Purification and Characterization of Esterase Enzyme from *Aspergillus Versicolor*

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ABSTRACT: *Esterase is a biotechnologically important enzyme as it hydrolyzes water-soluble short-chain fatty acid esters. We tried to isolate and purify the esterase enzyme from Aspergillus versicolor in this investigation. The enzyme was purified with ammonium sulfate precipitation, dialysis, and column chromatography. The enzyme was salted out, with maximum specific activity at 60 to 70 percent of saturation during precipitation. The column chromatography was performed with Sephadex G-75 to purify the esterase from Aspergillus versicolor and was able to achieve the purification fold of 6.9 nM. The partially purified enzyme was analyzed in SDS-PAGE and showed a single 32-kDa band. The partially purified esterase enzyme was checked for its optimum conditions for maximal enzyme activity. This enzyme has a huge industrial potential which makes a significant contribution to eco-friendly approaches such as textile, food, and agrochemical industries as well as for bioremediation.*

KEYWORDS: *Esterase; Partial purification; Sephadex G-75; SDS-PAGE; Characterization.*

INTRODUCTION

Enzymes are biocatalysts that catalyze biological and metabolic reactions [1, 2]. The global market for enzymes is expected to grow at a rapid rate in recent years [3-6]. Microorganisms can be cultivated in huge quantities in a limited time as well as genetic changes in bacterial cells may be made to improve enzyme synthesis. They are the primary source of enzymes [7-11]. Furthermore, because of their dynamic and consistent nature, microbial enzymes have more importance when compared with enzymes from animals as well as plants [12]. Lipolytic enzymes (phosphor lipases, lipases, and esterase) are important enzymes in the hydrolysis of peptides, fatty acid esters, and acylglycerols.

Extremophile enzymes appear to be the most promising for industrial use [13]. According to the Enzyme Commission classification [14], lipases and esterase are hydrolase enzymes that catalyze the production and hydrolysis of ester bonds. The biodegradation of polyurethane (PU) is mediated by two well-known enzymes: esterase and lipase. Esterase is found in abundance in living things and can be isolated from bacteria, plants, and mammals [15,16]. Esterase [17] catalyzes the breakdown of fat as well as the formation of fatty acid esters. Enantioselectivity, as well as area specificity, is desirable features in microbial esterase,

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and they have a lot of industrial potential [17, 18]. Esterase is becoming increasingly important in a variety of domains, including quantification, targeted synthesis, manufacturing, and purification, and is produced by experts from microbes, animals, as well as plants [19,20]. Esterase is used in the dairy sector, as well as in the beverage industry to make wine, beer, alcohol also fruit juices. Esterase is utilized as a catalyst in the transesterification process to convert low-value fats as well as oils to higher-value products.

Esterase has a wide range of biotechnological uses, including laundry detergent additives as well as stereo-specific biocatalysts in pharmaceutical manufacture [42]. But most industrial applications need extreme conditions, which can cause enzyme inactivation. Novel esterase having improved catalytic efficiency as well as particular characteristics appropriate for certain reaction circumstances are in high demand in this regard [22]. Halotolerant bacteria, which can survive in saline settings, are attractive candidates for finding new enzymes among the extremophiles [21, 22]. In combination with protease and amylase, esterase has a wide range of uses. The current study focuses on the isolation of esterase and its characterization from *Aspergillus versicolor*.

EXPERIMENTAL SCTION

Sample Collection:

The soil samples were collected from a plastic waste disposal site near Kozhikode, Kerala- state, India for isolating the microbe. Samples were collected in sterile polyethylene bags. Large particles (gravel, wood bits, plastic, and paper bits) were removed from the soil sample by sieving. Polyurethane films were prepared by mixing 0.5 percent polyurethane within tetra hydrofuran and pouring it onto Petri plates. The plates were then kept in the dark at room temperature for 6 months, after which the films were removed from the plates, washed with sterilized distilled water and buried in soil for six months, and then a large soil pot filled with mineral salt medium (Dispotassium phosphate 0.5g, Monopotassium phosphate 0.04g, Sodium chloride 0.1g, Calcium chloride dehydrate 0.002g, Ammonium sulfate 0.2g, magnesium sulfate heptahydrate 0.02g, ferrous sulfate 0.001g and 2g glucose were mixed with this solution. This solution was autoclaved and cooled before adding to the soil pot. After six months, the PU film was removed from the soil pot and washed with distilled water to remove the impurities. The washed polyurethane (PU) films were shifted to sabouraud dextrose agar (SDA) containing petriplate and incubated at 37°C for a week.

