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RESEARCH ARTICLE

A novel organic-solvent and detergent resistant esterase from *Bacillus* sp. isolated from Bazangan Lake

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ABSTRACT

This study reports biochemical features of a novel esterase of *Bacillus* sp. HP 96 isolated from Bazangan Lake in Iran. The optimum pH and temperature for 53.4 kDa-purified esterase were 8 and 40 °C, respectively. The HPLC technique was used to examine the main compound produced by the enzyme on nitrophenyl derivatives. V_{max} and K_m of esterase towards 4-nitrophenyl butyrate (*p*-NPB) were 75.12 μmol.min^{-1} mg^{-1} and 4.66 mM, respectively. Organic solvents (15, 30% *v/v*) did not have a significant effect on the stability of the purified enzyme. The *Bacillus* sp. HP 96 esterase showed a high compatibility towards various ionic liquids and detergents. In conclusion, the characterized esterase has potential to be used in detergent formularizations and biotechnological processes under harsh conditions.

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KEYWORDS
Esterase; HPLC; 4-nitrophenyl butyrate; detergent; organic solvent

Introduction

Recent developments in enzymology have interested many researchers throughout the world for the production of industrial enzymes from microorganisms, especially bacteria. Hydrolytic enzymes, such as proteases, amylases, and lipolytic enzymes have a significant role with a wide range of applications in various industries, especially food processing, detergent, pharmaceutical, dairy, and beauty products (Bouacem et al. 2015; Soleymani et al. 2017; Mechri et al. 2019). Among all of these enzymes, the most significantly used ones are lipolytic enzymes (Kirana et al. 2016). Lipolytic enzymes are known as α/β-hydrolases (Ramnath et al. 2017). There are three main feature differences between esterases (EC 3.1.1.1) and lipases (EC 3.1.1.3) (Bornscheuer 2002). The first feature is the length of the acyl ester chain: 10 carbon atoms or more for lipases and 2–8 for esterases. The physicochemical nature of the substrate is the second criterion. Lipases hydrolyse emulsified substrate; while esterases are hydrolysing the monomeric substrate in aqueous solutions and they can catalyse the ester synthesis and trans-esterification in a water-free medium. Enzymatic kinetics is the third feature, which is from "interfacial Michaelis-Menten" type for lipases and classical Michaelis–Menten type for esterases (Chich et al. 1997).

Colorimetric methods can be used to measure the activity of lipolytic enzymes. Measuring the coloured-end product that comes from substrate hydrolysis needs designing a suitable chromogenic substrate. *Para*-nitrophenyl (*p*-NP) derivatives are often used to detect esterase activities. The range of measured activities depends on chemical groups, which attach to the phenol part and this group could attach to different aliphatic chains (Max et al. 2012). Esterase activity is specified by synthetic substrates, such as α- or β-naphthyl and *p*-NP esters, because the natural substrate is unknown for most of the esterases (Bhavith et al. 2014). Owning to more excellent stability, broader availability and lower production cost, microbial esterases are more significant than esterases, which are extracted from plant and animal sources (Kirana et al. 2016). Esterases do not require cofactors, and this property makes them attractive biocatalysts. Esterases are valuable in a variety of commercial applications including pharmaceuticals, dairy products, cosmetics, leather, paper, textiles and detergents (Coughlan et al.

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2015). Increasing interest in the discovery and characterization of new esterases with microbial origins causes this enzyme to be used in different sectors of industry. Purification of proteins is essential in worktime with enzymes because it provides the needed material for structural, functional and kinetic investigations (Sharma-Chander and Kanwar-Shamsher 2012). The extracellular enzymes can be purified by sequential fractionation, concentration, and chromatography techniques (Sharma et al. 2016). The primary purpose of this project was to extract and characterize a novel esterase from indigenous bacteria from the salty water of Bazangan Lake.

Materials and methods

Ultrafiltration Discs (10 kDa) were obtained from Millipore. *p*-NPP, *p*-NPA, *p*-NPB (*p*-nitrophenol palmitate, acetate and butyrate) and lipase from *Candida rugosa* lipase (CRL) were bought from Sigma-Aldrich, St. Louis, MO. Ion exchange Q-sepharose was prepared from GE and culture media from Himedia (Mumbai, India). [HMIm][Br], [EMIm][Br], [BMIm][Cl] and [BMIm][Br] were kindly prepared by Tarbiat Modares University (Tehran, Iran).

Screening and isolation of esterase producing organisms

Water samples and soil were collected from Bazangan Lake in September of 2017. Of 1 mL liquid sample was diluted in 9 mL deionized water and 100 μL of resulting suspension was inoculated on the nutrient agar plates. The bacteria were incubated for 48 h at 37 °C to isolate single colonies, then transferred to the nutrient agar plates supplemented by 0.001% (*w/v*) rhodamine B and 3% (*v/v*) olive oil (Rabbani et al. 2013). The optimized temperature of the bacteria was specified in a medium of 3.0% (*v/v*) olive oil, 0.5% (*w/v*) peptone and 0.5% yeast extracts (Asoodeh and Ghanbari 2013). The medium was incubated by shaking for 48 h at 50, 42, 37 and 25 °C. The samples were centrifuged at 8000 × *g* and 4 °C for 20 min. The OD_{600} nm and OD_{410} nm were determined to evaluate the growth of the bacteria, and the esterase activity.

Identification of the lipolytic strain

The DNA from the elected strain was extracted by SinnaGen Kit (Iran, Tehran). Primers of 1492 R and 27 F were used to the PCR amplified of the *16 s rDNA* genes. The quality and size products were determined with gel electrophoresis (1% (*w/v*) agarose gels) (Joseph et al. 2012). The PCR products were analysed by Macrogen Company (Seoul, South Korea). The closest phylogenetic relatives were determined by using MEGA version 5 program (Lane et al. 1985). The *Bacillus* sp. HP96 strain was deposited at the GenBank database with the accession number of MHO46775.

Esterase purification

In this study, the optimized medium was incubated at temperature of 37 °C for 20 h. For doing so, one litre of a medium was separated by a separator funnel. This medium included oil and water-soluble compounds, and the outlet was transferred via a filter paper. After centrifuging at 4 °C, the cell-free crude enzyme was provided. To precipitate proteins, ammonium sulphate was gradually added to reach 85% (*w/v*) saturation. The precipitation was isolated by centrifuge and dissolved in Tris–HCl, 20 mM. To eliminate residual ammonium sulphate, the enzyme needs to be dialysed against Tris–HCl buffer for 20 h at 4 °C. After that, Q-sepharose ion-exchange chromatography was used to fractionize the crude enzyme. The elution was conducted with a linear NaCl gradient in 20 mM Tris–HCl buffer (pH 8.0). After collecting fractions, the protein content and enzyme activity were measured at 280 and 410 nm, respectively.

