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Biochemical Characterization of A Novel Thermophilic Esterase Isolated from Shewanella sp F88

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Abstract

The main objective of this study was to purify and characterize an esterase from *Shewanella* sp F88. The enzyme was purified 41-fold and an overall yield of 21 %, using a two-step procedure, including ammonium sulfate precipitation and Q-sepharore chromatography. Molecular weight of the enzyme was 62.3 kDa according to SDS-PAGE data. The enzyme showed an optimum activity at pH 6.5 and 58 ˚C. Evolution of substrate specificity demonstrated that this thermostable enzyme had the highest activity towards para-nitrophenol acetate (*p*NPA, C2). Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) of pNPA-hydrolyzing reaction were 12.6 mM and 550 $U.mg^{-1}$, respectively. Enzyme activity was declined in the presence of metal ions (2 and 5 mM), including Fe^{2+}, Ca^{2+}, Cu^{2+}, Zn^{2+}, Mg^{2+} and Mn^{2+}. The half-lives of purified esterase was 70 and 31 min at 60 °C and 80 °C, respectively. In conclusion, the enzyme is a novel thermostable lipolytic enzyme characterized from *Shewanella* species.

Keywords: Esterase; Thermostable; Lipolytic enzyme; Characterization; *Shewanella*.

Introduction

Esterases and lipases are carboxylic ester hydrolases (EC 3.1.1), and are classified into more than eight families [1-2]. Lipolytic enzymes are widely used in food, fat and oil industries, detergents, paper manufacturing, as well in the production of fine chemicals and pharmaceutical compounds [3]. Theses enzymes are found in most of living organisms, including plants, animals, and microorganisms [4]. Thermostable enzymes are commercially significant for their potential use in various industries. They are important due to their stability under high temperature conditions [5]. More attention is being paid nowadays to introduce novel thermal lipases/esterases. due to their importance in biotechnological applications.

Thermophilic esterases have been isolated from many genera, including *Sulfolobus solfataricus* [6], *Thermoanaerobacter tengcongensis* [7], *Geobacillus kaustophilus* HTA426 [8], *Bacillus subtilis* [9], *Bacillus licheniformis* S-86 [10], *Bacillus cereus* [11], *Thermotoga maritime* [12], *Thermus thermophilus* HB27 [13], *Staphylococcus arlettae* JPBW-1[14] and *Anoxybacillus flavithermus* [15].

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Shewanella species are widespread in various environments, but the characterization of lipolytic enzymes from *Shewanella* has not been described so far. In this study, we extracted a novel esterase from *Shewanella* sp F88. The biochemical characteristics of the enzyme such as pH and temperature profiles, thermostability, substrate specificity as well as metal ion-interactions revealed that it is very likely to be considered as a novel esterase.

Materials and Methods

Materials

*p*NPA, *p*NPB, *p*NPP (para-nitrophenol acetate, butyrate and palmitate, respectively) and porcine pancreatic lipase (Type VI-S) were prepared from Sigma (Louis, MO, USA). Q-Sepharose Fast Flow was bought from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Culture mediums were obtained from Himedia (Mumbai, India). DNA extraction and PCR reagents were purchased from Fermentas (life science, USA).

Isolation of Microorganism

Water samples were collected from the Ferdows hot mineral spring in the east of Iran. The samples were cultivated on nutrient agar plates and incubated at 50 °C for 48 h. The positive stains was selected based on lipolytic activity on agar plates comprising 1% (w/v) peptone, 1% (w/v) olive oil, 2.8 % (w/v) nutrient agar powder and 0.001% (w/v) Rhodamine B [16]. Lipase production was carefully monitored with the UV light at 350 nm. A lipolytic strain, which produced a fluorescent orange halo around colonies in agar plates containing Rhodamine B, was selected for enzyme production. To obtain the enzyme, a colony-representing enzyme activity was cultured in a medium (500 ml) containing 3% (v/v) olive oil along with nutrient broth media containing peptic digestion of animal tissue 0.5 % (w/v), beef extract 0.15 % (w/v), yeast extract 0.15 % (w/v) and sodium chloride 0.5% (w/v) at 50 °C by shaking at 150 rpm for 45 h. The growth of the selected strain and activity of extracted enzyme was recorded every 2 h to determine the optimum time of enzyme production.

