Purification and Characterization of an Extracellular High Molecular Mass Esterase from *Bacillus pumilus*

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Abstract: Esterases (EC 3.1.1.x) represent a diverse group of hydrolases catalyzing the cleavage and formation of ester bonds and are widely distributed in animals, plants and microorganisms. The esterase was purified from cell-free culture broth to homogeneity by size exclusion chromatography with 7.7 fold purification and 22.5% yield. The *Mr* of the purified esterase of *B. pumilus* was 17 kDa by SDS-PAGE. The esterase appeared to be a novel decameric protein as it possessed a single band of *M*r 17 kDa in SDS PAGE and 170 kDa in Native PAGE. A Lineweaver-Burk plot was calibrated to determine K_m (3.94 mM), V_{max} (49.02 mM/mL/min), K_{cat} (138.89 sec^{-1}), K_{spec} (35.28) and K_{si} (26.58 mM) values of purified esterase of *B. pumilus* for its substrate *p*-nitrophenylacetate. Energy of activation (Ea) of purified esterase of *Bacillus pumilus* was 2.61 $Jmol^{-1}$as determined with the help of an Arrhenius plot. The DMSO drastically inhibited the activity of purified esterase while benzene and propan-2-ol were fairly tolerated by the esterase. The potential protease inhibitors such as PMSF, EDTA, SDS and DTT were found to decrease the activity of purified esterase. Thermostability of the purified esterase was checked at 45°C. Purified esterase lost more than 50% of its initial activity at 45°C after 6.5 h of incubation in a water-bath under shaking.

Keywords: Esterase, Bacillus pumilus, extracellular, decameric protein, inhibitors, thermostability.

INTRODUCTION

Lipolytic enzymes, including esterases (EC 3.1.1.1) and lipases (EC 3.1.1.3), are among the most frequently used groups of biocatalysts in industry and they are widely distributed in nature. An esterase preferably catalyzes the hydrolysis of carboxyl ester linkages composed of short chain fatty acids only in aqueous solution, and they can also catalyze the ester synthesis and trans-esterification in water-free or water-restricted medium [1, 2]. Esterases do not require cofactors and this property makes them attractive biocatalysts [3]. Esterases are valuable in a variety of commercial applications in the production of food (including dairy products), pharmaceuticals, detergents, textiles, paper, animal food, leather and cosmetics [4]. The common use of this enzyme in various sectors of industry is stimulating increasing interest in the discovery and characterization of new esterases of microbial origin.

Purification of proteins is very important when working with enzymes because it provides the material required for structural, functional and kinetic studies. The extra-cellular enzyme of microbial origin can be purified by consecutive fractionation, concentration and chromatography techniques [5, 6]. The ease of the purification of enzyme might be influenced by the culture conditions, medium composition, the phase of growth the cells are harvested in, any delays in processing and the number of steps involved in its purification till a polished product is obtained.

MATERIALS AND METHODS

Chemicals

All the chemicals were of analytic grade and were used as received.

Production of Bacterial Extracellular Esterase

The esterase producing isolate *B. pumilus* was grown in the (broth) in a medium containing (g/L) yeast extract (5.0), potassium chloride (0.6), sodium nitrate (3.0), magnesium sulphate heptahydrate (0.6), dipotassium hydrogen phosphate (0.1), ferrous sulphate heptahydrate (0.01), and cotton seed oil (10 mL/L) without agar. The seed culture was transferred (7.5 %, v/v) to 50 mL production broth (250 mL Erlenmeyer flask) kept for 24 h under shaking (110 rpm) at 37˚C.

Esterase Assay

Esterase activity was assayed in the culture broth as well as purified column chromatographed fractions by the method of Winkler and Stuckmann (1979) by measuring the micromoles of *p*-nitrophenol released from *p*-nitrophenylacetate [7].

Procedure

To 2.9 mL of Tris-HCl buffer (0.05 M, pH 8.0), added 60 µL of the substrate (*p*-NPA, 10 mM).

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Incubated the reaction mixture at 37^{o}C in a water bath for 10 min in order to remove the turbidity and 40 µL of enzyme was added thereafter. The reaction mixture was again incubated at 37^{0}C in water bath for 20 min. The reaction was stopped by chilling at -40^{0}C. The amount of *p*-nitrophenol (*p*-NP) released was measured at A_{410} (Perkin Elmer UV/VIS Spectrophotometer Lambda 12) after bringing the tubes to room temperature.

