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Isolation and partial purification of lipase from *Erythrina indica*

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ABSTRACT

Lipases (triacylglycerol acyl hydrolase) are enzymes that catalyze the hydrolysis of triacylglycerol's at the oil-water interface to release glycerol and free fatty acids as well as their ability to catalyzevarious types ofsynthetic reactions in non-aqueous environment. These include acidolysis, alcoholysis, aminolysis, esterification, inter-esterification. The Lipase used in each application is selected based on its substrate specificity and stereo specificity as well as temperature and p^H stability. Lipasehas attracted more attention in recent years because of its diverse biotechnological applications. Lipase occurs widely in nature, but plant lipase is significant because of its substrate selectivity/specificity. Lipase is one of the most important enzymesused in many food processes, pharmaceuticals, detergents and biofuel industries. Italso Known as Indian coral tree or Tropical coral tree or Tiger's clow or Moochy wood tree or Variegated coral tree, sunshine tree, coral bean, Pangara (Marathi). It grows wild throughout the costal forest of India. Bark, root, leaves and fruits are used in fever, liver ailment. Rheumatism, relieve joint pain, and to kill worms.

Approaches: The Lipase produced by Erythrina Indica seeds was partially purified and characterized in terms of optimal p^H and temperature for activity as well as substrate specificity.

Keywords: Erythrina Indica seeds, protein estimation by assay, Dialysis Spectrophotometry, Gel Filtration, PAGE electrophoresis, optimum pH, optimum temperature, effect of metal ions, effect of surfactant and detergents, wash performance analysis

INTRODUCTION

Enzymes are biocatalyst that increases the rate of reaction. The first enzyme to be determined was urease in 1926 by James B. Sumner. Enzymes are proteins have an active site consisting of catalytic and binding sites. The binding site formschemical bond with specific ligands. Enzymes can increase the rate of reaction 10^{17} fold and showing specificity towards their substrate. The catalytic activity of an enzyme is regulated by other molecules or ions and by feedback inhibition as seen many biosynthetic pathways. In absence of enzymatic catalysis biochemical reactions are very slow that they would not occur mild conditions of temperature and pressure that are compatible with life. Lipases are among the most important classes of industrial enzymes. In recent years the demand of lipolytic enzymes has been increased due to its potential application in various manufacturing processes industrial goods such as detergent industry, food industry and cosmetics flavor enhancers and in pharmaceutical industry. Lipase may be used to produce fatty acids, aroma and flavor compounds lubricants and solvent esters, polyesters amides, thiol esters and bio modified fats.

Lipases are widespread in nature and present in animals, plants, and microorganisms. In plants lipase activity has been identified various tissues but relatively high concentration is found in seeds. Seeds are generally rich in triacylglycerol, which serve as compact source of energy for new plant.

Aims and objectives:

The identification of novel sources of lipases with unique patterns of reaction selectivity remains a strategic objective of lipase studies. Lipases are produced by plants, animals, bacteria and moulds. Manufacture of lipases by microbial fermentation constitutes a process that is currently popular. A less explored avenue is that of lipase extraction from natural sources. So we focus on the isolation of the lipase from easily available plant source; i.e., Erythrina Indica seeds.

METHODOLOGY

Materials:

Seed collection: seeds of Erythrina Indica were purchased from local markets.

Chemicals and biochemical's: acetic acid, acetone, acrylamide, ammonium sulphate, blue dextran bovine serum albumin, cadmium chloride, calcium chloride, cobalt chloride, comassie brilliant blue, copper chloride, copper sulphate, Disodium hydrogen phosphate, ethanol, ferrous sulphate, folin phenol reagent, glacial acetic acid, glycine, hydrochloric acid, lead oxide, magnesium chloride, mercuric chloride, papain, pepsin, p-nitro phenyl palmitate, potassium chloride, sephadex G100 , sodium acetate, sodium carbonate, sodium chloride, sodium dodecyl sulphate, sodium hydroxide, sulphuric acid, tetrametyl ethylenediamines, tris buffer, zinc chloride.