Enzyme Assay

The enzyme activity was assayed by a colorimetric method using *p*NPA (para-nitrophenol acetate) as substrate. To assay the activity, 0.1 ml of extracted enzyme was added into 0.8 ml of 50 mM phosphate buffer (pH 6.5) and 0.1 ml of 20 mM *p*NPA as a substrate and incubated for 20 min at 45 ˚C (standard assay conditions). After 20 min of incubation, 0.3 ml of 50 mM Na_2CO_3 was added to stop the reaction, and the enzyme activity was measured at 410 nm using a UV-visible spectrophotometer. One unit (U) of lipolytic activity was defined as micromole(s) of para-nitrophenol released by hydrolysis of para-nitrophenyl ester per one milliliter of soluble enzyme per minute [17]. Other substrates such as *p*NPB and *p*NPP at 20 mM concentration were also used to determine substrate specificity of the enzyme. Michaelis-Menten constant (K_m) and maximum velocity for the reaction (V_{max}) were determined based on Lineweaver-Burk double-reciprocal plot (1/V-1/[S]) in 50 mM phosphate buffer pH 6.5 at 45 °C.

Molecular Identification of the Strain

The positive lipolytic strain was identified using 16S rDNA sequence analysis. Forward primer 5´-AGTTTGATCCTGGCTCAG-3´ and reverse primer 5´-GGCTTACCTTGTTACGACTT-3´ was used to amplify 16S rDNA of the isolated strain. DNA amplification was performed using a Techne FT Gene 2D thermocycler under the following conditions: denaturation at 93 °C for 5 min followed by 35 cycles at 93 °C for 45 s, 50 °C for 45 s, 72 °C for 90 s and a final extension at 72 °C for 5 min [18]. The DNA sequencing was determined in Pasteur Institute (Tehran, Iran). Similarity searches of sequences were performed by the use of BLAST program (http://www.ncbi.nlm.nih.gov/BLAST). Ten 16S rDNA gene sequences from GenBank were selected based on the highest homology, and CLC Main Workbench 6 software (Aarhus, Denmark) was used to construct the phylogenetic tree.

Enzyme Purification

The isolated strain for enzyme production was cultivated in nutrient broth (500 ml), which was supplemented with 3% (v/v) olive-oil at 50 °C and 150 rpm for 18 h. Afterward, the culture medium was centrifuged at 7,000 g and 4 °C. The resultant supernatant was precipitated by 85% of ammonium sulfate, and then centrifuged at 8,700 g for 15 min. The precipitate was dissolved in minimum volume of 25 mM Tris-HCl pH 7.5 (buffer A), and dialyzed against the same buffer at 4 °C for 24 h. The dialyzed sample was loaded into a previously equilibrated Q-sepharose column (anion-exchange chromatography resin) with buffer A. Samples were fractionated by different stepwise gradient of NaCl (0.1-0.8 M) in buffer A. The protein eluted from the column was detected at 280 nm. Each fraction was assayed to find the major fraction containing enzyme. Active fractions were concentrated using a 10 kDa ultra-membrane (Bedford, MA, USA). Quantification of the protein concentration was accomplished by Bradford method [19]. Bovine serum albumin (BSA) was also used as a standard protein to estimate protein concentration.

SDS-PAGE and Zymography

To estimate the molecular weight and visualize enzyme activity, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and zymogram methods were used [20]. For gel electrophoresis, staching gel 5% and separting gel 10% were used. After running the SDS-PAGE, the gel was stained for 2 hours using a solution containing 45% distilled water, 45% methanol, 10% acetic acid and 2 mg Coomassie brilliant blue R-250. The gel was destained for approximately 30 min with an aqueous solution of 10% acetic acid, 45% distilled water and 45% methanol.

Non-denaturing PAGE was carried out to analyze the enzyme when it is still in folded state [16]. Before staining for enzyme activity on the gel, it was washed with Triton X-100 for 30 min and then washed twice in 10 mM phosphate buffer (pH 6.8) for 15 min. Afterward, the washed gel was laid onto a plate containing 1 % agar, 3% olive oil and 0.01% rodamin B for 24 h. The location of the enzyme band was detected under a UV light source.

