Purification and Characterization of An Esterase from *Halobacillus trueperi* whb27

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A Gram-positive bacillus designated as strain whb27 with high esterase hydrolysis activity was screening from 35 strains of moderately halophilic bacteria isolated from brine and sediment of saltern in Weihai, China. Phenotypic characterization and phylogenetic analysis based on 16S rDNA sequence comparison indicated that the strain was *Halobacillus trueperi*. Whb27 grew optimally at 7% NaCl, pH 7.5 and 28°C. A halophilic esterase was purified to homogeneity from the crude extract of whb27 by ammonium sulphate fractionation followed by ion exchange and gel permeation chromatography, with a 42-fold purification. The molecular mass of the enzyme was about 35 kDa as assessed by polyacrylamide gel electrophoresis. The enzyme showed maximum activity in the presence of 2.5M NaCl. The optimum temperature and pH were 42°C and 8.0, respectively. The Ba^{2+} and Fe^{2+} ions inhibited the activity of the esterase while Ca^{2+}, Mn^{2+} and Zn^{2+} ions stimulated its activity. In the presence of EDTA, SDS, DMSO, glycol and acetone, the esterase still displayed some activity, but the activity could be inhibited significantly by phenyl methyl sulphonyl fluoride (PMSF). One interesting point is that organic solvents like ethanol, glycerol, propylene glycol, PEG4000 stabilized or enhanced enzymatic activity, making the enzyme very useful in some industrial process.

Key words: Halophile; *Halobacillus trueperi*; Esterase; Purification; Characterization.

Enzymes have attracted much attention in recent years because of their wide range of application in physiology, analytical chemistry and industrial area and the enzymes from microorganisms is of great value due to their broad biochemical diversity, feasibility of mass culture and ease of genetic manipulation[1]. A considerable amount of effort has been dedicated to the study of extracellular salt-tolerant enzymes from moderately halophilic bacteria. Moderately halophilic bacteria are able to grow over a wide range of salt concentrations with optimum growth at 0.5–2.0 M. The halophilic enzymes perform identical functions as their non-halophilic counterparts but require salt, usually NaCl or KCl in the 1–4 M range, to keep their activity, solubility and stability[2-4]. Enzymes with optimal activity at high-salt concentrations are useful for many harsh industrial processes, where concentrated salt solutions otherwise inhibits many enzymatic conversions[5-7].

Microbial lipolytic enzymes also called esterases or lipases, catalyzing both the hydrolysis and synthesis of ester compounds, are among the most important hydrolytic enzymes and have been studied extensively. This kind of enzymes can be

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utilized in laundry, food, oil chemistry, fine chemistry, pharmaceutical and paper industries, as well as in biodiesel production, waste treatment and other biotechnological applications[8-10]. However, in spite of a growing interest in the use of halophilic enzymes for biotechnological applications, there are only a few reports in the literature about their production and characterization. Therefore, screening of halophiles with lipolytic activities could facilate the discovery of novel esterases with unique properties.

In our screening program for finding novel esterases, we determined the lipolytic activity of 35 strains isolated from the saltern in Weihai (China). Our results showed that one of the holipholic strain, *Halobacillus trueperi* whb27 displayed high lipolytic activity. The esterase (E.C.3.1.1.1) was purified to homogeneity, and its physic-chemical property was also studied.

MATERIALS AND METHODS

All the chemicals used were of analytical grade

Strains and culture conditions

The halophilic strains were isolated from brine and sediment at Shuangdao Saltern in Weihai, Shandong Province, China. The bacterial strains were routinely cultured in modified Gibbons medium consisting of (g/L): MgSO4 $7H_2O$, 20.0; KCl, 2.0; trisodium citrate, 3.0; casamino acids, 5.0; and yeast extract 10.0. The pH of the medium was adjusted to 7.5 and the salinity was adjusted to 7.0 prior to autoclaving. Agar plates contained 1.5 % (w/v) agar. The culture was incubated aerobically on a rotary shaker set at 180 rpm under the desired combination of salt concentration, pH and temperature. Growth status was determined by measuring the OD value of the culture at 600 nm.