Isolation and identification of selected microbe

After the incubation, microbes grown on the SDA plates were isolated and used for screening esterase production. The maximum esterase-producing microbe was identified up to the species level by molecular sequencing. The selected microbe was used for further study.

Production medium

The isolated fungus was grown in a production medium to produce the maximal enzyme. The production medium consists of peptone (5.0 g/L), $C_6H_{12}O_6$ (1 g/L), yeast extract (1 g/L), NaCl (0.5 g/L), Mono potassium phosphate (1 g/L), DDL4, (0.5 g/L), and dipotassium phosphate (1 g/L) maintained at pH 8.0. The isolate was cultured in conical flasks (250 mL) with a working capacity of 100 mL and incubated for 24 hours in an incubator under static conditions at 37°C. After 24 hours of incubation, the extracellular enzyme was recovered in a liquid fraction by centrifugation (Eppendorf Centrifuge 5425 Model) for 5 minutes with a relative centrifugal force of 10×10^3.

Enzyme assay

The hydrolysis of P-nitrophenyl butyrate was used to determine the esterase activity [18]. For this, 100 µl of the sample was mixed with 900 µl of substrate solution containing 113 µM of p-nitrophenyl butyrate. The reaction was carried out for 30 minutes at 30°C and 410 nm; the released p-nitrophenol was detected. One esterase unit was described as the quantity of enzyme that released 1 µmol of p-nitrophenol per minute [22-24].

Partial purification of enzyme

Ammonium sulfate precipitation

The culture supernatant containing extracellular esterase was analyzed for pH, and total proteins and subjected to ammonium sulfate precipitation. Pre-weighed ammonium sulfate was continuously added to the culture supernatant to reach 30, 50, and 80% saturation. The sample contained ammonium sulfate lumps broken, and salt was gradually added. To ensure complete equilibration between the dissolved and aggregated proteins, stirring was continued

for a further two hours after the ammonium sulfate was completely dissolved. Each precipitate fraction was separated through centrifugation at 10,000 rpm for 10 min. to pellet out the precipitated protein. At a low volume, the precipitates were reconstituted in 0.1 M Tris-HCl buffer (pH 8.0). Ammonium sulfate will present in large amounts in the dissolved precipitate and it has to remove either gel-filtration/de-salting columns or dialysis using the same buffer. The Precipitated sample was dialyzed in 0.1 M Tris-HCl buffer (pH 8.0) twice for 6–8 hours on the fractions.

Size-exclusion chromatography

The dialyzed material was applied to a Sephadex G-75 column (2 x 20 cm) pre-equilibrated with 0.1 M Tris-HCl buffer at a flow rate of 0.5 mL/min. The test fractions' were analyzed in UV-Vis Spectrophotometer (Systronics) by measuring the absorbance at 280 nm. The enzyme activity in the fractions was calculated by using the *Immanuel et al.* approach [20], and the protein content was calculated using Bradford's method [22,24]. The samples that consistently demonstrated the presence of protein and enzyme activity were combined, lyophilized, and dissolved in 0.1 M Tris-HCl (pH 8.0) for further analysis. The enzyme activity was determined by measuring the esterase activity in each fraction (100 µL) [20]. The positive fractions were combined, lyophilized, and mixed with the minimum volume of buffer (Tris – HCl, pH 8.0). Manually obtained protein peaks were lyophilized, and diluted in a small amount of Tris-HCl buffer (pH - 8.0), and the esterase activity, as well as the protein content, were measured.