Esterase activity assay

The hydrolysis of *p*-NPA was used for assaying esterase activity. The reaction was performed under the condition of the final volume of 0.6 mL of a mixture containing 0.05 mL enzyme solution, 0.05 mL of *p*-NPA 10 mM (in ethanol) and 0.5 mL Tris–HCl buffer 20 mM. The reaction was incubated at 40 °C for 30 min. A solution of 50 mM Na_2CO_3 (0.15 mL) was used to stop the reaction. The amount of free p-NP was evaluated by reading the absorbance of 410 nm (Sharma and Kaur 2008). Bovine serum albumin (BSA) was utilized to determine protein concentration.

Zymography and gel electrophoresis

SDS-PAGE with separating gel 15% (*w/v*) and stacking gel 7.5% (*w/v*) was used to examine the molecular mass of the esterase. Sample buffer contained 1% SDS in 0.25 M Tris–HCl buffer (pH 6.8), 10% glycerol, 5% β-mercaptoethanol (β-ME) and 0.05% bromophenol blue. For 10 μL of sample buffer was added to 40 μL of protein sample and boiled in water bath for 5 min.

The gel was visualized by Coomassie Brilliant Blue R-250. The molecular mass of esterase was estimated with the help of Vivantis protein ladder (Davis, CA, USA). For zymography analysis, the gel was incubated in renaturation buffer containing 2.5% Triton X-100 (Tris–HCl, 20 mM, pH 8) for 35 min at 25 °C. Afterward, the gel was put on a 2% agar plate containing 3% olive oil, 0.001% (*w/v*) rhodamine B, and was incubated for 24 h at 37 °C. After exposing the gel to UV light, an orange band on the plate indicated the enzyme activity.

Statistical analysis

All data were analysed utilizing one-way ANOVA Tukey post test to determine statistical significance. The level of significance was set at $p<.05$. The results of esterase activity were expressed as mean ± standard deviation (SD). Experiments were performed in triplicate.

Biochemical properties of the esterase

Effect of pH, temperature on the esterase activity

The esterase activity was assayed at 40 °C with different pH values (3.0–10.0) by using Tris–HCl buffer (pH: 8–10, 50 mM), sodium phosphate buffer (pH: 5.5–8.0, 50 mM) and sodium acetate buffer (pH: 3–5.5, 50 mM). The temperature range between 30 and 80 °C with an interval of 5 °C was applied and the effect of temperature was performed on the esterase activity. CRL was used as control. Formula $t_{1/2} = \ln 0.5/Kd$ was used to calculate the half-life value; Kd is an inactivation constant, which is estimated from the slope of the thermal inactivation curve. The determination of half-life was tested at optimum temperature and residual activity was assayed at 30, 40, 50, 60 and 70 °C for 8 h.

The effect of inhibitors, detergents and metal ions on the esterase

In the presence of different metal ions (at 1, 5 and 10 mM) , including Zn^{2+}, K^{+}, Cu^{2+}, Ca^{2+}, Fe^{2+}, Ba^{2+}, Mn^{2+}, Hg^{2+}, Mg^{2+}, Ni^{+}, Na^{+} and Co^{2+}, the enzyme activity was determined. A sample without any salt was taken as control and the enzyme activity was expressed as relative activity. The effects of detergent on the esterase activity were investigated with 1% and 5%, cetyltrimethylammonium bromide (CTAB), Triton X-100, Tween 80 and sodium dodecyl sulphate (SDS). The effects of inhibitors on the esterase activity were investigated with 1, 2 and 5 mM concentrations of ethylenediaminetetraacetic acid (EDTA), 1,10-phenanthroline, NaN_3 (sodium azide), dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), 5,5-dithiobis-2-nitrobenzoicacid (DTNB) and 2-ME. The activity of the esterase was taken as 100% at the beginning of the reaction.

The effect of ionic liquids and organic solvents on the performance of the esterase

Various organic solvents, such as toluene, methanol, ethanol, isoamyl alcohol, chloroform, hexane, isopropanol and acetone were provided at 15 and 30% (*v/v*) and were used to check their effects on the activity. The esterase activity was measured by 0.05 mL enzyme solution, mixed with 0.05 mL of 10 mM *p*-NPA in ethanol, 0.1 mL organic solvent and 0.4 mL buffer. Moreover, the effect of different ILs ranging from 2 to 10% (*v/v*) was investigated at optimum temperature and pH 8.0 on the esterase activity and the remaining activity was analysed by spectrophotometry method.

Determination of kinetic parameters

The lipolytic activity of esterase was studied at substrate concentration 0.01 M by using various substrates, such as methyl4-nitrobenzoate (C2), 4-nitrophenyl butyrate (*p*-NPB) (C4), acetate (C2) and palmitate (C16). K_m and V_{max} were determined by Eadie–Hofstee, Lineweaver–Burk and Michaelis–Menten.

Substrate specificity, the analysis of enzymatic reaction products

HPLC method was used to examine substrate specificity. The enzymatic reaction was accomplished with $0.0 - 3.0$ mg/mL of substrate in Tris–HCl buffer (20 mM, pH 8.0). The substrate hydrolysis was initiated by adding the esterase and incubating at 40 °C. C_8 reverse-phase Macherey–Nagel column (0.46×25 cm, Duren, Germany) was applied to measure *p*-NP concentration. A mixture acetonitrile and H_2O (50:50 *v/v*) was used as elution solvent at flow rate of 1 mL/min. Approximately, 20 μL of sample was subjected into the RP-column and the elution was monitored by recording the absorbance at 415 nm. Each experiment was repeated three times. A sample without enzyme was used as a control.

