameters viz. use of different cross linkers, concentration of enzyme, etc. for effective enzyme immobilization was carried out. Biochemical characterization of both the native and the immobilized enzyme was studied. Further, the reusability of the immobilized enzyme was also evaluated.

Keywords : Lipase, enzyme immobilization, buckwheat

I. INTRODUCTION

Fagopyrum esculentum commonly known as buckwheat belongs to *Polygonaceae* family and is a common staple food grain in many countries in Asia, viz. Japan, Korea, and China. It is also cultivated and used as a fasting food in north Indian states with its Indian name kuttu. The proteins in buckwheat have significant biological effects and have balanced amino acid composition with high percent of the essential amino acids such as lysine and isoleucine [1].

Lipases are triacylglycerol acylhydrolases (E.C. 3.1.1.3). They catalyze the hydrolysis of carboxylester linkage of triacylglycerol to release glycerol and free fatty acids. Lipase activity plays an important role in quality deterioration of buckwheat flour [2]. The catalytic power of lipases increases significantly at the lipid-water interface, thus showing the phenomenon of "interfacial activation". In water-restricted environment, lipases catalyze reverse reactions, catalyzing various reactions like like interesterification, transesterification, hydrolysis, alcoholysis, acidolysis, or esterification reactions. Thus, immobilization of lipase presents a wide scope in various industries [3].

Immobilization is the key to optimize enzymatic processes in industries. Immobilization of enzymes renders the Industrial processes economically cheap and reusable. Moreover, it aids in the development of continuous processes and the termination of industrial processes becomes easy [4]. Chitosan has shown it versatile use in its shape and immobilization of different substances [5].

II. MATERIALS AND METHODS

A. Isolation of semi-purified lipase

Buckwheat seeds used as the source of enzyme was procured from local market of north Indian states. Lipase from 100g of Fagopyrum esculentum seed meal was extracted in 10mM Tris buffer pH 7.2 with EDTA and PMSF [6] at constant stirring with a magnetic

stirrer for 4 hours at 20°C. The latter was centrifuged at 10,000 rpm at 4°C for 30 mins. The supernatant was then subjected to ammonium sulphate precipitation, which was then passed onto a Hydrophobic Interaction matrix of 6FF phenyl sepharose (sigma) column for further purification. The extract was dialysed as and when required. This semipurified enzyme was used for immobilization and for its biochemical characterization.

B. Preparation of Chitosan beads

4 % chitosan (low molecular weight, obtained from SRL) solution was made in 2.0% aqueous acetic acid. The chitosan solution was dropped into an aqueous 2M NaOH solution using a syringe to form chitosan gelatinous beads. The chitosan beads were thoroughly washed with distilled water until neutrality was reached [7].

C. Activity measurement for free and immobilized enzyme

Lipase activity was assayed essentially as described by Winkler and Stuckmann [8] using para-nitrophenyl palmitate (pNPP) as substrate as follows: 0.3 ml of enzyme extract and 0.4 ml of Tris buffer (10 mM, pH 7.2) were pre-incubated at 30 °C for 5 mins. 0.3 ml of the substrate solution was added and the mixture was incubated at 30 °C for 20 mins. The reaction was arrested by adding 200 mM Tris buffer pH 9. The intensity of the yellow colour due to p-nitrophenol obtained due to lipase action was measured at 410 nm. For activity measurement for immobilized enzyme 0.3 ml was replaced with 5 beads. The substrate solution consisted of 1 ml of pNPP solution (0.3% in isopropanol) and 9.0 ml of tris buffer[10 mM, pH 7.2, containing 0.4% (v/v) Triton –X-100]. One unit of enzyme activity is defined as the amount of lipase liberating 1 μmol of p- nitrophenol per min.

