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PARTIAL PURIFICATION AND CHARACTERISATION OF LIPASE FROM *EUPATORIUM ODORATUM*

Deepa G. Muricken* and Neethu K. R.[1]

Department of Biochemistry*, Department of Biotechnology[1]

St. Mary's College, Thrissur.

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***Corresponding Author**
Dr. Deepa G. Muricken
Department of Biochemistry, St. Mary's College, Thrissur.

ABSTRACT

Eupatorium odoratum belongs to asteraceae family and this plant in grown widely in tropical climates. The present study utilizes this plant as a source of lipase enzyme which has wide range of applications in the food, detergent, and pharmaceutical sectors. Lipase enzyme is a naturally occurring enzyme found in the stomach and pancreatic juice. They function is to digests fats and lipids, helping to maintain correct gall bladder function. The rich foliage from this plant can be utilized as the enzyme source. Lipase was extracted from *Eupatorium odoratum* leaf and purified partially using ammonium sulphate precipitation, dialysis and ion exchange chromatography on DEAE cellulose column. The purified enzyme was characterized for its size, optimum temperature, pH and thermal stability. The purified enzyme had a specific activity of 0.0599 meq/min/g. The molecular weight was found to be 66 kDa as determined by SDS- PAGE. Enzyme showed a maximum activity at 60°C and at pH 7.0. Enzyme retained its 66.67% activity after two and half hours of incubation at 70°C.

KEYWORDS: lipase, *Eupatorium odoratum*, molecular weight, thermal stability, optimum temperature, pH.

INTRODUCTION

Lipases (EC.3.1.1.3, triacylglycerol acyl hydrolases) hydrolyze triacylglycerol at an oil-water interface to release free fatty acids and glycerol.Lipases are a subclass of the esterases. Lipases catalyze esterification, interesterification, acidolysis, alcoholysis and aminolysis in addition to the hydrolytic activity on triglycerides.[1] Lipases can be found in wide variety of plants, animals, insects and microorganisms. Plants lipases are mostly present in food reserve tissues. In animals, the lipases are found in pancreas, and on the surface of mucous cells of

the gastric mucosa. In insects, these enzymes are found mostly in plasma, salivary glands, muscles and fat bodies. Extracellular lipases have been isolated from many microorganisms including bacteria, yeast, and fungi. Lipases are widely used in detergent industries, food industries, pulp and paper industries, hydrolysis of fats and oils, and production of cosmetics and pharmaceuticals. Lipases are widely used in the processing of fats and oils, detergents and degreasing formulations, food processing, the synthesis of fine chemicals and pharmaceuticals, paper manufacture, and production of cosmetics, and pharmaceuticals.[2] Lipase can be used to accelerate the degradation of fatty waste[3] and polyurethane. Because of their metabolic effects, PUFAs are increasingly used as pharmaceuticals, neutraceuticals, and food additives.[4, 5] Many of the PUFAs are essential for normal synthesis of lipid membranes and prostaglandins. Microbial lipases are used to obtain PUFAs from animal and plant lipids such as menhaden oil, tuna oil, and borage oil. Free PUFAs and their mono- and diglycerides are subsequently used to produce a variety of pharmaceuticals including anticholesterolemics, antiinflammatories, and thrombolytics.[6, 7] In addition, lipases have been used for development of flavors in cheese ripening, bakery products, and beverages.[8, 9] Also, lipases are used to aid removal of fat from meat and fish products.[4]

Eupatorium odoratum is a fast-growing perennial and invasive weed native to South and Central America. It has been introduced into the tropical regions of Asia, Africa and other parts of the world. The extract of the leaf contain phenols, terpenoids, alkaloids and flavonoids.[10] The plant has been reported to possess wound healing, anti-microbial, and anti-inflammatory activities.[11, 12] But the presence of lipase in it is not yet reported. We made an attempt to purify and characterize lipase from it.

MATERIALS AND METHODS

Chemicals and consumables

Eupatorium odoratum leaves were collected from the suburbs of Thrissur, Kerala and was shade dried and used for the studies.

All chemicals used in general and reagents for protein purification and characterization are from Merck chemicals India. Coconut oil used as substrate is of food grade quality. DEAE cellulose from Bangalore Genei, India.