Isolation and purification of lipase from Erythrina Indica:

The 50 g seeds of Erythrina Indica were soaked in 200 ml water and kept for overnight at room temperature. The seed coats were peeled off and the soft cotyledons were homogenized in a mixer with saline (0.85% Nacl). The extract was filtered with cheese cloth kept for overnight. All further purification steps were carried out at 4-7^{0}c. The homogenate was mixed with 1-butanol (20ml/100ml homogenate) and stirred for 4 hours at 4^{0}c on magnetic stirrer. The butanol layer was removed by centrifuged at 8000 rpm at 4^{0}c for 20 minutes. The supernatant thus obtained were collected and subjected to fractional salt precipitation with solid $(NH_4)_2SO_4$.

The highest lipase activity was found in the 30-80% ammonium sulphate salt precipitation. This fraction was dialyzed overnight against distilled water, saline, phosphate buffer p^H=8.0.50mM. Mobile phase for purification is phosphate buffer P^H=8.0; 50mM in dialysis bags. Dialysis was carried out in dialysis bags made up of cellophane placed in a plastic beaker containing desired solution against which contents of the bag was dialyzed. 3-4changes gives to remove the ammonium sulphate. The dialysis bags were washed in hot water prior to each use.

The dialyzed enzymes were used as partially purified enzyme and used for enzyme characterization. The dialyzed sample was clarified by centrifugation and loaded onto cell hydrophobic interaction column using phenyl sepharose matrix for purification. Fraction were collected by decreasing ionic strength showing lipase activity then eluted in gel filtration chromatography using G-100 matrix.

Lipase assay:

The spectrophotometric method was used for determination of lipase activity. The activities were determined by para-nitro –phenyl palmitate as substrate and by measuring the release of p-nitrophenol at 410 nm.one unit of Lipase activity is defined as the amount of enzyme that hydrolyzes 1µmol of p-nitrophenol per minute

Lipase assay:

	Blank	EB(ml)	SB(ml)	Test(ml)
Buffer	2.0	1.3	1.7	1.0
Enzyme	-	-	0.3	0.3
Incubate 5 min at 50^0 c				
Substrate	-	0.7	-	0.7
Incubate for 20 minutes at 50^0c and then cool at 8^0c for 10 minutes				

Protein Estimation:

Protein concentration of soluble enzyme preparation was quantified by method of Lowry et al using Bovine Serum Albumin (BSA) as standard. Plot the graph of O.D. verses µg of BSA. Using the standard calibration the protein concentration of the sample is calculated.

Lipase characterization:

***Polyacrylamide Gel electrophoresis*:**

Enzyme purification or purity was checked on non-denaturing native PAGE using 10% gel concentration with slight modification on method described by Holt and Hartman. Page was performed at room temperature in Tris Glycine (p^H8.0). Gel was stained using the comassie Brilliant Blue R-250 staining solution. Total enzyme protein used for page was of 10 µg for crude as well as partially purified lipase.

***Gel filtration chromatography*:**

Molecular weight of lipase from seeds of Erythrina Indica was estimated by gel filtration chromatography using sephadex G-100. The void volume of the column was determined by Blue dextran. The BSA (M.W.66kD), Pepsin (M.W.35kD), Trypsin (M.W.23kD), Lysozyme (M.W.14kD) proteins was used as molecular markers. The molecular weight was calculated by plotting a graph of Log (molecular weight) Vs elution volume/void volume.

***Effect of p^H and Temperature*:**

For optimum p^H the activity of lipase was investigated within the p^H range of 4-10 using the acetate buffer (p^H=4-5, 0.05M), Phosphate buffer (p^H=6-8, 0.05M), Tris-Hcl (p^H=8-9), Glycine-NaOH buffer (p^H=9-10, 0.05M). 300µl of enzyme and 1 ml buffer of different p^H were incubated at 50^0c for 5 minutes 700µl of substrate was added and again incubated 50^0c for 20 minutes to start reaction. To stop the reaction cool at 8^0c. The absorbance was taken at 410nm. For optimum temperature, the enzyme assay was performed as discussed above expect that incubation period was done at temperatures from 20-90^0c.

***Effect of metal ions*:**

The purified enzyme was incubated with 10mM EDTA (1:1v/v) at p^H=7 for 3 hours at 37^0c.The enzyme then dialyzed against mille Q water. The enzyme activity was checked by pre-incubating the demetalized enzyme. For the study of activator and inhibitor action on lipase, metal ion compounds of Al^{3+}, Hg^{2+}, Cu^{2+}, K^+, Zn^{2+}, Co^{2+}, Cd^{2+}, Ca^{2+}, Mg^{2+}, Fe^{3+} were used with 5 mM concentration incubated for 60minutes with demetalized enzyme(1:1)and assay was then carried out. Absorbance was taken at 410 nm.