Results

Esterase production

By using of consecutive cultures, two pure isolates were obtained. They were named as small and big based on the colony's shape. The small strain was

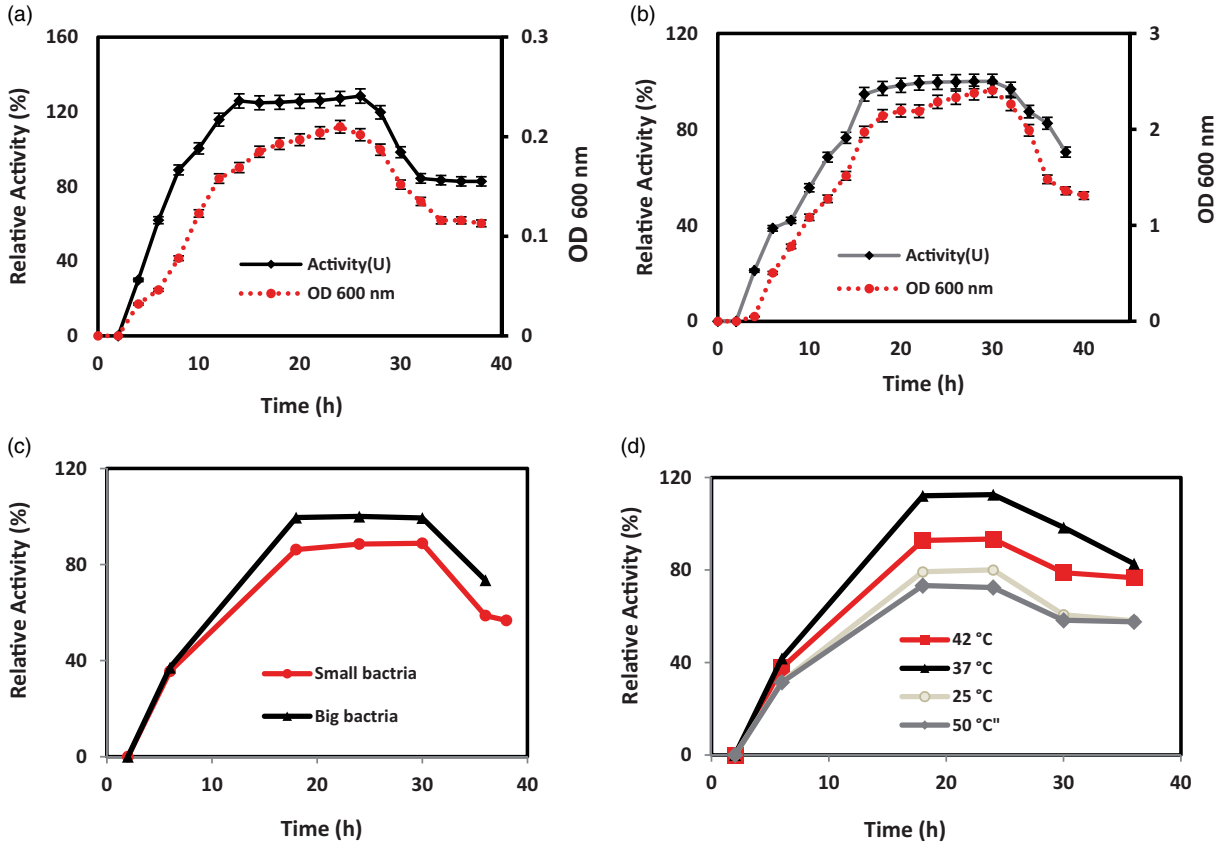

Figure 1. Growth curve and lipolytic activity of the two selected strains of bacteria (a). Big strain (*Bacillus* sp. HP96) (b). Small strain (*Serratia* sp. SP96). Comparison of lipolytic activity of the two selected strains (c). Effect of different temperatures on the activity of esterase obtained from *Bacillus* sp. HP96 (d).

Gram-negative, while big bacterial strain was Gram-positive. Appearing an orange fluorescent colour around the colony under UV light showed the lipolytic activity of bacteria (Figure 1(a,b)). Among the two strains, the big strain (hereby called *Bacillus* sp. HP 96) was selected because of its higher lipolytic activity (200 U/mg) for further analyses (Figure 1(c)). The selected bacterium was cultured at different temperatures of 50, 42, 37 and 25 °C for 48 h. The maximum enzyme production occurred after 18 h of the incubation and the optimum temperature of bacterial growth was 37 °C at OD_{600} nm. After 2 h, the logarithmic phase started and reached to the stationary phase near 16 h (Figure 1(d)). The esterase activity was improved in the stationary phase or the late logarithmic phase (Gururaj et al. 2016).

Phylogenetic analysis of the strains

With the help of analysing16S rDNA of the isolated strains, one strain was specified as *Serratia* sp. SP96 (MHO45859: NCBI Genbank accession number) had more than 99.5% similarity to family Enterobacteriaceae and another strain of *Bacillus* sp. HP96 (MHO46775: NCBI Genbank accession number) had more than 99% similarity to *Bacillus thuringiensis*. In addition, the phylogenetic tree confirmed that the strain of *Serratia* sp. SP96 was most closely related to *Serratia marcescens* (JOVM00000000: NCBI Genbank accession number), and the strain of *Bacillus* sp. HP96 was most closely related to *Bacillus cereus* (AP007209: NCBI Genbank accession number) (Figure 2(a)).

Esterase purification

Purification steps are summarized in Table 1. The ion-exchange chromatography showed that the esterase could be purified in the concentration of 0.15 M NaCl in 20 mMTris–HCl buffer with pH 8. The purified esterase was obtained by a yield of 80.81% and a 46.45-fold increase in the specific activity by comparing it with the initial crude enzyme. The molecular mass of 53.37 kDa was confirmed with the existence of a single band gel electrophoresis for the new esterase. In addition, the lipolytic activity of this esterase was specified with seeing a colour band in zymogram gel (Figure 2(b)).