Preparation of *Eupatorium odoratum* leaf extract

The plant leaves were collected and shade dried at room temperature. The dried plant leaves were finely powdered using an electric grinder. 10 gms of leaf powder was mixed in 100 ml 0.1M Tris-HCl buffer of pH 7 and stirred for 8-9 hours using a magnetic stirrer. It is then filtered through a muslin cloth and centrifuged at 10000 rpm for 10 minutes. Then the supernatant was collected and used as the crude extract.

Lipase activity of *Eupatorium odoratum* leaf extracts (plate method)

Lipase activity in *Eupatorium odoratum* leaf extract was analyzed using a simple method.[13] 0.1 % phenol red and 10 mM calcium chloride were mixed in 100 ml distilled water. The pH was adjusted to 7.3- 7.4 using sodium hydroxide. 2% agar was added to the media and mixed thoroughly. The media was poured into petriplates and allowed to solidify. Wells were punched on the agar and the leaf extract was poured to the wells. The plates were incubated at 37^0C overnight and the lipase activity was observed as yellow zone.

Lipase purification

100 ml 0.1 M Tris-HCl buffer extract of *Eupatorium odoratum* leaf were subjected to 80% ammonium sulfate precipitation in cold. After the precipitation the extract was centrifuged at 10,000 rpm for 10 minutes at 4^oC. The pellet was collected and dissolved in 5 ml distilled water. This mixture was dialyzed in water for 3-4 times and finally in 0.1 M Tris-HCl buffer pH 8.0. Further purification was carried out using ion exchange chromatography. The DEAE cellulose column was equilibrated with buffer (0.1 M Tris- HCl pH 8). 2 ml of ammonium sulfate precipitated sample was loaded to the equilibrated DEAE cellulose column.The enzyme was eluted by increasing the ionic strength of the elution buffer (0.1 M Tris-HCl pH 8.0 containing 0.1- 0.5 moles of NaCl). A step gradient using 0.1 to 0.5 M NaCl was performed. The eluents were collected in test tubes as 2ml fractions. Then lipase purification profile was plotted. The contents of the tubes of each peak were pooled separately and dialyzed against water. After dialysis the samples were concentrated. Lipase assay was carried out using plate method to find out which fraction contains the lipase enzyme. The sample which contains lipase was used for further experiments.

Molecular weight determination

After purification process the molecular weight determination was carried out using SDS-Poly acrylamide gel electrophoresis (SDS-PAGE). SDS–PAGE at alkaline pH (8.3) was done according to the method of Laemmli in a discontinuous buffer system.[14] After

electrophoretic separation, silver staining is used to detect proteins and molecular weight was analyzed by comparing with medium range protein marker.

Specific activity determination[15, 16]

Vegetable oil is used as the substrate to determine the specific activity of partially purified Lipase.2 ml of any clear vegetable oil was neutralized to pH 7.0. An emulsion was prepared by stirring it with 25 ml of water and100 mg bile salts. This substrate emulsion is used in assaying the activity of lipase. To 20 ml of substrate added 5ml of 50 Mm phosphate buffer (pH 7.0) at 35°C. The pH was noted and adjusted to 7.0. After adding the enzyme (0.5 ml), immediately record the pH and set the timer on. Let it be pH at zero time. At frequent intervals as the pH drops by about 0.2 units add 0.1 N NaOH to bring pH to the initial value. Continue the titration for 30-60 minutes. Note the volume of alkali consumed.

$$\text{Activity meq/min/g sample} = \frac{\text{Volume of alkali consumed} \times \text{Strength of alkali}}{\text{Wt. of sample in g} \times \text{Time in min}}$$

The weight of the sample (protein) was determined using biuret method.

Optimum pH

Optimum pH of lipase was determined. The activity of enzyme was assayed as mentioned earlier. To the substrate added buffers of different pH (6, 6.5, 7, 7.5, 8, 9.2, 9.6 and 10). Adjusted the initial pH of the reaction mixture as the pH of the buffer added. Add enzyme extract and immediately record the initial pH. 0.1 N NaOH was added to bring pH to the initial value during the titration period. A graph was plotted with pH on X axis and volume of alkali consumed on Y axis. Optimum pH was determined from the graph.