Esterase Activity

One unit (U) of esterase activity was defined as amount of enzyme required to release one micromole of *p*-NP from the substrate *p*-nitrophenylacetate (*p*-NPA) per minute by one mL of the enzyme preparation under standard assay conditions. Protein estimation was done by the method of Bradford [8].

Purification of Esterase

The purification of bacterial esterase was performed using techniques of acetone precipitation, dialysis and size exclusion chromatography (Sephadex G-200) in that order. The cell-free supernatant obtained after 24 h of esterase production was used for purification of esterase. Required amount of acetone (50% v/v) was added to the cell free broth to achieve maximum saturation. The contents were mixed and kept at -20ºC for 10 minutes to achieve maximum precipitation. Thereafter, the precipitates sedimented by centrifugation at 12,000 × g for 30 min at 4ºC were reconstituted in minimum volume of Tris buffer (0.05 M, pH 8.0). The protein precipitates transferred into a dialysis apparatus were extensively dialyzed against Tris buffer (0.05 M, pH 8.0) at a regular interval of 2 h so as to completely remove acetone. Finally, the esterase activity as well as protein content was assayed in the dialyzate. The dialyzate was stored at -20ºC until further use.

Sephadex G-200 Gel Permeation Chromatography

A column of pre-swollen Sephadex G-200 matrix (Sigma Chemical Co., U.S.A) was packed (*V*t = 200 cm^3) in a sintered glass column. Equilibration was done using 20 mL of start-buffer (0.05 M Tris buffer, pH 8.0) at a flow rate of 60 mL.h^{-1}. The dialyzed esterase was loaded on the column. The elution was performed with 30 mL of elution-buffer (0.05 M PBS buffer, pH 7.5). All fractions were assayed for both esterase activity as well as their protein content. The fractions showing esterase activity under a peak were pooled and quantified both for protein content as well as esterase activity. Fold purification as well as yield of esterase were determined at each stage of purification procedure. The purified esterase preparation was stored at -20^{o}C until subsequent use.

Determination of Molecular Mass [*Mr*] of Purified Esterase

The SDS-PAGE and Native-PAGE were performed to determine the purity, structure, and the subunit molecular mass of the purified bacterial lipase.

Characterization of *B. pumilus* Purified Esterase

Effect of Different Substrate on Purified Esterase

To study the substrate specificity of the purified esterase, different chromogenic substrates namely *p*-NPA, *p*-nitrophenylbenzoate (*p*-NPBz), *p*-nitrophenyllaurate (*n*-NPL) and *p*-nitrophenylpalmitate (*p*-NPP) were used. Each of the above substrates was prepared (10 mM) in *iso*-propanol. The reaction was performed using 40 µL of purified enzyme in Tris buffer (0.05 M) of pH 8.0 at 37ºC for 20 min.

Effect of Molarity of Tris Buffer on Purified Esterase

To study the effect of concentration of Tris buffer (pH 8.0), esterase was assayed at selected concentrations of Tris buffer (0.01, 0.03, 0.05, 0.07, 0.1, 0.15 and 0.2 M) with 5 mM substrate *p*-NPA. The reaction was performed separately using 40 µL of purified enzyme in Tris buffer (0.05 M) of pH 8.0 at 37ºC for 20 min.

Kinetic Study and Activation Energy of Purified Esterase

Critical kinetic parameter of purified esterase such as K_m, $V_{max,}$ $K_{cat,}$ *K*si and specificity constant were determined by measuring the reaction velocities at the different concentrations of the *p*-NPA *i.e* 1-30 mM. Activation energy (Ea) was determined from Arrhenius plot [9].

Thermostability of Purified Esterase

To examine the effect of temperature on stability of the enzyme, purified esterase (0.5 mL) was kept separately in test tubes for 2 h intervals at 45^{o}C. The activity measured immediately before incubation was defined as 100% of hydrolytic activity. The reaction was performed using 40 µL of purified enzyme and Tris buffer (0.05 M) of pH 8.0 for 20 min.