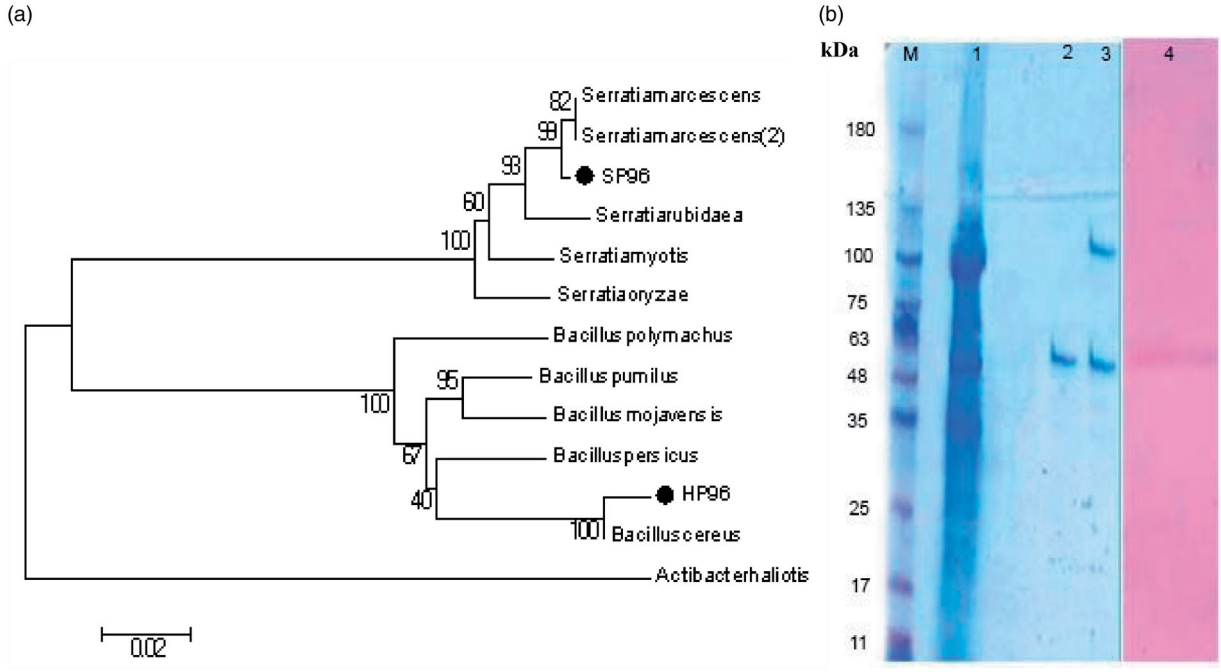

Figure 2. Phylogenetic tree based on *16S rRNA* gene sequence displaying the relationship between strain *Bacillus* sp. HP96 and *Serratia* sp. SP96 and other bacteria species (a). SDS-PAGE electrophoresis of a lipolytic enzyme from *Bacillus* sp. HP96 (b). Lane M: protein markers (11–180 kDa); Lane 1: crude enzyme; Lane 2: The purified enzyme from Q-sepharose; Lane 3: The precipitated fraction by 85% ammonium sulphate; Lane 4: represents homogeneity of the purified enzyme on the gel.

Table 1. Purification data of esterase from *Bacillus* sp. HP 96.

Purification step	Specific activity (U/mg)	Total protein (mg)	Total activity (U)	Fold	Yield (%)
Crude extract	4.30	28.45	122.5	1.0	100
Ammonium sulphate precipitation	75.66	1.5	113.5	17.59	92.65
Q-Sepharose	200	0.495	99	46.51	80.82

The effect of pH and temperature on esterase activity

Figure 3(a) shows the effect of various pHs on the esterase activity. Purified esterase was active over a broad pH range (5.0 < pHs >9.0). The highest lipolytic activity was observed for CRL and the esterase at pH 8.0. With the interval of 5 °C, the effect of temperature on the esterase activity was assayed from 30 to 80 °C. The maximum activity of novel esterase was at 40 °C and the esterase preserved 85% of its maximum activity at the temperature of 35–55 °C (Figure 3(b)).

Thermal-stability and half-life of the esterase

The stability of enzymes is a desirable characteristic and offers an advantage because of potential industrial applications. The purified esterase was almost entirely stable, at 30, 40 and 50 °C at the end of 2 h-incubation, 84, 77 and 72% of the original enzyme activity were retained, respectively. After 8 h-incubation at 60 and 70 °C, it was seen that *Bacillus* sp. HP96 esterase was not stable at high temperatures (Figure 3(c)).

The residual activity assay was carried out at 70, 60, 50, 40 and 30 °C. The esterase had a half-life of 210 min at 40 °C, also 150 min at 50 °C (Figure 3(d)). The maximum activity of CRL was at 37 °C and its half-life was100 min, whereas its activity decreased at 50 °C.

The effect of some metal ions on the esterase activity

Figure 4(a,b) shows the effect of some metal ions on *Bacillus* sp. HP96 esterase. Metal ions by changing the solubility of fatty acids can alter the catalytic function of the enzyme and increase or decrease or inhibit the enzyme activity. Our results showed that metal ions of Fe^{2+}, Ca^{2+}, Co^{2+} and K^{+} mildly increased the esterase activity at 10 mM, whereas the esterase activity severely increased with 5 mM Mn^{2+} and 10 mM Mg^{2+}. Similar results were observed for CRL.