Screening lipolytic activity of the strains

The strains were cultured for 2-7 days and the fermentation medium was collected by centrifugation at 10000 g for 10 min at 4°C. An aliquot of supernatant was removed to check lipolytic activity using the following two approaches: Rhodamine B-olive oil agar plates approach and a colorimetric method. The rhodamineB-olive oil agar plate approach was based on the spread plate technique[11, 12]. Briefly, growth medium contained 2.5% olive oil (w/v) and 0.001% rhodamine B (w/v) was spread into plates and holes with 3-mm-diameter, and the agar were punched and filled with cell-free culture supernatant. The plates were incubated for 16 h at 37°C and monitored with UV light at 350 nm. A colorimetric method[13] was also performed to check esterase-catalyzed hydrolysis activity. Briefly, 0.5 ml of the working substrate solution of PNPB (PNP-esters of butyrate) was added to 1 ml enzyme solution. Working solution contained: 1 ml of 15 mM substrate in 2-propanol and 9 ml of 66.67 mM Tris–HCl buffer, pH 8.0 NaCl 2.5 M. The enzyme reaction mixture was incubated at 42°C for 10 min and the OD value at 410 nm was measured using a spectrophotometer (Schimadzu UV-1205). One unit of enzymatic activity was defined as the amount of enzyme that liberated 1µmol of pNPP per minute under the test conditions. The enzyme activity was determined under standard assay conditions every 12 h for 1 week.

Morphological and physiological observation

Morphological and physiological characteristics of the isolates were studied either on nutrient agar or in nutrient broth. Standard biochemical tests were performed as described previously[14].

Phylogenetic analysis

Genomic DNA for PCR amplification was extracted from the cells using TIANamp Bacteria DNA Kit (Tiangen, China), following the manufacturer's instructions. The 16S rDNA gene was amplified by PCR using total DNA as the template and the sequences of the primers were as follows: forward primer, 16F27(5'-AGAGTTTGATCCTGGCTCAG-3'); reverse primer, 16 R1492 (5'-GGTTACCTTGTTACGACTT-3'). The PCR products were purified using TAKARA Agarose Gel DNA Purification Kit Ver.2.0 (Takara, Japan) and cloned into the pMD 18-T vector. After ligation, the plasmids were transformed into the competent cells of *E. coli* DH5α. The positive clones were identified by blue-white colony assay and its sequence was further confirmed by sequencing analysis. The multiple alignments were done by the ClustalW program. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4.0. The phylogenetic tree was constructed by the maximum parsimony method and bootstrap analysis (1000 replications) was used to validate the reproducibility of the branching patterns of the trees.

Optimization of growth conditions

The effects of temperature, pH and salt concentration on the growth status were determined using colorimetric method. The range of temperature in our experiment was 5, 10, 15, 25, 28, 30, 33, 37, 40, 45, and 55°C. The pH range was adjusted between 5 and 10 (5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10). The effect of salt on growth was studied by varying the NaCl concentrations (0–20%, w/v).

Esterase purification

All purification steps were performed at 4°C except specification. The strain was cultured in 2.5 M NaCl medium at certain conditions as described above. The cultured medium was collected by centrifugation at 10,000 g for 15min. Ammonium sulphate was added slowly to the cell free culture supernatant to reach 70% saturation. The solution was allowed to stand overnight at 4°C. The precipitate was collected by centrifugation at 10,000 g for 15 min, and dialyzed against Tris buffer (20 mM, pH 8.0) using dialysis tubing (Himedia, Mumbai, India) with 5 kDa cut off value. The dialyzed fraction was loaded to a DEAE-Sepharose Fast Flow (GE Healthcare Bio-Sciences AB) column at a flow rate of 0.2 µL/min, and washed with the same buffer. The bound proteins were then eluted by NaCl solution with gradient from 0 to 0.5 M in the same buffer. Protein concentrations were measured using BCA protein assay reagent (Pierce, USA), with bovine serum albumin as a standard. Fractions with high esterase activity were pooled and concentrated by lyophilization. The partially purified enzyme was loaded onto a sephadex-G-100 column (Pharmacia/ GE Healthcare Bio-Sciences AB) and eluted with Tris buffer (pH 8.5), and fractions with esterase activity were collected and lyophilized.