Polyacrylamide gel electrophoresis

Electrophoresis in the presence of a detergent Sodium dodecyl-sulfate (SDS) and a reducing agent mercaptoethanol was used to measure the molecular weight of proteins [25-27].

Assembling the plates

Glass plates are kept on a smooth surface and two spacers of the same thickness were inserted along the length of the rectangular plate to hold the longer glass plate in place. The shorter glass plate was then placed on the spacer, with the spacer and the glass plate's bottom aligned. In the casting stand, the plates were kept ready for casting the gel.

Casting the gel

Separating gel monomer was made by combining 30% acrylamide, 1.5 M Tris buffer, 10% Ammonium persulphate (APS) solution, and 10% Sodium dodecyl sulfate, with TEMED together. The Comb was assembled on the plates, and then APS with TEMED was added to the solution and immediately poured onto the plates. Then the gel was overlaid with 1 mL of an equal volume of methanol and water. The gel was allowed to polymerize for 45 to 1hr. The excess water was evacuated using strips of filter paper after complete polymerization. A stacking gel was made by combining 30% acrylamide, 0.5 M Tris buffer, 10% SDS, 10% APS, and TEMED and pouring it into the plates until it filled the sandwich and kept for 30 minutes for the gel to polymerize. The plates were then placed in the buffer chamber, and filled with running buffer.

Preparation and loading of samples

The sample buffer was combined with the protein standard and samples. The sample was then loaded into the wells after boiling for 5 min in a water bath. The well was rinsed with the buffer for few times before loading the sample and loaded with the prepared sample. The gel was made to run at a constant current of 30 mA with electrical leads linked to an appropriate power pack.

Staining and Distaining of the gel

After the run, the gel was removed from the glass plates; one end of the gel was cut to orientate the gel placed on a staining solution, and stained for 1– 2 h in a gel rocker. Then it was placed in a destaining solution. The gel was destained for few times until the protein bands were visible. The molecular weight of the esterase sample was estimated by comparing the molecular standard.

Characterization of purified esterase from aspergillus versicolor

pH optimum and pH stability

The partially purified enzyme was tested for the effect of pH by determining the enzyme activity at various pH ranges from 4 to 8. The enzyme fractions (100 µg) were pre-incubated in different buffers, citrate, phosphate, Tris Hcl, and carbonate-bicarbonate buffers with pH 4.0 – 8.0, respectively, for 15 min at 40˚C. After incubation, enzyme activity was tested in all the pH-maintained samples under standard assay conditions [28-32].

Optimum Temperature and Temperature Stability

At temperatures ranging from 30 to 70 °C, the influence of temperature on the activity of the partially purified esterase was examined. The enzyme was diluted appropriately and treated with the substrate (1-naphthyl acetate) for 15 minutes at various temperatures. The amount of 1-naphthol emitted was calculated using a calorimeter.

By incubating the partially purified esterase for 15 minutes at various temperatures (30°C to 70 °C), the temperature stability of the enzyme was calculated. The samples were rapidly cooled as well as analyzed at the ideal temperature. A calorimeter was used to determine the amount of 1-naphthol released [27-31].

Substrate Specificity

Substrate specificity was measured by testing the enzyme activity with different substrates such as P-nitrophenyl palmitate (p-NPP), p-nitrophenyl butyrate (p-NPB), p-nitrophenyl acetate (p-NPA), p-nitrophenyl formate (p-NPF), and p-nitrophenyl octanoate (p-NPO) [31].

Impact of organic solvents, activators, and inhibitors

Ethylenediamine tetraacetic acid (EDTA) salts, Sodium dodecyl sulfate (SDS), as well as phenyl methyl sulfonyl fluoride (PMSF), were tested to determine the enzyme activity at concentrations of 5 mM to screen for potential inhibitors and activators. Regular enzyme tests also included 1% and 2% concentrations of the detergents Tween 20, Tween 80, and Triton X-100. By adding another 25% organic solvent to the enzyme experiment, the capability of purified esterase enhances the hydrolysis of p-nitrophenyl butyrate within a water-restricted atmosphere. The residual activity was measured in all of these studies and compared to the control without any additions [31-33].