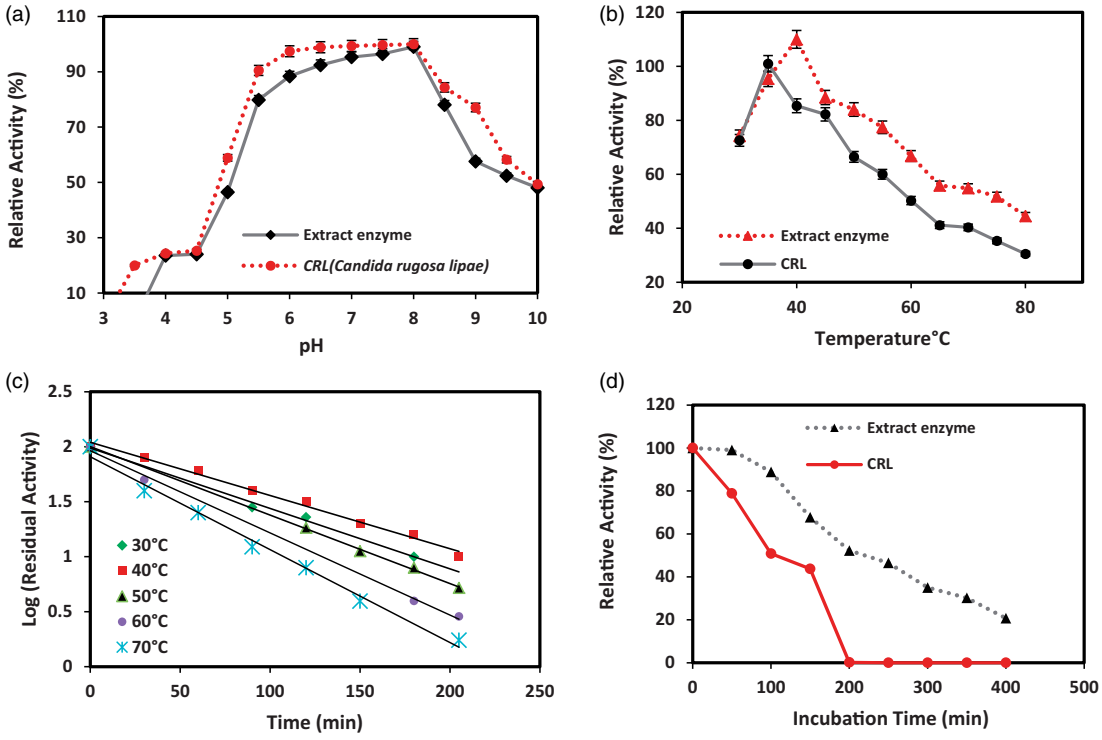

Figure 3. Effect of pH on the activity of the purified esterase (a). CRL (*Candida rugosa* lipases) (●) was tested as standard sample under the same conditions as the purified enzyme. The standard errors were less than 5% of the means. Temperature-activity profile and residual activity of the purified enzyme (b). In temperature-activity profile, the activity of the enzyme at maximum (40 °C) was taken as 100%. The CRL (●) was tested as standard enzyme that showed a maximum activity at 37 °C. Thermal-stability of the purified esterase was assayed at 30, 40, 50, 60 and 70 °C for 8 h (c). Sampling carried out at 30 min intervals. The residual activities were measured by standard assay conditions. Control with non-incubated enzyme was considered as 100% activity. Determination of half-life of *Bacillus* sp. HP96 and CRL in optimum temperature (d).

The effect of inhibitors and detergent on esterase activity

Table 2 shows the effect of inhibitors (EDTA, PMSF, NaN_3, 1, 10-phenanthroline, DTNB, 2-ME and DTT) at 1, 2 and 5 mM concentrations on the catalytic activity. The purified enzyme might be a metalloenzyme because it showed sensitivity to the inhibitory effect of EDTA. The disulphide bonds of the enzyme are reduced by DTT; also, DTT could prevent the creation of inter or intramolecular disulphide bonds between cysteine residues of esterase. The enzyme activity was stimulated by 96.55, 96.24, and 100%, respectively, in the presence of 5 mM 2-ME, NaN_3 and 1, 10-phenanthroline but remained nearly unchanged in the presence of DTNB. The unchanged effect of DTNB on the enzyme activity indicated that the enzyme might not have free SH group(s) in its structure.

Detergent making is common now in most countries with the help of enzymes. Table 3 shows the effect of detergents on the esterase activity. To check the effect of denaturing agents on the purified enzyme, each of the selected compounds (CTAB, SDS, Triton X-100 and Tween 80; 0.5 and 1% *w/v*, *v/v*) was included separately in the reaction mixture. The esterase of *Bacillus* sp. HP96 was found to be highly stable towards detergents. In reaction mixture, containing 1% *v/v* CTAB (cationic detergent), SDS and Triton X-100; the enzyme activity retained 96.24, 95.46 and 98.12%, respectively. The lipolytic activity was enhanced by SDS and CTAB. The esterase activity was inhibited in the presence of Tween 80 (1%), whereas the enzyme activity did not change by 0.5 and 1% *v/v* Triton X-100.

The comparison of the results of esterase with CRL showed that the reaction mixture containing 1% (*v/v*) detergent esterase towards ionic and non-ionic detergents was much more resistant (Table 3). This esterase demonstrated excellent compatibility towards the ionic and non-ionic detergents. It also suggests it has great potential for using in detergent formulations.

Organic solvents and ILs effects on esterase function

Figure 4(c) shows the influence of various organic solvents at the concentrations of 15 and 30% *v/v* on esterase activity. Non-polar organic solvents such as

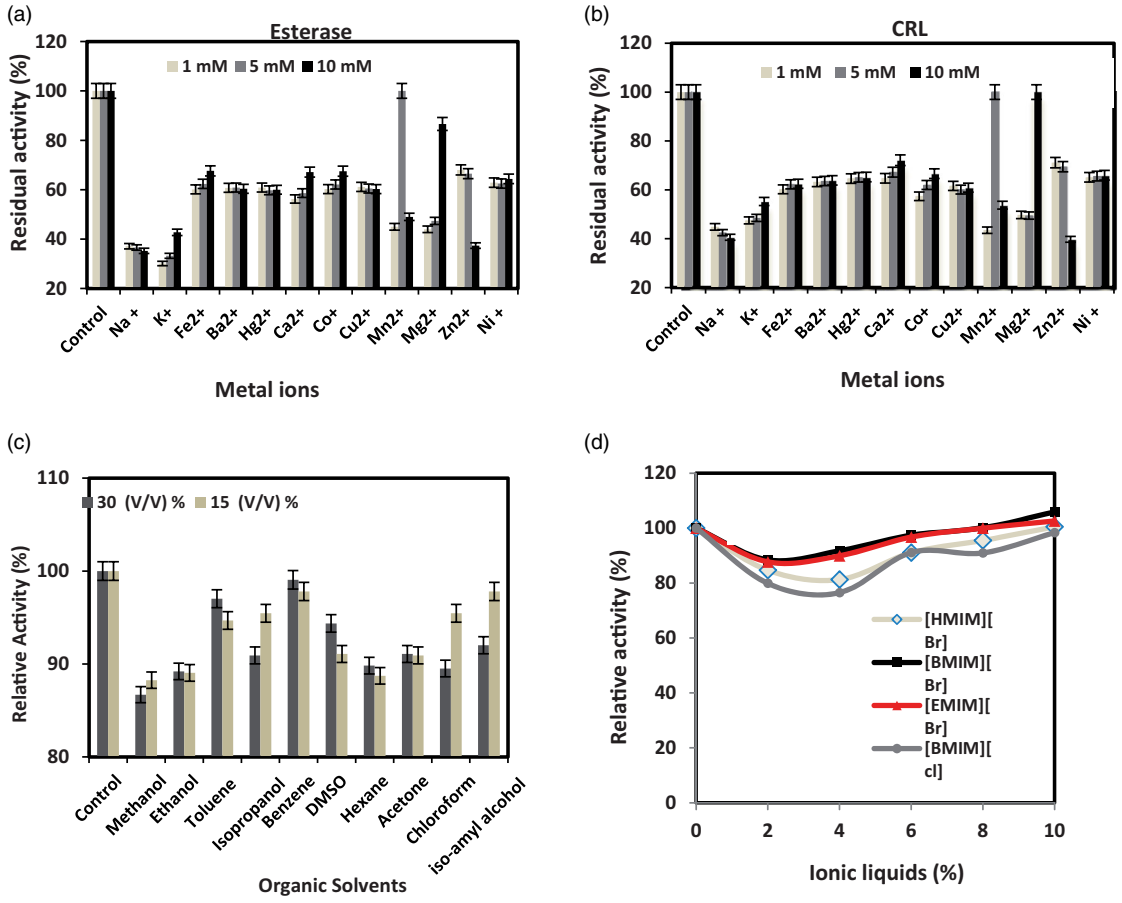

Figure 4. Effect of metal ions on the activity of esterase of *Bacillus* sp. HP96 and CRL. The residual enzyme activity was measured under the standard assay conditions. Activity without metal ions was taken as control (100%). The reaction mixture was incubated at 40 °C for 30 min in the presence of various metal ions at concentrations of 1, 5 and 10 mM. Effect of metal ions on the activity of esterase (a). Effect of metal ions on the activity of CRL (b). Activity of the extracted enzyme in various organic solvents at 15 and 30% (*v/v*) concentration (c). Influence of various imidazolium-based ionic liquids at 2–10% (*v/v*) concentrations on the activity of esterase from *Bacillus* sp. HP96 (d).