Determination of the molecular weight of the esterase

SDS-PAGE was performed to estimate the molecular weight of the purified esterase using 5% stacking gel and 12% resolving gel according to method of Laemmli[15]. Protein bands were detected by staining with 0.1% Coomassie brilliant blue R-250. Molecular weight was estimated by comparing the relative mobility of proteins of different molecular size using a standard molecular weight marker.

Physi-chemical properties of esterase from Whb27

Effects of pH, temperature and NaCl on the activity and stability of esterase

To study the effect of temperature on the activity of the purified enzyme, the reaction mixture was incubated at certain temperatures ranging from 22 to 52°C for 30 min, and the enzymatic activity was analyzed to determine the effect of temperature on the enzyme activity. Similar experiments were performed to determine the effect of pH or NaCl on the enzyme activity.

Effects of ions on activity of esterase

Purified esterase was dissolved in 100 mM Tris-Cl buffer (20 mM, pH 8.0) containing the following metal ions, Ba^{2+}, Ca^{2+}, Cu^{2+}, Fe^{2+}, K^{+}, Mg^{2+}, Mn^{2+} and Zn^{2+} respectively. After pre-incubated for 30 min at 42°C, the enzymatic activity was analyzed using the method as described above.

Effects of esterase inhibitors and organic solvent on enzymatic activity

To study the effect of different inhibitors of esterase on the purified enzyme, aliquots of the enzyme were pre-incubated with certain inhibitors, including PMSF (5 mM), SDS (10%, W/V), DMSO(10%, V/V) and EDTA (20 mM) at 42°C for 30 min. Residual activities were measured and compared with a control (without inhibitors).

The effect of organic solvent on the enzyme activity was also studied. Briefly, the enzyme solution was mixed with certain organic solvents (5%, V/V) and incubated at 42°C with constant shaking for 30 min. Residual activity of an aliquot was measured by the standard assay. Activity in the absence of any additives was taken as 100%.

RESULTS

Characteristics of the esterase-producing bacteria

Thirty-five strains of halophiles were selected for their potential to produce esterase activity. Significant lipolytic activity was observed in culture supernatants from 15 strains, as determined using RhodamineB-olive oil agar plate approach. Among these, strain whb27 exhibited prominent esterase activity (i.e., secreted

Table 1. Phenotypic properties of strain whb27 and selected reference organism

Characteristics	Strain whb27	*H. trueperi* whb45
Cell morphology	rods	rods
Colony color	yellow	yellow
Gram-reaction	+	+
Spore formation	+	+
Nitrate reduction	—	—
Hydrolysis of D-Glucose	+	+
Hydrolysis of gelatin	+	+
Starch hydrolysis	—	—
Voges-Proskauer reaction	+	NR
Indole	+	NR
Catalase	+	+
Oxidase	+	+
Growth temperature range(°C)	10 -45	4 -40
Optimal growth temperature	28	34
pH range	6.0–9.5	6–10
Optimal pH	7.5	7-9
Salt range for growth(%)	1-15	0-25
NaCl optimum(%)	7	7.5

+ Positive - negative NR not reported Data from Chen L[16]

Table 2. Summary of the purification of esterase from *H. trueperi* whb27

Purification step	Total protein(mg)	Total activity(units)	Specific activity(U/mg)	Purification fold	Recovery (%)
Crude extract	756	589	0.78	1.0	100
Ammonium sulphate precipitation	63.8	332	5.2	6.7	56.4
DEAENion exchange	9.1	193	21.2	27.2	32.8
Sephadex gel filtration	2.5	82	32.8	42.1	13.9

Purification procedure is described in Materials and Methods. Experiments were done in triplicate.