Effect of pH on enzyme activity

In the pH range of 3.0-8 at 45 °C, effect of pH on the enzyme activity was studied. For this purpose, different buffers, including 50 mM sodium acetate (pH 3-5.5), 50 mM sodium phosphate (pH 6-7.0) and 50 mM Tris-HCl (pH 7.5.0-8.5) were used. In order to analyze the pH-activity profile, the enzyme was incubated at 45 °C for 20 min in buffers of variable pH, and subsequently the residual activity was determined. The maximum activity of the enzyme was considered as 100% activity. In this experiment, porcine pancreatic lipase (PPL) was used as a control to compare the results.

Effect of temperature on enzyme activity

The temperature effect on the enzyme activity was evaluated in the range between 30 to 80 °C at the optimum pH. The activity of unheated enzyme was taken as 100% activity. Thermal inactivation of the enzyme was also assessed at different temperatures. After incubating the enzyme for 20 min at different temperatures (60, 70, 80 and 85 °C); samples were withdrawn at 10 min intervals, and immidiately cooled on ice and the remaining activity was assayed. The half-life values were estimated from the slope of thermal inactivation curve. To compare the thermo stability results, the PPL was also used as the reference sample.

Effect of metal ions

Under the optimum pH and temperature condition, the effect of metal ions on enzyme activity was studied. For this purpose, the enzyme and substrate were incubated in the presence of 2 and 5 mM of divalent metal ions ($CaCl_2$, $FeCl_2$, $CuCl_2$, $ZnCl_2$, $MgCl_2$ and $MnCl_2$) as well as EDTA. Afterward, the residual activity was assayed. The relative enzyme activity (%) in the presence of different concentrations of ions was calculated and compared to control without metal sample. The experiment was accomplished in triplicate.

Results and Discussion

Bacterial Growth

Positive strain which showed lipolytic activity on agar plate containing olive oil was cultured. The profile of bacterial growth indicated that the strain reached to a plateau phase at 50 °C after approximately 30 h. The results showed that the maximum enzyme productions were in between 18 h to 24 h (Fig. 1). After 9 h of incubation, the logarithmic phase was initiated, and reached to plateau after 30 h. The growth profile indicated that the enzyme production occurred before starting exponential growth of the strain. Therefore, enzyme collection was performed after 18 h of incubation.

Molecular Identification

The sequence of 16S rDNA gene was used for strain identification. The homology search showed that the strain had the most similarity to *Shewanella* species, therefore the isolated bacterium named *Shewanella* sp. F88. The 16S rDNA sequence of *Shewanella* sp F88 has been deposited in the NCBI GenBank with accession number JN402324. A total of 10 strains of *Shewanella* were initially compared based on differences in 16S rDNA sequence (Fig. 2). The sequence from a Shewanellaceae bacterium ACEMC 15-2 strain (accesion numer FM162911) was used as an outgroup. The 16S rDNA phylogenetic analysis organized the strains into one cluster. Overall genetic distances between the strains ranged from 0.00 to 0.060, whereas

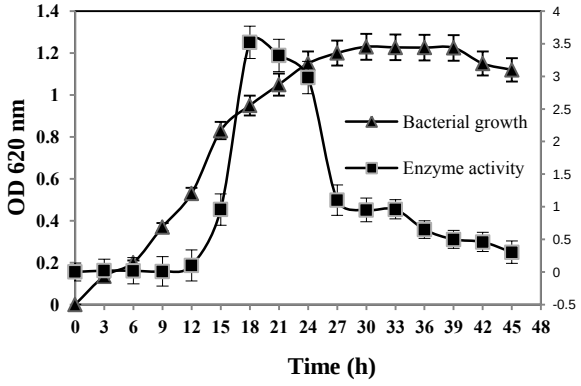

Figure. 1. Bacterial growth and enzyme activity profiles. The plot shows a great activity at 18 h of bacterial growth.