***Effect of surfactant and commercial detergents*:**

The detergent and surfactant solution were prepared as 1%w/v or v/v and then that solution were incubated in boiling water bath for 60 minutes to degrade the proteins present in detergent. The enzyme sample was incubated in presence of deproteinized surfactants viz. Triton X-100, tween-80, sodium taurocholate commercial detergents like tide, wheel, rin, surf excel, Ariel, ujala at 1:1 proportion for

1 hourat room temperature and then assay was carried out. Absorbance was taken at 410 nm.

***Wash performance analysis*:**
The wash performance of lipase was examined for removal of oily stains from cloths cotton swatches (2*2 cm) were stained with coconut oil. The cloth piece was dried and soaked separately in tap water, tap water with lipase, detergent with lipase.

Statistical analysis:
All experiments were conducted in triplicate and results were represented with standard deviation calculated by Microsoft excel program.

RESULTS AND DISCUSSION

Lipasecharacterization:
In the present investigation, an attempt has been made to isolate and purify the Lipase from Erythrina Indica seeds which is an important constituent in detergents. Erythrina Indica seeds were partially purified by acetone ammonium sulphate fractionation, followed by dialysis, hydrophobic interaction chromatography and gel filtration chromatography. Partial purification showed cut off of unnecessary proteins and was evidenced on native PAGE.

Hydrophobic interaction chromatography:

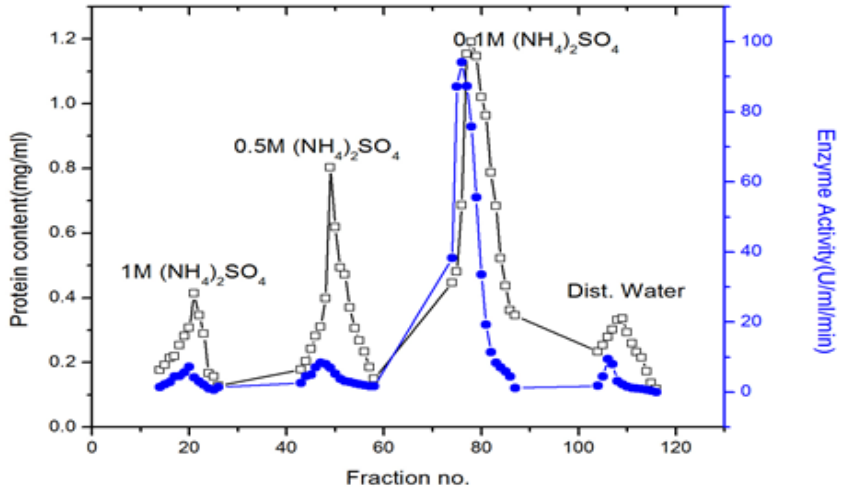

Fig 1: Elution Profile of Lipase by Hydrophobic interaction chromatography shows highest activity at 0.1 M $(NH_4)_2SO_4$.

Gel filtration chromatography:

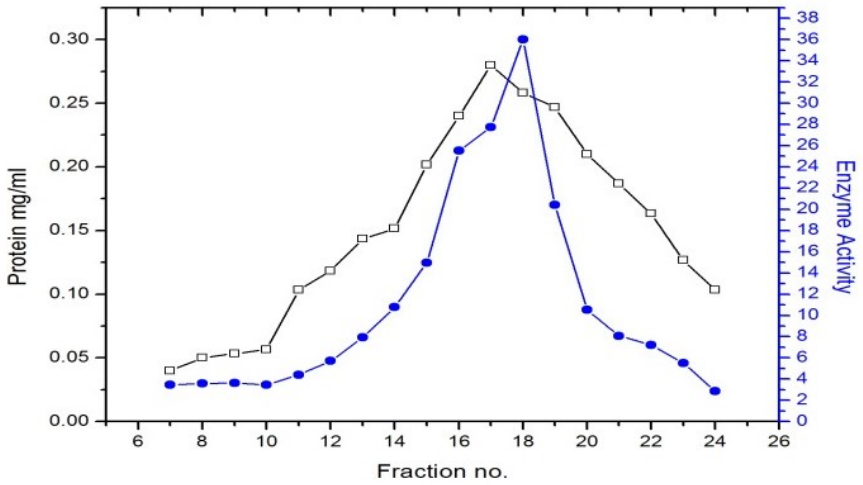

Fig 2: Elution Profile for gel filtration chromatography

Purification summary of lipase:

Stages of purification	Activity (units/ml/min)	Protein (mg/ml)	Specific activity	Fold Purification
Saline Extraction	120.7	60.00	2.011	1
Fraction A	111.1	39.40	2.8197	1.4021
Hydrophobic interaction chromatography	94.11	1.190	79.08	39.32
Gel filtration chromatography	36.00	0.280	128.57	63.94

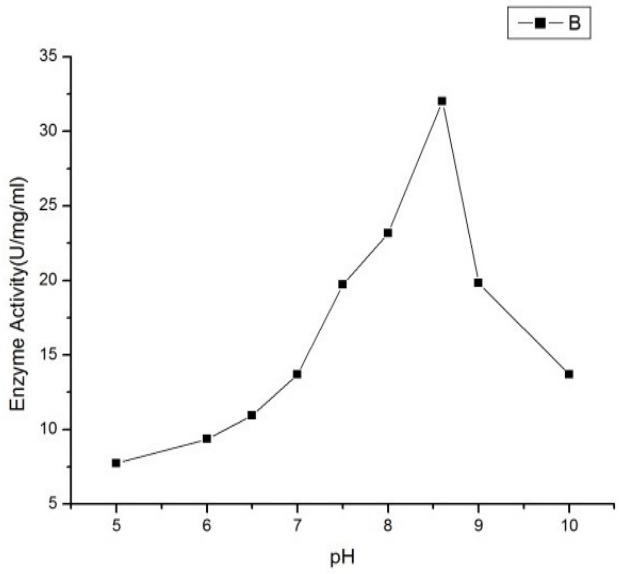

Fig 3:

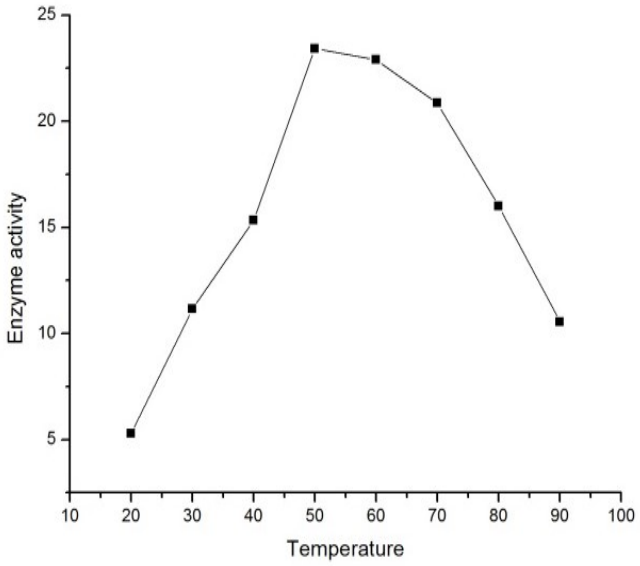

Fig 4:

Fig 3: Effect of p^H on Lipase activity from Erythrina Indica geminating seeds. Lipase assay performed at 50^0c and at various p^H values

Fig 4: Effect of temperature on activity of the Lipase of the Erythrina Indica Seeds .Lipase assay was performed at p^H 8 and at various temperatures.

From above table it concludes that after gel filtration chromatography it gives highest purification value 63.94 folds.

Effect of pH on lipase activity:

The enzyme activity increased with an initial increase in p^H and optimum activity noted at p^H8.6 suggesting alkaline nature of the enzyme. Further increased in p^H beyond optimum caused rapid decreased in enzyme activity (Fig. 3)

Effect of temperature on lipase activity:

The enzyme activity increased with an initial increase in temperature and optimum activity noted at 50^0c. Further increased in temperature beyond optimum caused rapid decreased in enzyme activity (Fig. 4)

Effect of Metal ions and inhibitors:

Metal ions as Ca^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Zn^{2+} and k^{+}at lower concentration shows highest activity on Lipase whereas at higher concentration the lipase activities were found to be inhibited. EDTA, Fe^{3+}, Cd^{2+}, $Hg2^{+}$ Co^{2+} inhibited the enzyme activity.