Optimum temperature

20 ml of oil emulsion was poured into a series of beakers. To it added phosphate buffer of pH 7 and enzyme extract. The pH was adjusted to 7.0. Then contents of each beaker were incubated in different temperatures (10^0C, 20^0C, 37^0C, 45^0C, 60^0C, 70^0C, 80^0C, 90^0C, and 100^0C). Note the pH of the mixture after 30 minutes of incubation. Bring the pH of the mixture to 7 by adding 0.1N NaOH. The temperature corresponding to the sample which consumed more amount of NaOH will be the optimum temperature of lipase.

Thermal stability of lipase

Thermal stability was initially assayed at 100^0C and later at 70 ^{0}C. Mixture of enzyme and phosphate buffer of pH 7 was incubated at 70 ^{0}C for different time periods. After incubation,

the reaction mixture containing enzyme was immediately cooled on ice. It was added to the substrate (pH 7) and noted the pH. NaOH was added to bring pH to 7 when there is reduction in pH during incubation. Continue the titration for 30 minutes. Check the difference in alkali consumption of each sample. Graph was plotted to determine the thermal stability.

RESULTS AND DISCUSSION

Lipase activity of *Eupatorium odoratum*

The *Eupatorium odoratum* leaf extracts were analyzed for the lipase activity. Preliminary studies shown that this leaf extract has lipase enzyme. In order to utilize this enzyme in various fields of industry we have to purify and characterize this enzyme. After the initial screening for lipase using phenol red in agar plates, color changes from pink to yellow denoted the presence of lipase. Here the indicator property of phenol red is being utilized. Phenol red is red in alkaline pH and yellow at acid pH. The small amount of free fatty acids released after lipase digestion of the oil sample lead to slight reduction in the pH. This denoted that the leaf extract have oil degrading activity or lipase activity. This is the first report for the presence of lipase from *Eupatorium odoratum.*

Partial purification of lipase

Crude enzyme extract was prepared in 0.1M Tris-HCl buffer. Purification of lipase from *Eupatorium odoratum* was carried out using 80 % ammonium sulphate precipitation followed by dialysis and ion exchange chromatography. After ion exchange chromatography in DEAE cellulose column, absorbance of the eluted sample was read at 280 nm and 2mL fractions were collected. The graph given below shows the absorbance of each fraction (fig. 1). Five peaks were obtained during the purification process. The peaks were pooled separately and concentrated to analyze the sample for the presence of lipase enzyme (fig. 2). All the peaks were screened for lipase activity and the plate containing peak 1 fractions showed the lipase activity. The fractions of peak 1 was concentrated and used for further studies.

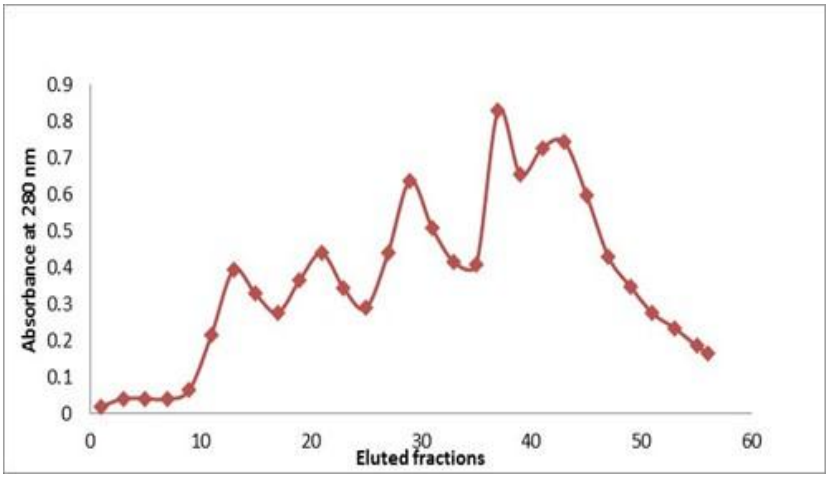

Fig 1: Purification profile of lipase on DEAE cellulose ion exchange Chromatography.