D. Optimization of immobilization of beads

i. Crosslinking with glutaraldehyde and epichlorohydrin: The beads were stirred on a magnetic stirrer for 1 h with 1%, 3%, 5% and 10% crosslinkers viz glutaraldehyde and epichlorohydrin. The crosslinked beads were washed with distilled water to remove excess crosslinker followed by 3 washings with the 10mM Tris buffer (pH 7.2).

ii. Immobilization of lipase on crosslinked beads: 5 g crosslinked beads were stirred for varying time of 1h, 2h, 3h, 4h and 5h; followed by washings with buffer to remove unbound protein. The activity of the immobilized enzyme was measured with the same lipase assay.

iii. Effect of temperature: The immobilized and free enzyme activity was measured at different temperature 10, 20, 30, 40, 50 and 60°C by incubating the assay at these temperatures.

iv. Temperature stability: The stability of both free and immobilized enzyme was measured at different temperature 10, 20, 30, 40, 50 and 60°C by pre-incubating the enzyme in buffer at different temperature for 1 h and then the activity was measured by the lipase assay.

v. Effect of pH: The immobilized and free enzyme activity was measured at different pH by carrying out the assay with different pH buffer viz. 3, 4, 5, 6, 7, 8, 9 and 10.

vi. pH stability: The stability of both free and immobilized enzyme was measured at different pH by pre-incubating the enzyme in different pH buffer viz. 3, 4, 5, 6, 7, 8, 9 and 10 for 1 h and then the activity was measured by the lipase assay.

vii. Effect of metal ions: The enzymes were pre-incubated with 5 mM metal solutions viz. Fe^{3+}, Pb^{2+}, Hg^{2+}, Mn^{2+}, Mg^{2+}, Ba^{2+}, Ca^{2+}, Sn^{2+}, Al^{3+}, Cu^{+}, K^{+}, Ni^{2+}, Cd^{2+}, Na^{+}, $Co^{2+}$and Zn^{2+} for an hour then the assay was performed to check the residual activity of the enzymes. All the required controls and blanks with and without metal solutions were maintained.

viii. Reusability of immobilized enzyme: The activity of the beads were measured at optimum temperature and pH once, then the beads were washed with buffer until all yellow colored pNP

product is removed to perform next cycle to check the reusability.

III. RESULTS AND DISCUSSION

A. Effect of different crosslinkers

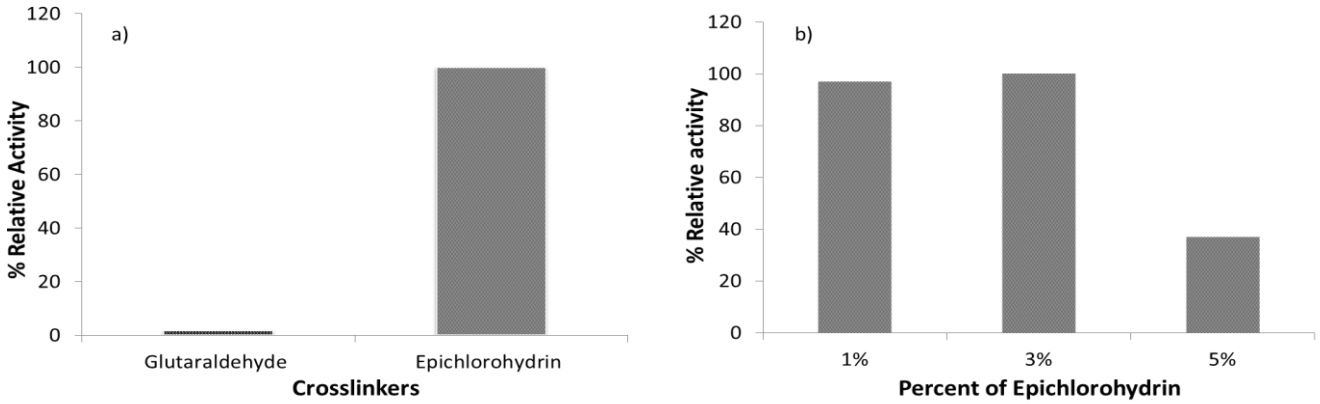

Figure 1. a) % Relative activity with different crosslinkers b) % Relative activity with different % of epichlorohydrin

Crosslinking with glutaraldehyde and epichlorohydrin at different percentages was carried out. 3% Epichlorohydrin shows maximum lipase activity in immobilized enzyme as shown in Fig. 1. This reflects the bonding of carbon atoms, disturbing the epoxide ring and the removal chlorine atom [9], compared to chitosan immobilization of peroxidase [7].