Table 2. Effect of inhibitors on *Bacillus* sp. HP 96 esterase and CRL (*Candida rugosa* lipases) activity.

	Relative activity (%)					
	1 mM		2 mM		5 mM	
Inhibitors	Esterase	CRL	Esterase	CRL	Esterase	CRL
Control[a]	100	100	100	100	100	100
NaN_3	77.77 ± 1	81.53 ± 1.22	89.82 ± 1.02	89.82 ± 1.65	96.24 ± 1	96.87 ± 1.32
DTT	92.64 ± 0.65	93.11 ± 1	90.89 ± 0.61	91.42 ± 1.34	89.20 ± 0.54	89.33 ± 1
EDTA	97.02 ± 0.58	97.80 ± 1.5	97.33 ± 0.42	97.49 ± 2.11	91.39 ± 0.55	92.01 ± 1.25
DTNB	97.80 ± 0.92	98.12 ± 0.7	97.96 ± 0.99	98.43 ± 0.75	97.90 ± 0.73	98.43 ± 0.81
PMSF	97.49 ± 0.62	98.59 ± 2	92.59 ± 0.6	99.06 ± 2.04	89.20 ± 0.66	93.89 ± 2.1
Beta-ME	91.39 ± 0.43	91.70 ± 1.5	96.08 ± 0.47	97.02 ± 1.64	96.55 ± 0.47	97.02 ± 1
Phenanthrene	92.17 ± 0.7	92.64 ± 2.9	98.59 ± 0.73	98.90 ± 2	100 ± 0.006	98.59 ± 2

[a]The enzyme solution without inhibitor was used as control. Enzyme samples were incubated with various inhibitors for 30 min and then remaining activity was assessed under the standard assay protocol. Results are presented as means ± standard deviation ($n = 3$).

toluene, benzene and DMSO (30% *v/v*) have improved the enzyme activity. It seems that in the presence of organic solvents of the closed form of the enzyme is converted into an open form, thereby stimulating the enzyme and increasing enzyme activity.

In this study, the *Bacillus* sp. HP96 esterase retained more than 90% of its own activity in the presence of hexane, acetone, chloroform, isopropanol and isoamyl alcohol. A lipolytic enzyme from *Bacillus licheniformis* MTCC 6824 retained its own activity in the presence

of ethanol and methanol, while chloroform inhibited this enzyme (Chakraborty and Paul-Raj 2008). A recombinant esterase from *Sulfolobus solfataricus* P2 had the least activity in the presence of ethanol and methanol (15 and 30%) (Shang et al. 2010).

Molten salts at low temperatures (ILs) were investigated due to their peerless chemical features such as viscosity, non-volatile nature and solubility. Figure 4(d) indicates that the enzyme activity has a direct relation with the concentration of different ILs. The stimulatory influence of Br-containing ILs on esterase activity was as follows: [BMIm][Br] > [EMIm][Br] > [HMIm][Br] > [BMIm][Cl]. The results revealed that the esterase activity was enhanced with the increase in alkyl chain length of the imidazolium ring of ILs and had greater stability in ionic liquids than in traditional organic solvents. ILs could block non-polar active site of the isolated esterase with the help of their alkyl groups. Worth mentioning, ILs are made the most exploitation for esterases catalysed reactions (Fan et al. 2018).

Table 3. Effect of detergents and oxidant agent on esterase and CRL activity[a].

	Relative activity (%)			
	Concentration 0.5 (%)		Concentration 1 (%)	
Detergents	Esterase	CRL	Esterase	CRL
Control[a]	100	100	100	100
SDS	90.61 ± 0.75	87.16 ± 1.5	95.46 ± 0.71	90.24 ± 1.76
CTAB	90.76 ± 0.52	82.80 ± 1.98	96.24 ± 0.32	93.71 ± 2.06
H_2O_2	97.01 ± 1.22	92.24 ± 0.7	97.02 ± 0.98	96.21 ± 0.81
Triton X-100	98.02 ± 0.64	92.4 ± 2	98.12 ± 0.69	97.15 ± 2.42
Tween 80	96.87 ± 1	95.46 ± 1.37	91.7 ± 1.02	90.85 ± 1.52

[a]The enzyme was incubated with various concentrations of detergents of at 40 °C for 30 min, and assayed under standard assay conditions. The enzyme incubated without detergents and oxidizing agents was used as a control. Each value presented here is an average of three replicates.

Substrate specificity and kinetic parameter

By using different *p*-NPs with acyl chain lengths C2, C4, and C16, the specificity of the enzyme was examined (Figure 5(a)). *p*-NPP with C16 (longer chain length) changed a little, whereas *p*-NPA was hydrolysed quite well. Results displayed that *p*-NPB was an appropriate substrate for the purified esterase; therefore, *Bacillus* sp. HP96 esterase prefers short fatty acid esters as the substrate. By increasing the substrate concentration, the interaction of the active site with its specific substrate increased; thereby, the active sites of the enzyme are saturated. The effect of substrate concentration (*p*-NPB) on esterase activity was determined via Michaelis–Menten, Lineweaver Burk