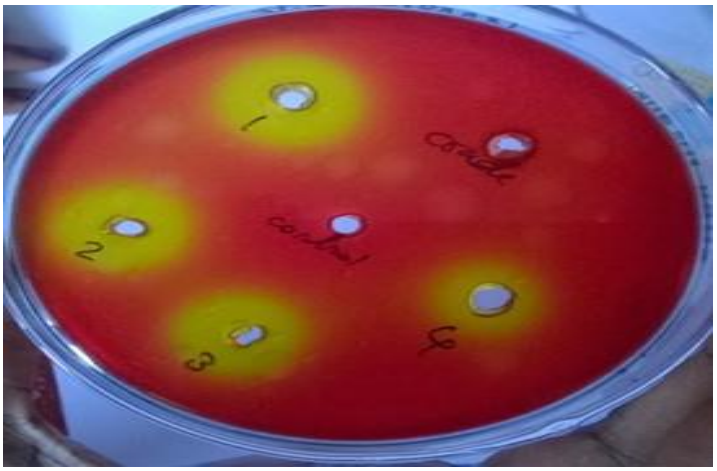

Fig 2: Lipase assay for fractions of peak 1.

This peak showed the lipase activity conforming the presence of lipase in peak 1. The buffer is used s control and is not showing any activity. The crude enzyme extract showed very slight activity. 100 µl of samples are used for the assay.

Specific activity of lipase

The specific activities of enzymes are determined during purification stages. During the purification process specific activity increases. The specific activity of lipase was determined after ion exchange chromatography. The mixture of substrate and enzyme was titrated with 0.1N NaOH for 30 minutes. NaOH consumption of the sample was found to be 220 µl. NaOH consumption increases as lipase activity increases due to the formation of free fatty acids. The protein concentration was determined using biuret method (fig. 4) was found to be 12.25 mg/ ml. The specific activity was found to be ± 59.9 $\times 10^{-3}$meq/min/g.

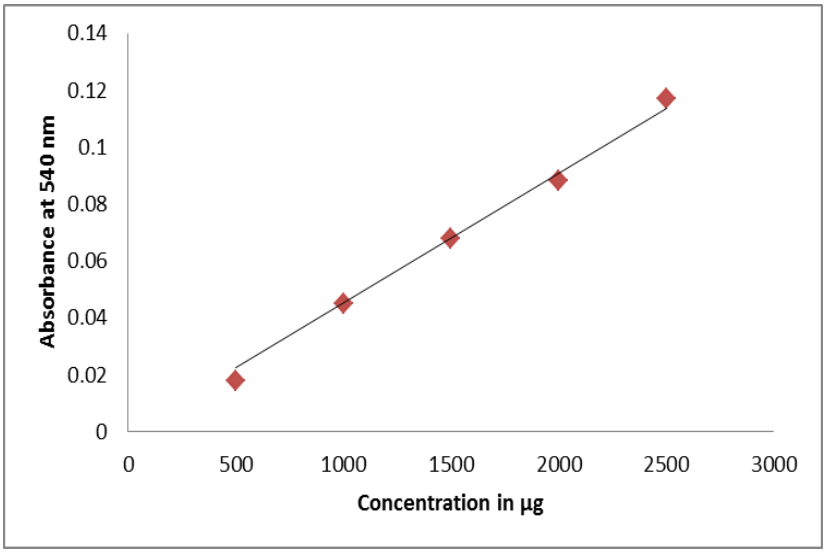

Fig 3: Standard graph using BSA for biuret method.

Determination of molecular weight

The enzyme was characterized for its molecular weight and purity. The molecular weight was determined using SDS-PAGE electrophoresis. The purified peak fractions of peaks 1 to 5 were concentrated separately and dialyzed. The concentrated samples were loaded on to SDS- PAGE to check for the purity and also to analyze the molecular weight of the protein. Since the purified protein was low in concentration silver staining was performed for the visualization of proteins.

The molecular weight of lipase in plants ranges between 19000 to 270000 Da.[17] Our protein is also giving a molecular weight within the range. The samples (concentrated fractions of 5 peaks) were loaded on to SDS-PAGE. The protein band corresponding to the molecular weight of approximately 35 KDa was present in every samples but did not showed lipase activity in assays.The concentrated fractions of peak 1 was found to possess lipase activity. The band in the sample corresponding to molecular weight of 66 KDa was more prominant in sample 1. Silver staining was carried out to obtain clear bands (fig;4). From the gel images, it was seen that the approximate molecular weight of the partially purified lipase enzyme is 66 KDa.