B. Effect of temperature on lipase activity and stability

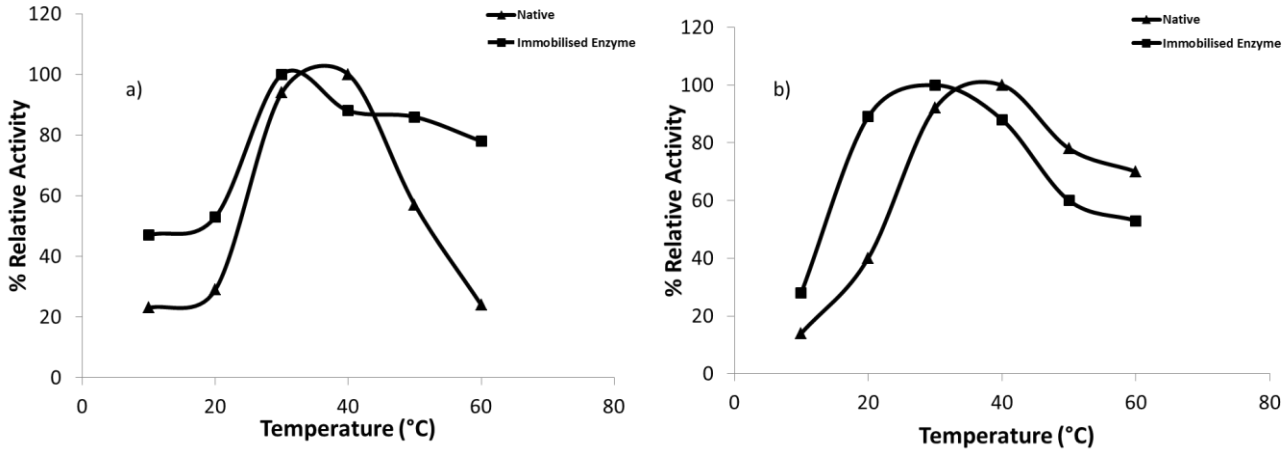

Figure 2. A) Effect Of Temperature On Native And Immobilized Lipase Activity B) Temperature Stability Of Native And Immobilized Of Lipase

As seen in the above Figure 2 a) shows the optimum temperature of native enzyme as 37°C and optimum temperature of immobilized enzyme as 30°C. As seen in the graph the immobilized enzyme has more than 80% relative activity at temperature higher than 30°C. As seen in b) the immobilized enzyme is stable in the range of 25-40°C and the native enzyme is stable in the temperature range of 30-45°C.