Effect of Organic Solvent on Purified Esterase

The purified esterase was assayed in the presence of different (2%, v/v) organic solvents *viz*, DMSO, benzene, *p*-xylene, toluene, *n*-pentane, *n*-hexane, *n*-heptane, *n*-octane, ethanol, decanol, propan-1-ol, propan-2-ol, butan-1-ol, but-2-ol, decan-1-ol, phenol and actonitrile. The reaction was performed using 40 µL of purified esterase in Tris buffer (0.05 M) of pH 8.0 at 37ºC for 20 min.

Effect of Denaturing/ Chelating Agents on Purified Esterase

To study the effect of denaturing/ chelating agents on *B. pumilus* esterase, each of the selected compounds (PMSF, DTT, EDTA and SDS; 10 mM) was included separately in reaction mixture. The reaction was performed using 40 µL of purified enzyme in Tris buffer (0.05 M) of pH 8.0 for 20 min.

RESULTS

Precipitation of Crude Esterase

The extracellular esterase (4.68 U/mL; 0.527 mg protein/mL) secreted by *B. pumilus* in the culture broth (200 mL) was subjected to 50% acetone precipitation. After acetone precipitation, an activity of 28.12 U/mL with 1.65-fold purification of esterase was observed (Table **1**). The precipitates were reconstituted and dialyzed for 24 h against Tris buffer (0.05 M, pH 8.0). After dialysis, an activity of 17.9 U/mL with 2.0-fold purification was recorded.

Sephadex G-200 Gel Permeation Chromatography

The purification of dialyzed esterase on Sephadex G-200 column resulted in a single peak. A total of 31 fractions were collected (1 mL each) by elution with PBS buffer (0.05 M pH 7.5). The esterase was recorded in 8-17 fractions. The fractions showing both good protein content (A_{280}) as well as esterase activity were pooled. This chromatographic step resulted in 7.7 fold purification of enzyme with a yield of 25.9% (Table **1**). The size exclusion column purified esterase was further evaluated for its homogeneity on gel electrophoresis.

Electrophoretic Characterization of Sephadex G-200 Column Purified *B. pumilus* Esterase

The analysis of purified bacterial esterase under reducing and denaturing SDS-PAGE (15%) and non-denaturing Native-PAGE (12%) revealed that the esterase possessed a single band of *M*r17 kDa (Figure **1**) and a 170 kDa, respectively (Figure **2**). The esterase of *B. pumilus* appeared to be decameric protein of 17 kDa monomeric subunit.

Effect of Different *p*-Nitrophenyl Acyl Esters Substrates on Purified Esterase of *B. pumilus*

To examine the effect of selected *p*-nitrophenyl acyl esters varying in their C-chain length *i.e. p*-NPA, *p*-NPBz, *p*-NPL and *p*-NPP each of these substrates was separately included in the reaction mixture. The purified esterase showed maximum activity towards *p*-NPA, followed by *p*-NPBz and *p*-NPL in that order (Figure **3**).

Effect of Molarity of Tris Buffer on Purified Esterase of *B. pumilus*

To study the effect of the variation in the molarity of Tris buffer (pH 8.0 on the esterase activity, the concentration of the 0.05 M Tris-buffer (a part of the reaction mixture) was adjusted separately to 0.01, 0.03, 0.05, 0.07, 0.1, 0.15 and 0.2 M (Figure **4**). The maximum activity of purified esterase (38.0 U/mL) was recorded at 0.05 M of Tris-buffer.

Kinetic Study of Purified Esterase of *B. pumilus*

The rate of reaction (V_{max}) and K_m using the best colorimetric substrate *p*-NPA were studied by employing 1-30 mM of *p*-NPA concentration in 0.05 M Tris-buffer (pH 8.0) under shaking at 37^{o}C (Figure **5a**). A Lineweaver-Burk plot was calibrated to determine

Table 1: Summary of Step-Wise Purification of *B. pumilus* Esterase

Purification stage	Volume (mL)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Fold purification	Yield (%)
CFE	200.0	93.6	105.4	8.8	1.0	100.0
Acetone precipitation	5.0	140.6	9.6	14.5	1.6	15.0
Dialysate esterase	5.0	146.3	8.1	17.9	2.0	15.6
Sephadex G-200	10.0	242.2	3.5	67.7	7.7	25.9

CFE: Cell Free Extract.