Effect of salinity

Various sodium chloride (NaCl) concentrations (0 to 5M) were analyzed to calculate the effect of salinity on enzyme activity. Enzymes were treated with glycine ($C_2H_5NO_2$) at 50 mM as well as Sodium hydroxide buffer at different Sodium chloride concentrations at 70 ° C for 3 hours [31].

Fig. 1: Microscopic view of lactophenol stained fungal strain.

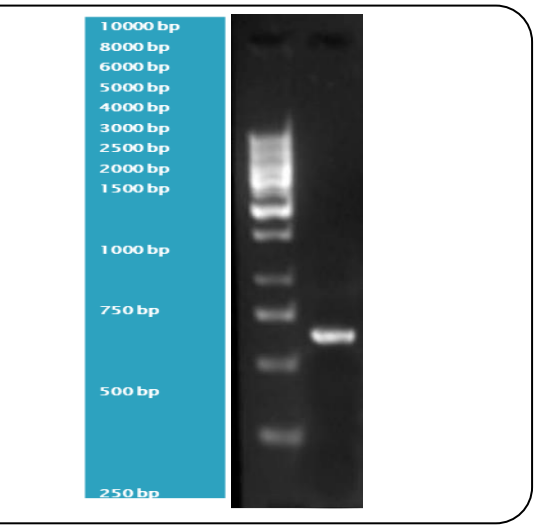

Fig. 2: RAPD PCRs with universal fungal primers.

RESULTS AND DISCUSSION

Isolation of microbe

The isolated fungi from garbage soil samples which have the potential to degrade the ester-based Polyurethane were identified by sequencing. The DNA was isolated from the fungal sample and it was amplified in the polymerase chain reaction. A comparison of the amplified sequence with the sequence already available in the GenBank was done and the results showed high sequence similarity (98%) with *Aspergillus versicolor VS2*. The Sequence was submitted to GenBank and the accession number was OL614816 (Fig. 2).

Enzyme assay

Precipitation of ammonium sulphate

The culture filtrate was subjected to ammonium sulfate precipitation and saturated for 30, 50, and 80%

Table. 1: Purification steps with activity, protein content; specific activity, recovery and purification fold in each step.

Stages of Purification	Activity of Total Esterase (U)	Total Protein Content (mg)	Specific Activity (U/mg)	Fold purification (nM)	Recovery (%)
Crude esterase	1182	52	22.7	1	100%
Ammonium sulphate precipitation	1096	16	68.5	3.12	93%
Dialysis	1006	12	83.3	3.47	84%
Sephadex G-75 chromatography	316	21	150	6.9	25%

and protein content and enzyme activity were assessed in all the fractions. In *the culture supernatant of Aspergillus versicolor*, the initial enzyme activity of crude esterase was *found to be* 1.18 U/mL with a protein content of 0.19 mg/mL. After ammonium sulfate precipitation, the specific activity and the purification fold of the enzyme were found to be 68.5 U/mg and 3.12 nM respectively (Table 1). This fraction precipitate was dialyzed overnight against the same buffer (Tris – HCl, pH 8.0) about 2 to 3 times and checked for protein and enzyme activity and found to be 12 mg/mL and 1006 U/mL respectively. This fraction precipitate was dialyzed overnight twice or three times against the same buffer (Tris-HCl, pH 8.0), and protein and enzyme activity results showed 12 mg/mL with 1006 U/mL respectively and yield 3.47-fold purification of the enzyme with 84% recovery.

Size exclusion chromatography

The dialysate sample from the above step was further purified by applying column chromatography by packing the column with Sephadex G-75 (2 x 20 cm) and eluted with 0.1M Tris-HCl buffer of pH – 8.0. 2 mL, fractions were collected and each fraction was examined for total protein concentration and enzyme activity (Fig. 3). After Sephadex G-75 column chromatography, the specific activity was found to increase to 150 U/mg and the fold purification of the enzyme was identified as 6.9 nM.