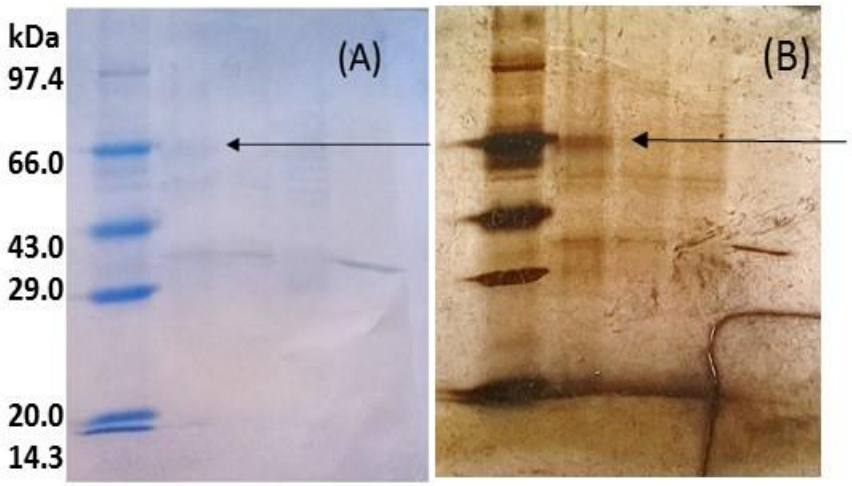

Figure 4. SDS PAGE profile of lipase purification. (A) Coomassie staining of purified samples. (B) Silver staining of purified samples.

Optimum pH

Usually lipases are having optimum pH in neutral to alkaline range. Jatropha lipase was showing optimum pH at pH 9.0. Acid lipases were also isolated and characterised.[18, 19] Therefore we analysed pH optimum in the range pH 6- 10 using different buffers. The activity was analysed using lipase assay described earlier. The purified enzyme had a pH optimum of 7. Below and above the optimum pH, enzyme activity is lower (fig 5). The enzyme gave better result at alkaline pH than acidic pH. Hydrogen ions influence the enzyme activity by altering the ionic charges on the amino acids (particularly the active site), substrate, ES complex etc.

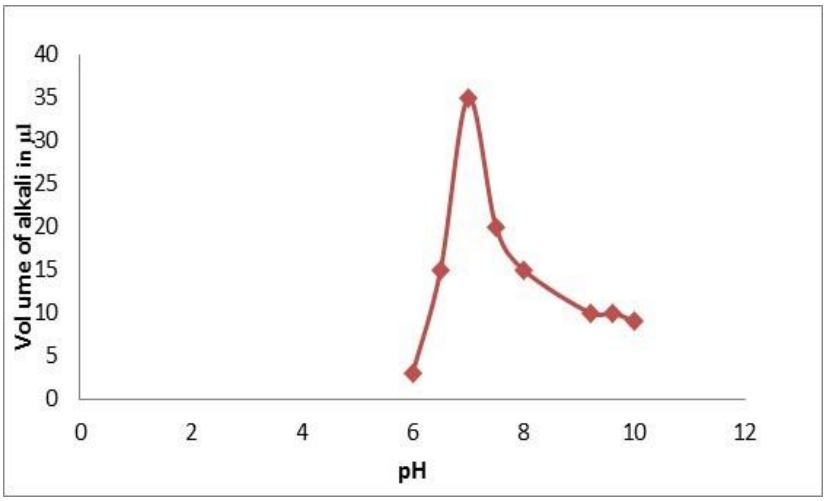

Fig 5. Effect of pH on lipase activity. Data points are average of three determinations.

Optimum temperature

Optimum temperature is the temperature at which we get maximum velocity for the enzyme-substrate reaction. Most of the lipases are having optimum temperature in the range 37-45^{0}C. Alkaline lipase from a thermophilic *Bacillus* showed an optimum temperature at 50°C.[20] Lipases vary in their optimum temperature. Lipase from rice bran showed maximum activity at 80°C.[21] Temperature studies for *Eupatorium odoratum* lipase were done at pH 7 where we got maximum activity for our enzyme. Purified enzyme was incubated at different temperatues before performing the assay. The optimum temperature was found as 60 ^{0}C. Activity of the enzyme increases with increase in temperature upto 60 ^{0}C and then declines (fig: 6). The number of successful collision between molecules increases with increase in temperature. That is why the activity increases with increase in temperature. But very high temperatures denatures enzymes.