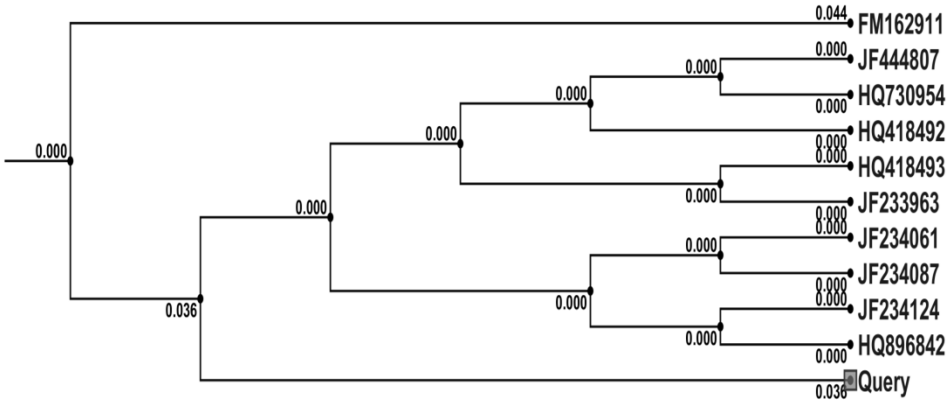

Figure. 2. Phylogenetic tree of 16S rDNA gene sequences. The isolated strain is represented by *Shewanella* sp F88 (Query). Accesion number of the each sequence had been typed in the end of the corresponding branch. The sequence of FM162911 (*Shewanellaceae bacterium* ACEMC 15-2)was used as an outgroup. The relevant strain for other accession numbers are as follows: JF444807(*Shewanella* sp. 24), HQ730954(*Shewanella* sp. J25), HQ418492 (*Shewanella xiamenensis* sp. H2), HQ418493(*Shewanella xiamenensis* sp. H3), JF233963 (uncultured bacterium clone ncd2700a08c1), JF234061(uncultured bacterium clone ncd2701e03c1), JF234087(uncultured bacterium clone ncd2701h10c1), JF234124(uncultured bacterium clone ncd2702c10c1) and HQ8968429 (*Shewanella* sp. enrichment culture clone lzh-2).

the degree of similarity within the strains ranged from 94 to 100%. The isolate showed 94% 16S rDNA nucleotide identity to *Shewanella* sp. J25 (accession number HQ730954) as well as other cluster's members. To the best of our knowledge, this study is the first report of lipolytic enzyme characterization from *Shewanella* species. Bahobil purified a protease from *Shewanella putrefaciens* EGKSA21 [21]. It has been reported that some species of *Shewanella* such as *S. hanedai* produce and secrete lipolytic enzyme into their media [22].

Enzyme Purification

Purification of the desired enzyme bound on Q-sepharose column (1 × 10 cm) was carried out using a stepwise-gradient of sodium chloride (0.1 to 1 M). The pure enzyme was eluted at 0.4 M sodium chloride in buffer A at pH 7.5 (Fig. 3). Specific activities of the enzyme and purification folds were provided in Table 1. After a three-step process, the purified lipase with a 41-fold of purification and 21% yield was achieved.

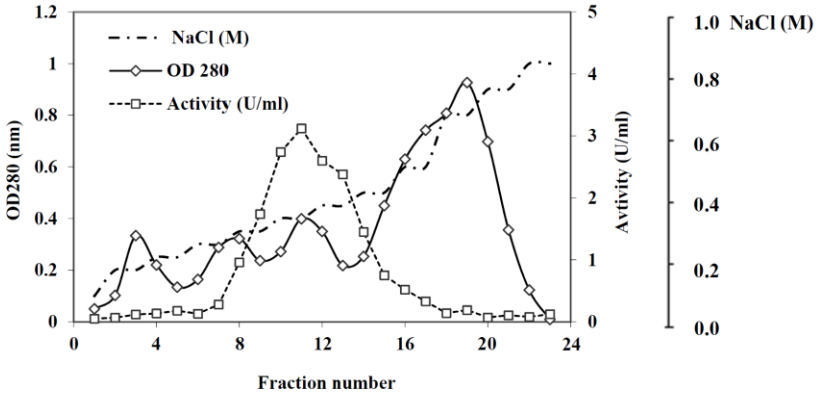

Figure. 3. Ion-exchange chromatography plots. The maximun enzyme activity was eluted in fraction number 11 by 0.4 M sodium chloride in buffer A which described in material and methods.