C. Effect of pH on lipase activity and stability

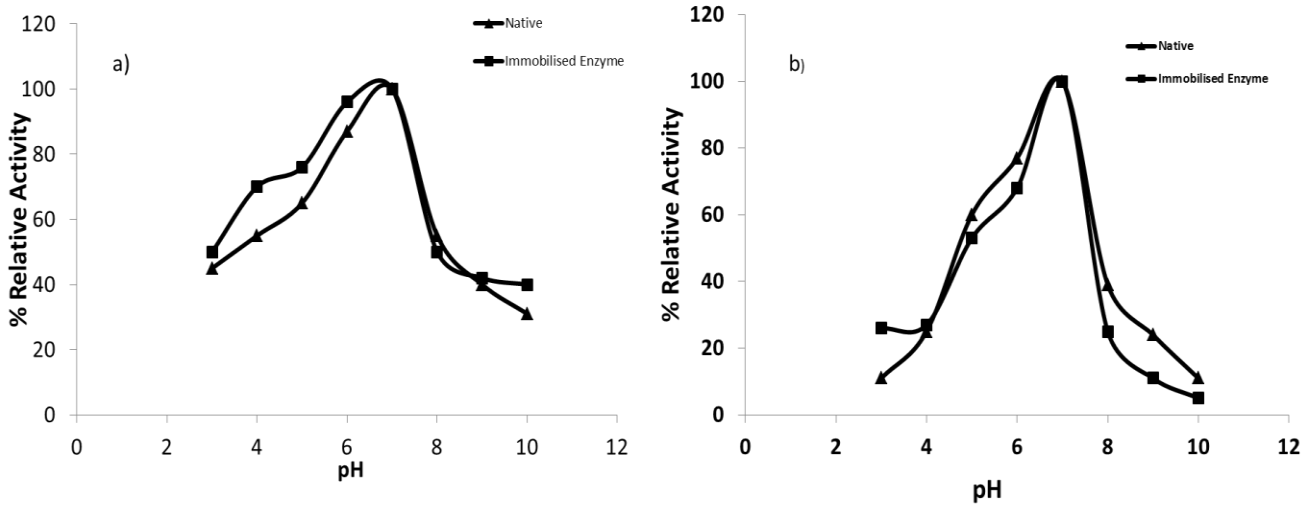

Figure 3 a) Effect of ph on on native and immobilized lipase activity b) ph stability of native and immobilized of lipase

As seen in the above figure 3 a) & b) the effect of different pH and stability of enzyme in terms of their relative activity shows the optimum pH and maximum stability for both immobilized and native enzyme is pH 7.

D. Effect of different metal ions on lipase activity

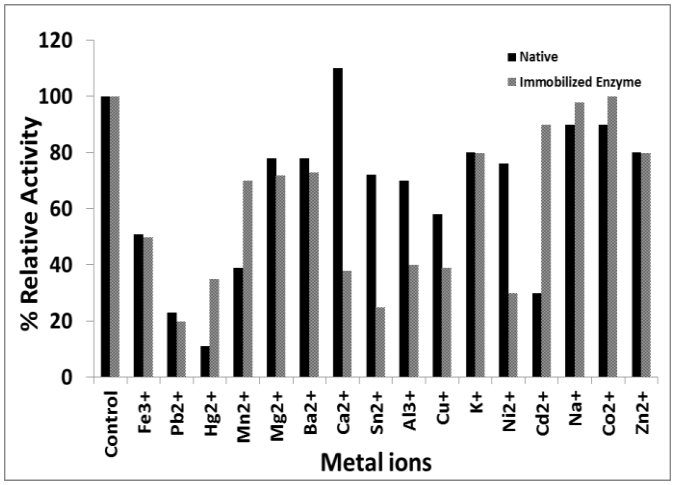

Figure 4. Effect of metal ions

As seen in the figure 4 the activity of native enzyme was maximally inhibited by Hg^{2+} ions, whereas the immobilized enzyme was maximally inhibited by Pb^{2+} ions.

E. Reusability of immobilized lipase on chitosan beads

Reusability is the most significant parameter to evaluate the application of immobilized enzymes in industries [10].

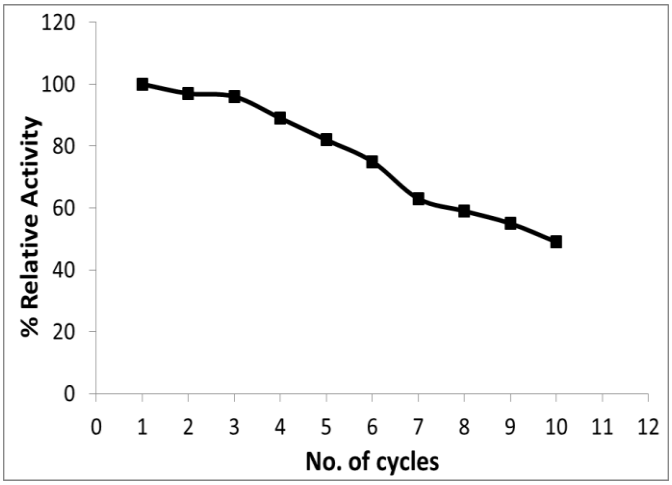

Figure 5. Reusability of the immobilized enzyme

Figure 5 shows that the immobilized enzyme retains almost 80% the lipase activity till 4-5 cycles of reuse of the same beads.

IV.CONCLUSION

The present study showed that the immobilization of semi-purified lipase enzyme on chitosan beads was successful in adsorbing and reusing the enzymes for repeated cycles with limited leaching with 3% epichlorohydrin.

Also, the inhibition of enzyme activity in presence of metal ions opens avenues for development of a biosensor based on this immobilized enzyme for sensing of lead and mercury toxicity studies.

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