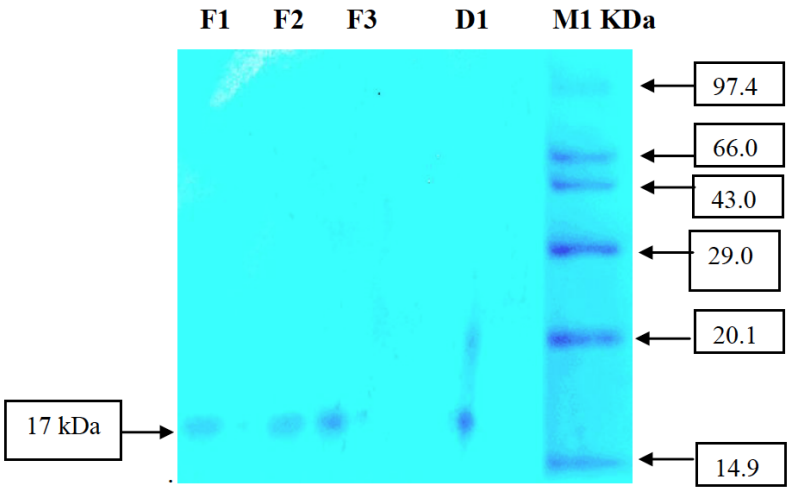


Figure 1: Pattern of esterase of *B. pumilus* on 15% SDS-PAGE.

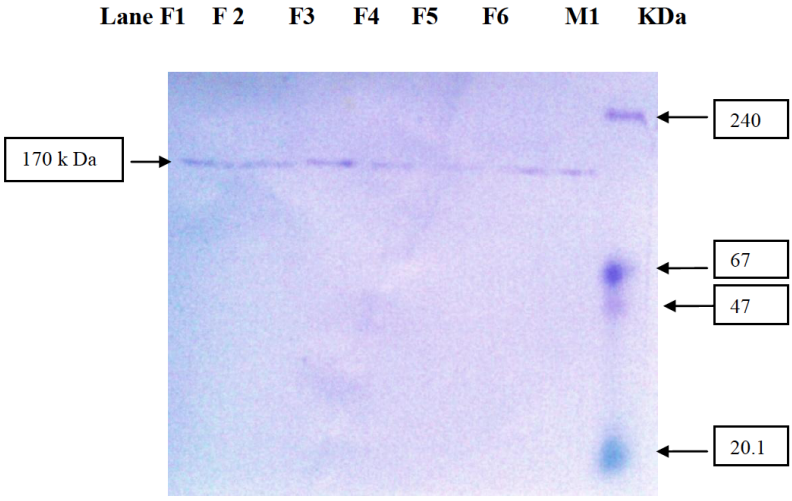


Figure 2: Pattern of *B. pumilus* on 12% Native-PAGE.

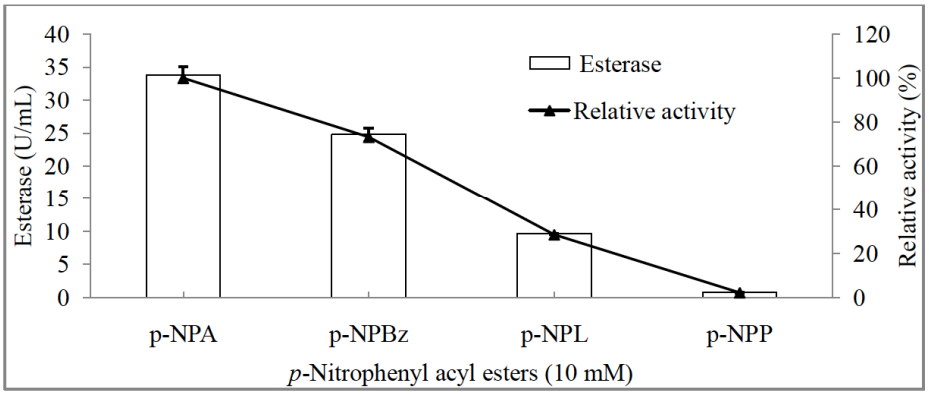


Figure 3: Effect of different *p*-nitrophenyl acyl esters substrates on purified esterase of *B. pumilus.*

K_m (3.94mM), V_{max} (49.02 mM/mL/min), K_{cat} (138.89 sec^{-1}), K_{spec} (35.28) and K_{si} (26.58 mM) values of purified esterase of *B. pumilus*. Energy of activation (Ea) of purified esterases of *B. pumilus* was 2.61 $Jmol^{-1}$ calculated with the help of Arrhenius plot (Figure **5b**).