SDS-PAGE

The samples purified by using ammonium sulfate precipitation, dialysis, and gel permeation chromatography were loaded in SDS-PAGE to observe the band separation. In each step of purification, the appearance of bands was reduced and finally, the sample eluted from gel permeation chromatography indicates a single band in SDS-PAGE indicating the monomer protein. The molecular weight of the enzyme was found

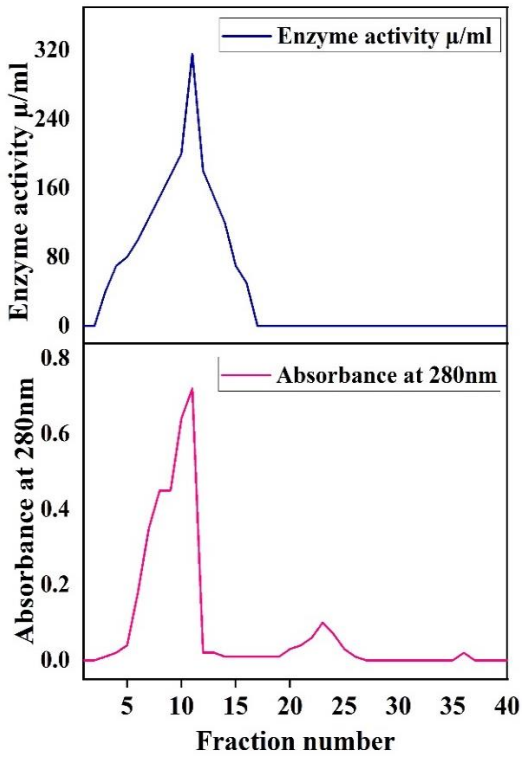

Fig. 3: The figure shows the fractionation pattern of a partially purified esterase enzyme by gel filtration chromatography.

to be 32 KDa identified by comparing it with the standard protein marker (Fig. 4).

The result is similar to another investigation where the molecular weight of pure prbA esterase isolated from *E. cloacae* EM was determined as 55 kDa [34]. In another study, the molecular mass of purified esterase isolated from *Aspergillus westerdijkiae* was determined to be 32 kDa [5, 35].

Purified esterase characterization

pH Optimum and pH Stability

The impact of pH was investigated at 40°C with pH ranging from 4 to 8. The enzyme had a pH range of

4.0 to 8.0, with an optimum pH of 6.0 to 7.0. The highest activity (560 U/mL) at pH 7.0 was considered to be 100% and optimum. It retained 90% (526 U/ml) of its activity even at pH 8.0, indicating its alkalinity stability.

At pH 4.5 and 5.0, the activity was found to be 453 U/mL and 510 U/mL, respectively. It was active at pH 4.0 (acidic) and it demonstrated a steady decrease in activity with 392 U/mL. The enzyme was found to have high stability at very high pH ranges, which is important in the detergent industry (Fig. 5).

The optimal pH for most of the esterase enzymes extracted from animals and plants animals was found to be between 7.0 and 9.0. *Sorghum, barley*, and *Mucuna seeds* had an ideal pH of 7.0 [27,38,39], while *finger millet, Synadenium grantii,* with *Mucuna seeds*, had an optimum pH of 7.5 [24, 35-38]. The pH value between 4.0 and 9.0 was found to be optimum for a majority of plant esterases.

Temperature optimum and stability

The purified esterase was analyzed for optimum temperature conditions by incubating at different temperatures. The optimum temperature for enzyme activity was studied by keeping the enzyme (pH 7) from 30˚C to 70˚C. The highest esterase activity observed at 45˚C was (1182 U/mL) (Fig. 6). The number of units obtained at 45˚C was taken as a basis for comparing the activities at other temperatures. The relative activity was 97 % (1098 U/mL) at 45 ℃ and 89% (1059 U/mL) at 40 ℃, respectively. It demonstrated a substantial decrease at 60°C, with just 30% (400 U/mL) activity remaining. Enzyme activity was found to decrease at low temperatures and it was found to be active even at higher temperatures.