Table 1. the summary of purification steps of the esterase

Purification step	Volume (ml)	Lipase (U/ml)	Protein(mg/ml)	Specific activity (U /mg)	Fold	Yield (%)
Crude extract	200	2.48	6.8	0.36	1	100
85% ammonium sulfate	14	10.35	3.4	3.04	8.45	29.2
Q-Sepharose	5	21.12	1.5	14.8	41.12	21.3

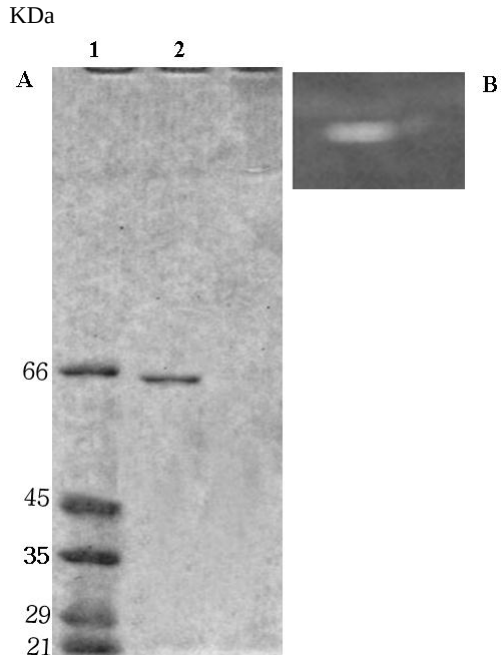

Figure. 4. SDS-PAGE (**A**) and zymogram (**B**) analysis of the isolated enzyme. In (A): Lane 1, indicates protein ladder (Sigma, USA); lane 2, the purified sample. The enzyme showed a molcular weight of 62.3 kDa. (B): About 10 µg of the purified esterase was analyzed on 10% native PAGE. It was appeared as the light band on the background.

SDS-PAGE and zymogram

After purification, according to SDS-PAGE, a sharp band with a molecular weight of 62.3 kDa was observed (Fig. 4A). Zymogram also showed the activity of the enzyme on gel electrophoresis (Fig. 4B). Most of the reported lipolytic bacterial enzymes have a molecular weight of 28 to 81.9 kDa [23-24]. The molecular weight of our purified enzyme was near to that reported for *Rhodococcus sp*. MB1 (65 kDa)[24].

Optimum pH and temperature

For an optimal pH, the enzyme activity was examined at different pH values (pH 3.0-8.0) using *p*NPA as substrate. The enzyme exhibited high levels of activities in the pH range of 6.2 to 7.5 with very low activity below pH 5.0. Specially, the enzyme presented over 70% of its maximal activity in the pH range of 6.2-6.8 (Fig. 5a). The optimum pH of the isolated enzyme and PPL was found to be 6.5 and 8, respectivley. The pH-dependent activity of our esterase indicated probably existence of one histidine residue having a pKa near to 6.5 which may be involved in catalytic triad of the enzyme active site as observed in other esterases [24, 25], although further study is needed to confirm such conclusion .

Our results showed that the enzyme was active over a broad range of temperature (45 °C to 75 °C), and the maximum activity of the isolated enzyme and PPL after incubation for 20 min, were observed at 58 °C and 37 °C respectively (Fig. 5b). In Fig. 6, thermal inactivation of the isolated enzyme at different temperatures was shown. The esterase from *Shewalenlla* showed a half-life of 70 and 31 min at 60 °C and 80 °C, respectively. No activity was observed for PPL at 60 °C and 80 °C (data not shown). The optimal temperature of the isolated enzyme was higher than that of family VIII alkaline esterase [2], while the optimum temperature was lower in comparison with esterases isolated from *Thermus thermophilus* HB27 (80 °C at pH 8) [13] and *Thermus scotoductus* SA-01 (80 °C at pH 7) [26].

Substrate Specificity

The enzyme exhibited very narrow substrate specificity as it showed 100 % and 70% its activity towards two para-nitrophenyl esters (*p*NPA and *p*NPB respectively), whereas 15% activity towards long-chain one (pNPP) (Fig. 7a). The preference to hydrolysis short-chain esters by the enzyme, demonstrates that it is an esterase rather than a lipase. These results are supported by some reported studies of thermophilic enzymes. Most of characterized lipolytic enzymes have a higher affinity to short or medium chain-length substrates (C2–C10), e.g., two esterases EstA and EstB