Thermostability of Free Esterase at 45°C

Thermostability experiments of purified esterase were studied by incubating the esterase at 45°C for

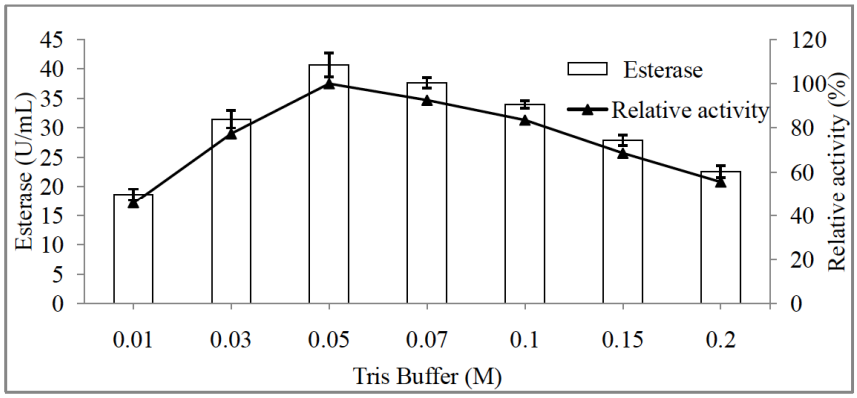
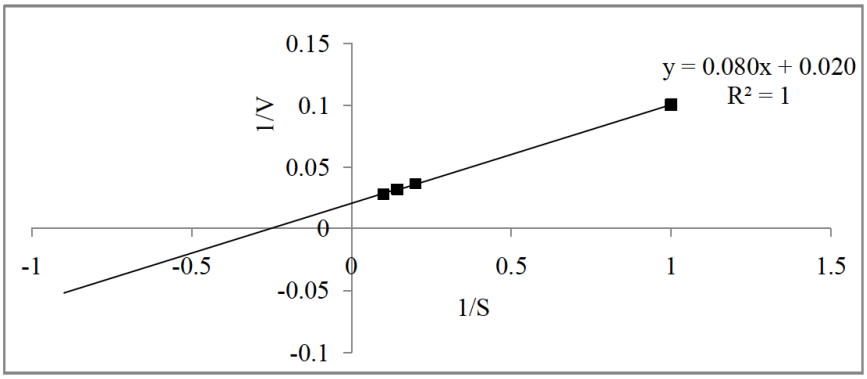
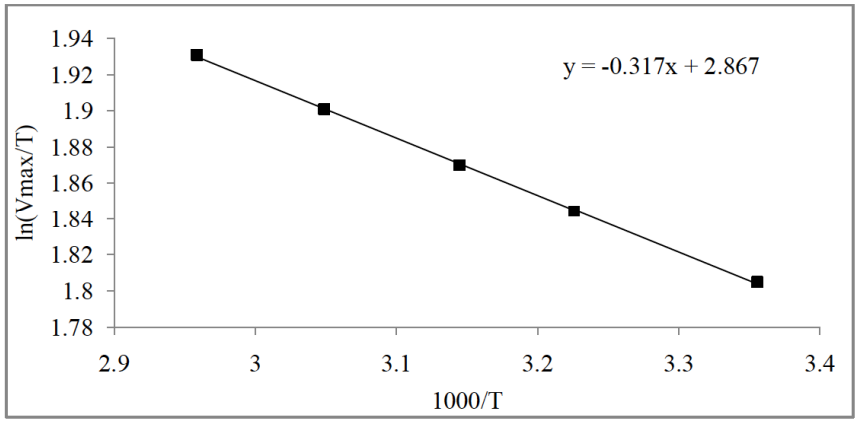


Figure 4: Effect of concentrations of Tris buffer on purified esterase of *B. pumilus*.



a



b

Figure 5: a) Lineweaver-Burk plot for the purified esterase of *B. pumilus* for *p*-NPA. **b**) Arrhenius plot for determination of energy of activation of purified esterase produced from *B. pumilus.*

14h (Figure **6**). Purified esterase lost more than 50% of its initial activity at 45°C after 6.5 h of incubation under shaking.