In another study, purified esterase from *Caesalpinia seed* was found to be most active at 45℃ and stable up to 60℃. The plant esterases with optimum temperatures in the same range were seen in *Synadenium grantii, finger millet, barley, M. pruriens* as well as *Cucurbita pepo* [27, 38, 39].

Effects of Various Substrates on Purified Esterase Activity

The substrate specificity of the esterase enzyme was determined by incubating with different substrates. P-nitrophenyl butyrate (p-NPB) had the highest esterase activity of 42.85 U/mg, while with p-nitrophenyl acetate (*p*-NPA) the enzyme activity was reduced to 13.34 U/mg and found to be the lowest. Enzymes are typically quite selective in terms of the reactions they catalyze as well as the substrates they catalyze. This specificity is due to complementary structure, charge, as well as

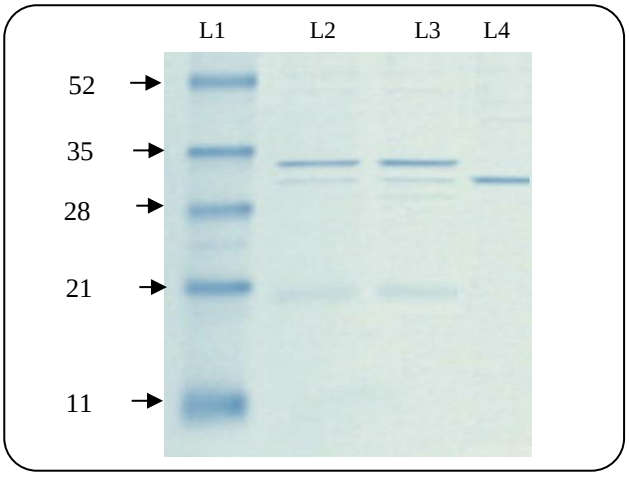

Fig. 4: SDS-PAGE picture with samples and Protein marker.

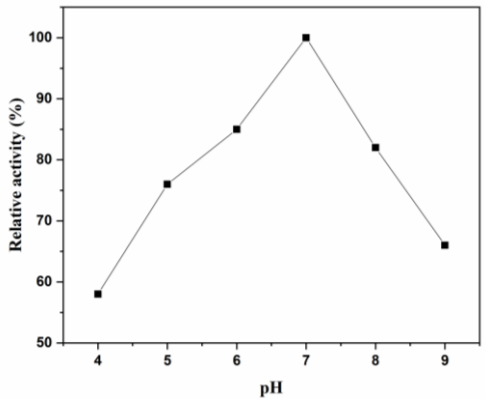

Fig. 5: Effect of pH on partially purified enzyme.

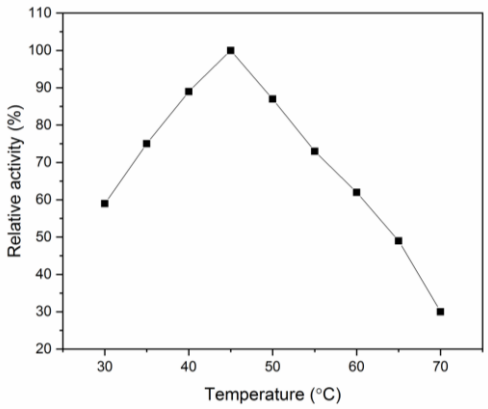

Fig. 6: Effect of temperature on partially purified enzyme isolated from Aspergillus versicolor.

Table 2: Effect of substrates.

Substrate (20mM)	Specific Activity
p-NPP	15.65±0.96
p-NPA	13.34±0.51
p-NPB	42.85±0.36
p-NPF	32.98±0.38
P-NPO	17.16±0.23

Table 3: The activity of esterase is affected by activators, inhibitors, and organic solvents.