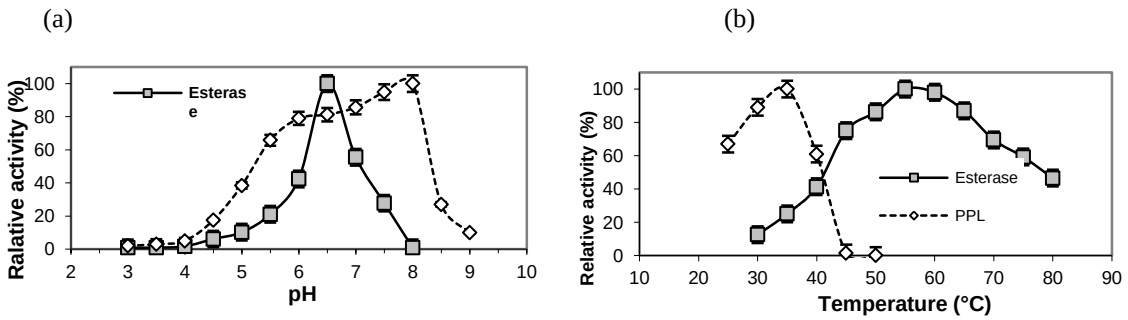

Figure. 5. The pH and temperature on activity of isolated esterase and PPL. In the (**a**) effect of pH on the enzyme activities were shown. (**b**) Effect of numerous temperatures on the enzyme activities.

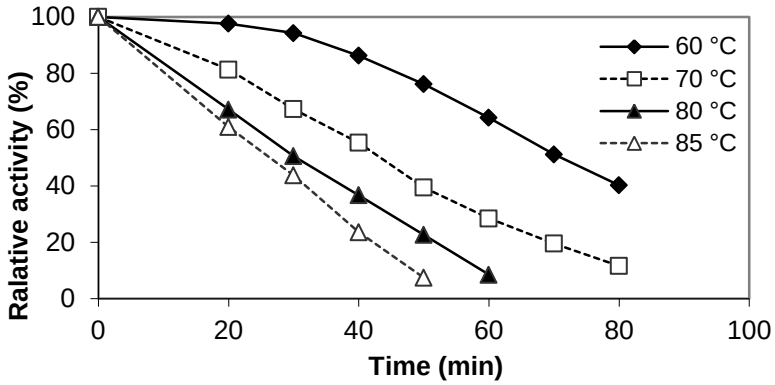

Figure. 6. Thermal inactivation of isolated esterase at various temperatures (60, 70, 80 and 85°C) for different times.

from *Picrophilus torridus* have shown the preference for C2 esters [27]. The maximum activity towards p-nitrophenyl butyrate (C4) has been reported for an esterase from *Thermus scotoductus* SA-01 [26]. EST53 from *Thermotoga maritime* [12] and E34Tt from *Thermus thermophilus* HB27 [13] have exhibited the greatest affinity towards p-nitrophenyl decanoate (C10). The esterase encoded by ORF PF2001 from *Pyrococcus furiosus* showed the best tendency towards 4-methylumbelliferyl-heptanoate (C7) [28] as well as the maximum affinity of carboxylesterases from *Sulfolobus solfataricus* P1 was towards p-nitrophenyl octanoate (C8) [29]. In Fig. 7b, K_m and V_{max} values of the isolated enzyme towards *p*NPA as substrate were obtained to be 12.6 mM and 550 $U.mg^{-1}$, respectively. The resulted K_m value was lower than that of *Bacillus subtilis* DSM402 (BS2), which showed a 119 mM K_m value towards *p*NPA (C2) as substrate [9]. The recombinant Est25 esterase exhibited the highest activity towards *p*NPA (K_m = 1.0 mM and V_{max} = 63.7 $U.mg^{-1}$) [25]. The K_m and V_{max} values for a thermostable esterase from *Bacillus* sp towards *p*NPB (C4) as substrate were 62.89 µM and 833.33 $U.mg^{-1}$ protein, respectively [23]. For the most industrial enzymes, the K_m values are in the range of 10^{-1} to 10^{-5} M [23].