Effect of Organic Solvents on Purified Esterase of *B. pumilus*

To study the effect of each of the selected organic solvents on purified esterase, each of the selected organic solvents was pre-incubated with enzyme at 37°C for 10 min. All the tested solvents drastically inhibited the activity of the B. pumilus esterase while benzene, n-heptane, n-octane, n-hexane and propan-2-ol were fairly tolerated by the esterase (Table **2**).

Effect of Denaturing/ Chelating Agents on Purified Esterase of *B. pumilus*

To study the effect of PMSF, EDTA, DTT and SDS on esterase activity, these were preincubated

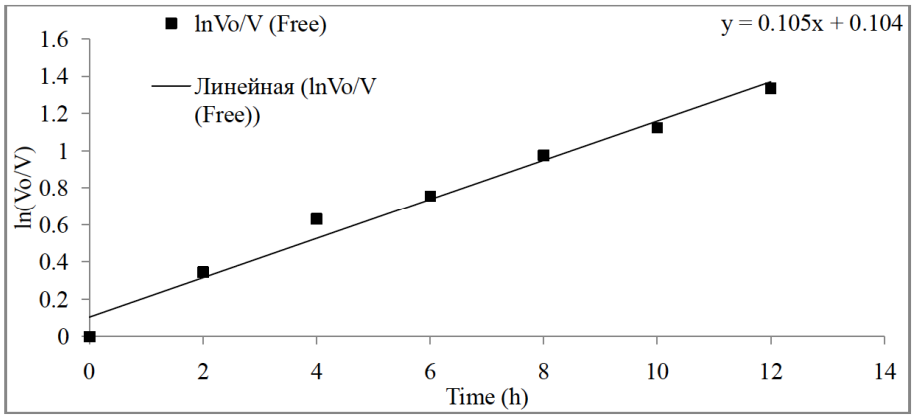


Figure 6: Thermostability profile of purified esterase of *B. pumilus* incubated at 45^0C.

Table 2: Effect of Organic Solvents on Purified Esterase of *B. pumilus*

Solvents	Log (*p*) value	Free esterase (U/mL)	Relative activity (%)
Control		40.67	100
DMSO	1.22	12.86	32
Benzene	2.13	27.32	67
p-Xylene	3.1	22.48	55
Toluene	2.73	25.07	62
n-Pentane	3.25	24.00	59
n-Hexane	3.76	25.68	63
n-Heptane	4.27	26.59	65
n-Octane	4.78	26.48	65
Ethanol	-0.58	22.86	56
Propan-1-ol	0.28	25.07	62
Propan-2-ol	-0.19	26.71	66
Butan-1-ol	0.839	26.50	65
Butan-2-ol	0.68	23.67	58
Decan-1-ol	4.57	16.78	41
Phenol	1.12	19.02	47
Acetonitrile	-0.33	18.76	46

separately at 10 mM concentration with the purified esterase at 37°C. The residual esterase activity was assayed thereafter. The SDS strongly inhibited the activity of purified esterase (Figure **7**).

DISCUSSION

Esterases are ubiquitous enzymes which usually prefer to hydrolyze the esters of short chain fatty acids. Over the years, there has been a tremendous increase in the use of microbial ester hydrolases as biocatalysts in biomedical applications because of their excellent capabilities to carry out regio-, stereo-, and enantio-specific reactions [10, 11]. In the last couple of years, major emphases were laid on purification of microbial esterase(s). In the present study, an esterase from *B. pumilus* was purified to homogeneity by Sephadex G-200 chromatography with 7.7-fold purification and high (25.9%) yield. The molecular weight of esterase was found to be ~170 kDa by using native PAGE and ~17 kDa by SDS-PAGE. In a previous study an esterase from *B. pumilus* showed molecular weight of190 kDa, by native PAGE and 40 kDa by SDS-PAGE [12]. It seems the esterase(s) of *Bacillus* spp. have great diversity as they vary in their molecular masses and possibly their biochemical properties too. The highest