Substance	Concentration	Relative activity (%)	Concentration	Relative activity (%)
Control	–	100	–	100
Mercury (II) chloride	5 mM	0	10 mM	0
Magnesium sulfate heptahydrate	5 mM	97	10 mM	61
Sodium chloride	5 mM	123	10 mM	94
Potassium chloride	5 mM	120	10 mM	102
$ZnSO\ 4^{b} \cdot 7H_2O$	5 mM	12	10 mM	8
Ethylene diamine tetraacetic acid	5 mM	47	10 mM	14
Sodium dodecyl sulfate	5 mM	113	10 mM	80
Phenylmethyl sulfonyl fluoride	5 mM	0	10 mM	0
$C_{26}H_{50}O_{10}$	1%	61	2%	28
Polysorbate 80	1%	42	2%	22
Triton X – 100	1%	45	2%	21
Methanol	25%	39		
Acetone	25%	54		
Isopropanol	25%	26		

a: The average of duplicates is used to calculate the results. b: To keep away from the precipitation of the reaction, the enzyme assay used 50 mM Tris buffer at pH 7.0, instead of phosphate buffer.

hydrophilic/hydrophobic features of enzymes with substrates. The purified enzyme was highly selective for p-NPB and had only a minor relative activity against p-NPA (Table 2).

In a previous study, p-nitrophenyl butyrate (*p*-NPB) caused a significant reduction in the activity of the esterase formed through *Geodermatophilus obscurus G20* [40]. *Salimicrobium sp. LY19*, a halotolerant isolate, has the highest efficacy against p-nitrophenyl butyrate [41]. Another research found that a cold-adapted esterase had the highest activity against p-nitrophenyl butyrate at 18°C with pH 6.5.

Effect of activators, inhibitors, and organic solvents

The effect of activators, inhibitors, and organic solvents on enzyme activity was determined. Table 3 summarizes the effects of potential inhibitors as well as activators on esterase activity. At both concentrations (5 and 10 mM), phenylmethylsulphonyl fluoride, EDTA, Hg2+, Zn2+, Ni2+, Tween 80, Tween 20 as well as Triton X–100 inhibited the enzyme. The concentration of enzymes without inhibitors was adjusted at 100. At concentrations of 5 and 10 mM, the enzyme was fully inhibited with PMSF. At the lowest concentrations, Na+, as well as K+, activated the enzyme. SDS is an anionic detergent, it improves the activity of enzymes around 5mM rather than inhibited at the maximum concentration. In the presence of 25% organic solvent, the recovered esterase was capable of hydrolyzing P-nitrophenyl butyrate, with acetone being more active [42, 43].

Halo-stability

The purified esterase exhibits excellent stability and approximately 100 percent activity was stored when

Table 4: Effect of salinity.

Molarity of Sodium chloride	Specific activity(U/mg)	Relative activity (%)
Control	775.1	100
1 M	1841.73	234.6
2 M	1678.61	216.3
3 M	1172.26	159
4 M	1109.4	153.4
5 M	765.81	107.8

incubated with 10 M sodium chloride. While combined with 1M Sodium chloride, the activity of the enzyme increases by around two times and then reduces slightly up to 3 M sodium chloride (Table 4). Esterase activity was increased by 1 M sodium chloride and activity was maintained up to 5 M sodium chloride, indicating that it was more halotolerant than previously described esterase [31].

CONCLUSIONS

Although most esterases have an acidic optimal pH, fungi and bacteria both have alkaline esterases. When temperatures below 40 ℃ are used for biocatalytic processes to maintain the labile substrates. In this study, we discussed the purification of the esterase from *Aspergillus versicolor* by using the ammonium sulphate precipitation, dialysis, and Sephadex G-75 chromatography technique. Monomer-purified esterases with a molecular mass of 32 kDa belonging to the family of fungal esterases. The protein from *Aspergillus terreus*, which is phylogenetically related to *Aspergillus versicolor* and belongs to the *Aspergillus genus Terrei* division, has the maximum identity with the investigated esterase (55.3%). The significant inhibition through PMSF as well as Hg^{2+}, respectively, supports the existence of a large serine residue in the enzyme's catalytic region as well as the significance of the sulfhydryl group within catalysis or in the enzyme structure. The current study is primarily concerned with the manufacture of an esterase by *Aspergillus versicolor* and its characterization.

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