Effect of surfactant and commercial detergents:

Lipase exhibits highest activity in the presence of commercial detergentslike tide, wheel, rin, surf excel, Ariel, ujala

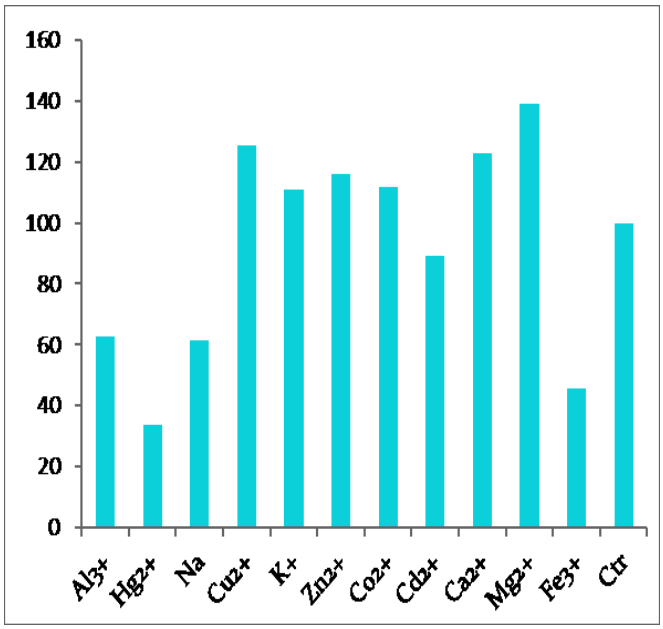

Fig 5:

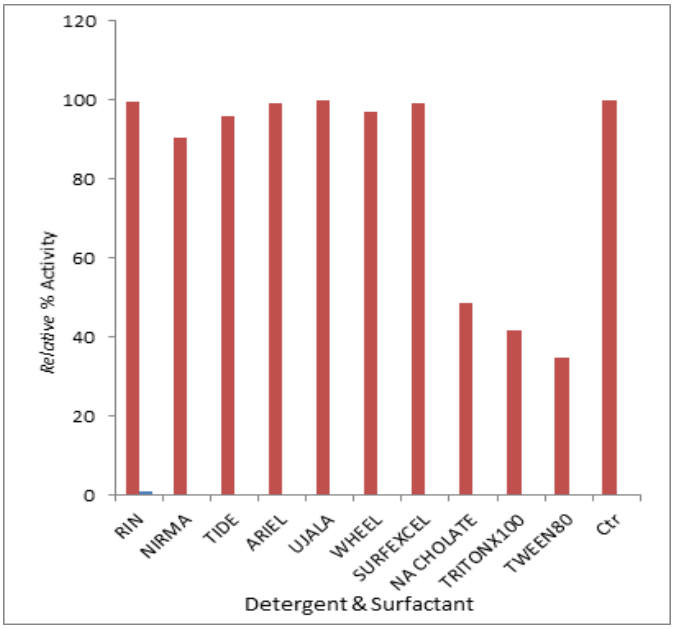

Fig 6:

Fig 5: Graph shows enzyme activity vs metal ions on Lipase.
Fig 6: Effect of surfactant and detergents on Lipase activity.

DISCUSSION

Lipase was isolated from Erythrina Indica seeds and purified. The results clearly indicate that Lipase of Erythrina Indica seeds are well suited for use as an additive in detergents formulation. Maximum activity at alkaline condition is compatible with harsh washing conditions. Maximum activity of lipase showed at temperature 50^0 c. the current thrusts for novel enzymes that tolerate oxidative stress makes the present lipase of high commercial value.

CONCLUSION

Lipases of Erythrina Indica seeds has so far shown the properties best suited for use in detergents and has superior properties to all existing Lipases. Lipase was isolated from Erythrina Indica seeds with saline and purified by dialysis, hydrophobic interaction chromatography and gel filtration. 63.94 folds purification was obtained. The enzyme shows maximum activity at p^H=8.6 and temperature at50^0c.Lipase of Erythrina Indica seeds has so far shown the properties best suited for use in detergents.Metal ions as Ca2+, Co2+, Cu2+, Mg2+, Zn2+ and k+ at lower concentration shows highest activity on Lipase whereas at higher concentration the lipase activities were found to be inhibited. EDTA, Fe3+, Cd2+, Hg2+ Co2+ inhibited the enzyme activity. Thus, the lipase was successfully isolated and its characterization was carried out.

Conflicts of interest: The authors stated that no conflicts of interest.

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