The effect of metal ions on activity

The effect of metal ion concentrations (2 and 5 mM) on enzyme revealed that the activity decreased in the presence of different metal ions, including $CaCl_2$, $FeCl_2$, $CuCl_2$, $ZnCl_2$, $MgCl_2$ and $MnCl_2$ (Fig. 8). The enzyme showed more inactivation in the presence of Zn^{2+} and Fe^{2+} ions (5 mM) with respect to others. A 93% esterase activity was observed at 5 mM concentration of EDTA. Our finding indicates that the enzyme does not catalyze a cofactor demanding hydrolytic reaction, which is confirmed by previous reports [16]. Divalent ions such

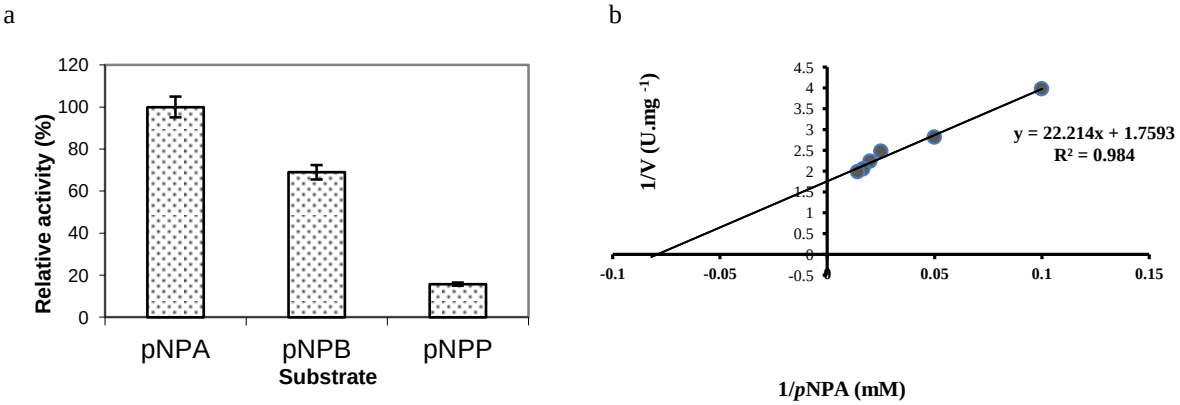

Figure. 7. Substrate specificity and kinetic parameters of the purified esterase. (**a**) The enzyme showed the maximun acticity towards *p*NPA as substrate rather than *p*NAB and *p*NPP. (**b**) Double-reciprocal Lineweaver-Burk plot for determining kinetic parameters (K_m and V_{max}) of *p*NPA.

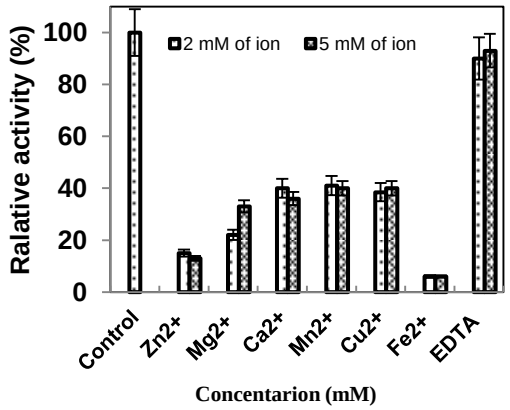

Figure. 8. Effect of divalent metal ions on the enzyme. The activity of esterase without any reagent was taken as 100%.

as; Mg^{2+}, Co^{2+} and Cu^{2+} led to decrease in esterase enzyme from *Pseudoalteromas* sp 643A, but the enzyme activity was more stimulated (30%) by Ca^{2+} ions [30]. *Pseudoalteromas* sp 643A enzyme exhibited a Ca^{2+}-independent activity. This finding has been described for some reported lipases such as a lipolytic enzymes isolated from *Bacillus pumilus* B26 [31] and *Streptomyces fradiae* k11 [32]. The calcium-independent lipase which is likely to function effectively in the presence of chelating agents (such as EDTA), is usually found in the laundry detergents. Some metal ions may affect the bonds between side chains of amino acids in the enzyme active site and act as non-competitive inhibitors, or by stabilizing or destabilizing of enzyme conformation modulate the enzyme activity [33].

The above-described features of the isolated enzyme have not been reported previously elsewhere for *Shewanella* genus. The data indicates that the enzyme is a thermophile and metal-sensitive enzyme in its own type acting at a limited range of pH and a broad range of temperature, as well as the enzyme has a high affinity towards the small chain para-nitrophenyl esters, which corresponded to the new features of the esterase subfamily. The novel esterase displayed a reasonably high thermal stability, so future research may be relevant for a better understanding of its catalytic capabilities. The thermostable esterase enzymes such that described here are commercially significant for their potential use in industries.

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