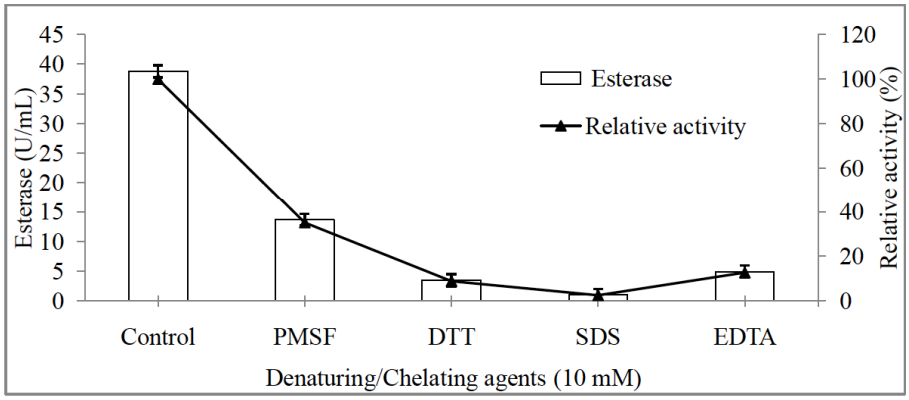


Figure 7: Effect of denaturing/ chelating agents on purified esterase of *B. pumilus.*

activity of the purified esterase of *B. pumilus* was towards *p*-NPA (10 mM), a short chain ester, and least towards *p*-NPP which is a relatively long chain ester. An esterase prefers water-soluble substrates composed of short carbon-chain fatty acids while aromatic compounds are difficult to degrade by the most microbes/microbial enzymes. An esterase from halotolerant isolates *Salimicrobium* sp. LY19 exhibited maximum activity towards *p*-NPB [13]. The optimum pH of Tris HCl (0.05 M) buffer for assay of esterase of *B. pumilus* was found to be 8.0 in the present study. In a previous study, *L. brevis* NJ 13 also showed highest activity towards at pH 8.0 of 10 mM Tris HCl buffer [14]. The characteristics Lineweaver-Burk plot was calibrated to determine K_m (3.94 mM) and V_{max} (49.02 mM/mL/min) values of purified esterase of *B. pumilus.* In a recent study, a novel thermotolerant esterase from *Thermos* spp. showed K_m of 18.32 mM and V_{max} of 96.15 U/mg [15], so present study lipase is better over this because of its lower *K*m. Among various organic solvents, benzene (67%), *n*-decane 1-ol (66%), propan 2-ol (66%), *n*-heptane (65%), *n*-octane (65%) and *n*-buatne 1-ol (65%) had lower inhibitory effect on the esterase activity of *B. pumilus* while other organic solvents strongly inhibited the esterase activity. In a previous study, esterase activity was potentially inhibited by dichloromethane, *tert*-butanol and methanol [14]. All the denaturing anionic detergents *viz*. PMSF, DTT, SDS and EDTA in the present study had severe inhibitory effect on esterase activity of *B. pumilus*. The present study provided a mesophilic bacterial isolate *B. pumilus* that produced an extracellular esterase of *M*r (~ 170 kDa) that was highly reactive towards relatively shorter length (*p*-NPA) ester at an alkaline pH and temperature 37°C. This enzyme appears to be a novel multimeric alkaliphilic bacterial esterase. The esterase showed good stability in the presence of many commonly used solvents including benzene, propan-2-ol, butan-1-ol, *n*-heptane and *n*-octane that reflected its amenability to perform synthesis of esters in such organic solvent system as well as in majority of industrial applied biocatalysis. By considering all these properties, this esterase may be further explored for the synthesis of short-chain esters which have a broad range of industrial/ commercial applications in foods, drugs, as fragrances & flavor compounds and in cosmetics. The biocatalytic potential of *B. pumilus* esterase may be improved by using immobilized form of enzyme to economize the synthetic process(es) for the synthesis of short-chain fatty acid esters.

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CONFLICT OF INTEREST

The authors declare no conflict of interests publishing this article in this journal.

ETHICAL STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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