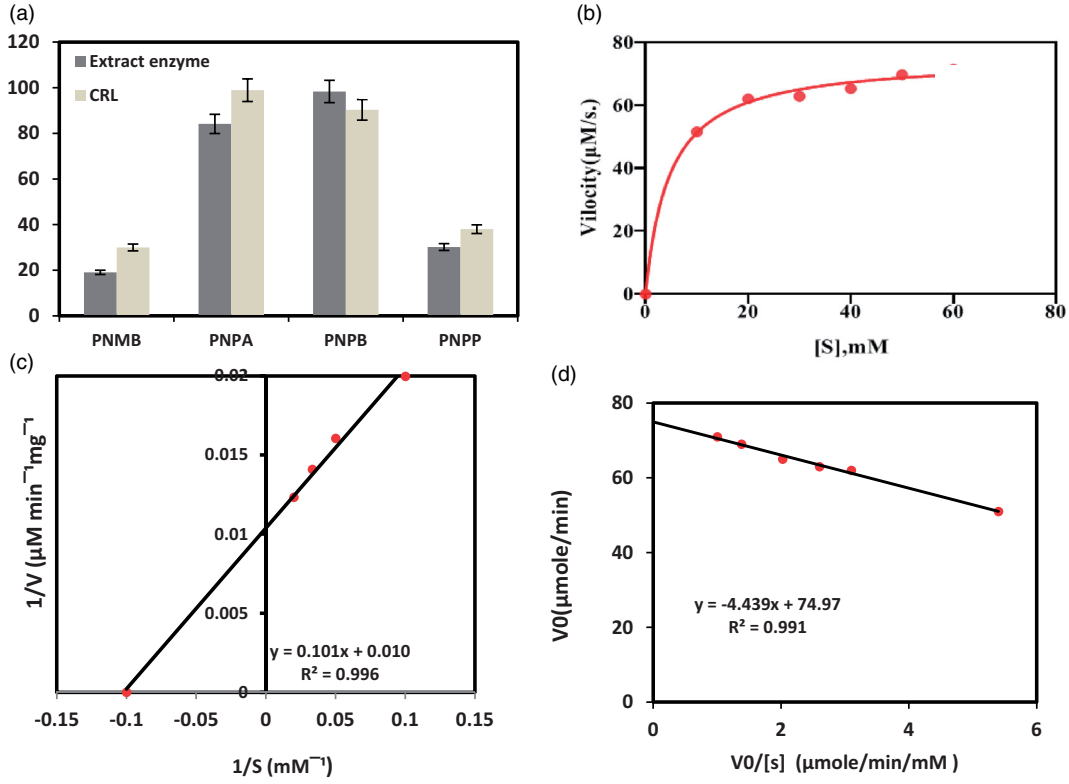

Figure 5. The activity of *Bacillus* sp. HP 96 esterase against different *p*-nitrophenyl esters (*p*-NP) (a). Esters of various lengths were assayed at 40 °C in 10 mM and 20 mM Tris-buffer (pH 8.0). Michaelis–Menten (b); Lineweaver–Burk plots (c); and Eadie–Hofstee diagram (d). The value of kinetic parameters was calculated by GraphPad Prism version 8 software using 10–60 mM of *p*-NPB concentration in 20 mM Tris-buffer (pH 8.0) under shaking at 40 °C.

plots and Eadie–Hofstee diagram (Figure 5(b–d)). V_{max}, K_m and V_{max}/K_m values are shown in Table 4.

The kinetic constants of K_m showed that a small quantity of *p*-NPB could saturate the enzyme when the K_m had a low value. V_{max}/K_m ratio was moderate for long-chain esters, but it was small for short-chain esters. *p*-PNA was used as the best substrate for CRLs and the V_{max}, K_m and V_{max}/K_m parameters for CRL were found to be 103 μmol min^{-1} mg^{-1}, 10.43 mM and 9.87, respectively.

The results indicated that the affinity (K_m) of CRL for *p*-NPA was lower than the affinity of the esterase for *p*-NPB; therefore, it was found that *p*-NPB was an appropriate substrate for the isolated esterase.

Table 4. K_m and V_{max} values of the enzyme determined by Michaelis–Menten, Lineweaver–Burk plots and Eadie–Hofstee diagram for *p*-NPB.

Plots	K_m (mM)	V_{max} (μmol min^{-1} mg^{-1})	V_{max}/K_m
Michaelis–Menten	4.66	75.12	16.11
Lineweaver–Burk	10	100	10
Eadie–Hofstee	4.43	74.97	16.88

Utilization of HPLC for substrate specificity analysis

Figure 6 depicts the results of HPLC analysis. The absorbance of *p*-NP at 415 nm increased when the *p*-NP ester conversed into *p*-NP and its corresponding acyl group. There are two peaks that one of them occurred at 5.66 min, which was related to *p*-NP; another peak occurred at 15.88 min and was referred to *p*-NPB. To calculate the amount of p-NP, the area of peaks was used straightly. The concentrations of *p*-NP were found to be 2.18 mg/mL, 1.77 and 1.20 mg/mL following the activity of CRL, control and esterase, respectively. The peak area ratio (%) of *p*-NP/*p*-NPB was obtained to be 50.5, 30.9, and 29.2% for *Bacillus* sp. HP96 esterase, CRL and control (without enzyme) that showed the efficiency of the isolated esterase was higher than standard enzyme (CRL).

Results obtained by HPLC confirm the results of the previous steps and show that *Bacillus* sp. HP96 esterase prefers short fatty acid esters as the substrate and highlights the highest hydrolytic activity of the purified enzyme towards *p*-NPB.