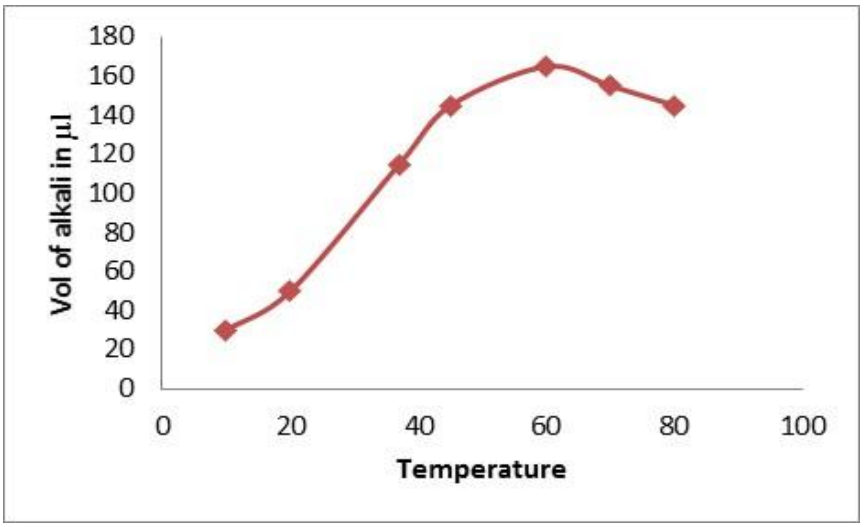

Fig 6. Effect temperature on enzyme activity.The optimum temperature was observed at 60^{0}C. Data points are average of three determinations.

Thermal Stability of Lipase

Usually lipases are not stable at high temperature. Exception of this is rice bran lipase.[21] We analysed our lipase for its thermal stability.The sample was incubated at 100 ^{0}C initially and the assay was performed but our enzyme was not stable at this temperature and activity was lost. Therefore we performed further assays at 70^{0}C for different time intervals and lipase activity was assayed. The enzyme is completely stable at 70^{0}C for half an hour. After that the activity decreases slightly. But the enzyme retained its 66.67% activity even after two and half hours (fig: 7) of incubation at 70 ^{0}C.

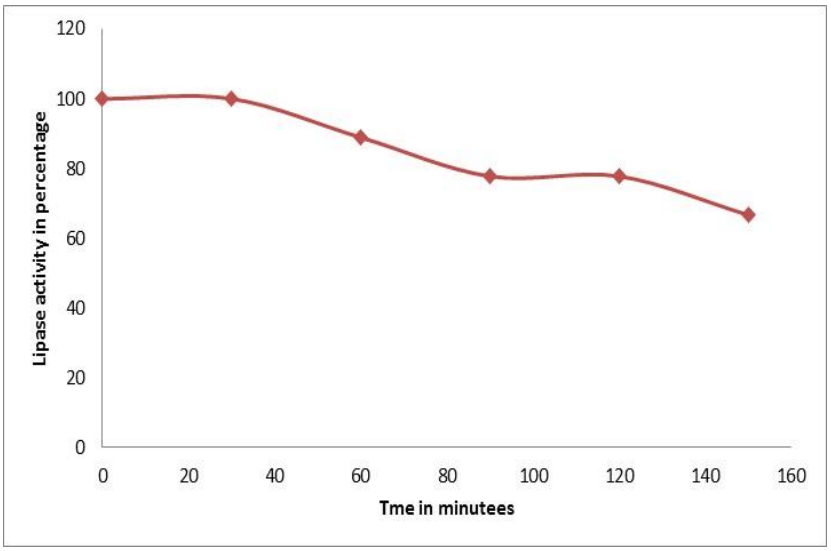

Fig 7. Activity of lipase at 90^0C for different incubation periods. . Data points are average of three determinations.

CONCLUSIONS

Lipases are not only used in chemical, pharmaceutical, food and detergent industries but also used to perform biotransformation. Our study aimed at purification and characterization of the purified lipase from *E. odoratum.* We determined the molecular weight and specific activity of purified lipase. Molecular weight analyzed from SDS PAGE was 66 kDa and specific activity was 59.9×10^{-3} meq/ min/g. The enzyme acted maximally at 60^0C and had an optimum pH of 7. It also retained most of its activity at 70^0C. Due to its optimum temperature at 60ºC and neutral optimum pH this enzyme can be applied in many industries. Lipase is one among t^{0}he most versatile enzyme used in industry; our enzyme can also be added as a new candidate in this field.

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