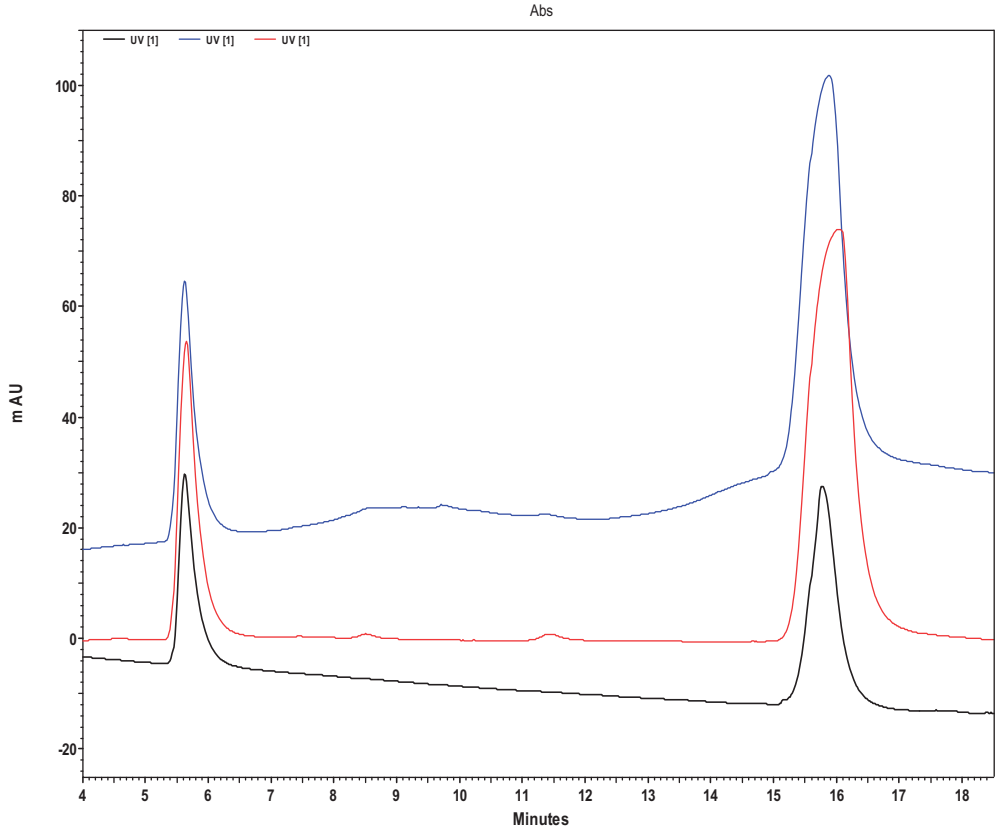

Figure 6. HPLC analysis of *p*-NP-derivatives hydrolysed by esterase. Using the peak area of releasing *p*-NP, the affinity of HP96 esterase towards *p*-NPB was assessed. In the chromatogram, the black trace represents products of HP96 esterase, while the chromatogram of spontaneous hydrolysis of *p*-NPB products is shown in blue (control) and CRL is shown in red. Analysing the products showed two main peaks occurred at 5.66 and 15.88 min that were related to *p*-NP and *p*-NPB, respectively.

Discussion

In this study, a *Bacillus* strain was isolated from Bazangan Lake that produced a new extracellular esterase. The enzyme was purified by Q-sepharose chromatography. The esterase of *Bacillus* sp. HP96 was archived by a purification fold of 46.45. Esterases isolated from *Salimicrobium* sp. LY19 (Xin and Ying 2013) and *Geobacillus* sp. TF17 (Ayna et al. 2013) showed a purification fold of 6.9 and 42.7. Molecular masses of esterase from various sources are the range of 20–70 kDa. The purified enzyme in this study had the molecular mass of 53.4 kDa, whose molecular mass was less than the esterase of *Salimicrobium* sp. LY19 (57 kDa) and more than the lipolytic enzyme of *Bacillus subtilis* RRL 1789 (52 kDa) (Kaiser et al. 2006).

Optimum pHs of esterases in different studies were between pH 5-9. Herein, the purified enzyme revealed an optimal pH of 8 that was consistent with those reported for *Bacillus subtilis* RRL1789 (Kaiser et al. 2006). The optimum pH values for esterases obtained from *Bacillus* sp. K91(Ding et al. 2015) and *Bacillus subtilis* (Eggert et al. 2000) were 7.5 and 5, respectively.

A maximum temperature of *Bacillus* sp. HP96 esterase was 40 °C. This feature was similar to two esterases of *Cucurbita pepo* Ell and Elc with an optimum temperature 40 °C. Previous studies reported a various optimal temperatures for esterases obtained from *Bacillus* sp. (45 °C) (Fahmy et al. 2008), *Bacillus licheniformis* S-86 (60 °C) (Torres et al. 2008), and *Fervidobacterium nodosum* Rt17-B1 (75 °C) (Yu et al. 2010). The purified esterase had a remarkable activity in the range of 25–50 °C.

Lipolytic activity of the esterase and CRL was inhibited nearly 37.4 and 39.7% in the presence of 10 mM Zn^{2+}. The same effects of Zn^{2+} on the activity of different esterases were observed for some isolated lipases, which had been isolated from *Bacillus* subtilis FH5 (Hasan et al. 2007) and *Bacillus licheniformis* MTCC 6824 (Chakraborty and Paul-Raj 2008). This study showed that K^{+} and Mg^{2+} ions (at 10 mM) increased the esterase activity by 42.7 and 86.6%, respectively. The same findings were observed for an esterase of *Anoxybacillus gonensis* G2 (Colak et al. 2005).

EDTA as a chelating agent had decreasing impacts on the activity of extracted enzyme and CRL thereby confirming the requisite of metal ions for the enzyme activity. A lipase of *Bacillus amyloliquefaciens* PS35 was also inhibited by EDTA (Kanmani et al. 2015). In comparison with other lipolytic enzymes, such as *Bacillus ubonensis* SL-4 (Yang et al. 2016), the initial activity of the enzyme was preserved more than 89% in the presence of PMSF (1, 2 and 5 mM). Taken together, it seems that our esterase similar to alpha/beta-hydrolases possesses a third catalytic site comprising serine residue in which the substrate was easily accessible to the active site and also can inhibit be PMSF. The non-ionic detergent of Triton X-100 inhibited the denaturation process of the esterase by reducing the interfacial tension at the oil/water interface. Stability in organic solvents is an advantage for the purified *Bacillus* sp. HP96 esterase. The tendency of using non-polar solvents for biocatalysts was more than polar solvents. Regarding this feature of the isolated esterase, it could be used in the field of ester synthesis.

The valuable range for K_m in biotechnology applications is from 10^{-1} to 10^{-5} M (Robinson 2015). The esterase of *Bacillus* sp. HP96 towards *p*-NPB revealed K_m and V_{max} values of 4.66 mM and 75.12 μmol $min^{-1} mg^{-1}$ by the use of the same substrate, K_m and V_{max} values were 0.056 mM and 19.38 μmol $min^{-1} mg^{-1}$ for an esterase extracted from *Geobacillus sp*. TF17 respectively (Ayna et al. 2013). Comparing results of these two esterases (*Bacillus* sp. HP96, *Geobacillus sp*. TF17) show that with a slight change in solution conditions, such as pH and temperature, the value of K_m esterase of *Bacillus* sp. HP96 can be improved.

To sum up, the results clearly demonstrated remarkable stability of esterase in the presence of many commonly used organic solvents, ionic liquids, detergents and metal ions. The isolated esterase revealed a higher efficacy in hydrolysing *P*-NPB in comparison to CRl. Owing to a high stability, a neutral pH range and the lipolytic activity at moderate temperature, the application of *Bacillus* sp. HP 96 esterase is suggested in the fields of detergent formulation and biotechnological processes.

Ethical approval

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

Disclosure statement

No potential conflict of interest was reported by the author(s).

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