Table 3. Effects of various metal ions on esterase activity

Metal ions and chemicals	Relative activity (%)
Ba^{2+}	31
Fe^{2+}	74
Cu^{2+}	82
K^{+}	104
Mn^{2+}	119
Mg^{2+}	93
Zn^{2+}	127
Ca^{2+}	146

Remaining activity was measured using the standard method with pNPP. Final enzyme activity was calculated relative to control activity (enzyme reaction without the reagent listed in the table).
All experiments were conducted in triplicate and the average of the 3 values is reported.

significant amounts of esterase). This strain was selected for further optimization of extracellular esterase production.

Colonies derived from strain whb27 were circular, raised, and orange and measured 2–3 mm in diameter after incubation for 2 d at 28°C on Gibbons agar. Based on morphological, physiological, biochemical, and 16S rRNA studies (Table 1), the strain was identified as *Halobacillus trueperi*. A 1460-bp segment of the 16S rRNA gene from this strain was sequenced and submitted to NCBI GenBank (accession no. FJ444973.1). A phylogenetic tree (Fig. 1) constructed by the maximum parsimony method indicated that strain whb27 was in the same clade within the genus *Halobacillus*, showing the highest similarity (99%)

Table 4. Effects of chemicals on esterase activity

Chemicals	Relative activity (%)
EDTA	88
SDS	89
DMSO	76
PMSF	48
Methanol	88
Glycol	67
Ethanol	95
Glycerol	95
Acetone	75
Propylene glycol	99
Polyethylene Glycol(PEG) 4000	112
PEG 6000	112
PEG 10000	83

Remaining activity was measured using the standard method with pNPP. Final enzyme activity was calculated relative to control activity (enzyme reaction without the reagent listed in the table).
All experiments were conducted in triplicate and the average of the 3 values is reported.

with *H. trueperi* XJSL8-7. The other related validated strain, *H. salinus* HSL-3, had a similarity of 97%. The levels of 16S rRNA gene similarity between strain whb27 and other species used in the phylogenetic analysis were lower than 95.0%.

Growth conditions affect the productivity of esterase

Strain whb27 could grow in the presence of 1%–15% NaCl with an optimum growth at 7% NaCl. The optimum temperature for growth was 28°C, and no growth was observed above 45°C or below 10°C. The optimum pH for growth was 7.5. Table 1 presented the growth status of the strain from pH 6.0–9.5. The enzyme activity was determined from the medium at the end of the exponential phase (48 h) and into the stationary phase. Maximum esterase production was observed at the late stationary phase (96 h).

Purification of esterase from whb27

The enzyme was purified to homogeneity by ammonium sulfate fractionation followed by ion exchange and gel permeation chromatography. A summary of the purification of esterase from whb27 is shown in Table 2. Approximately 42-fold purification of the crude enzyme was achieved, with a yield of 14%. Under optimal conditions, the activity of the final purified enzyme was about 32.8 U/mg protein. SDS-PAGE analysis of the purified esterase revealed a single band, and the apparent molecular weight of the purified esterase was about 35 kDa (Fig. 2).

Effects of pH, temperature, and salt concentration on esterase activity

The optimum pH for the activity of the enzyme was 8.0, and the enzyme was active over a broad range of pH values (6.5–9.5; Fig. 3). The purified enzyme showed quite good activity over a temperature range of 22–52°C, and optimum activity was observed at 42°C (Fig. 4). The effects of different salt concentrations on esterase activity were tested over the concentration range of 0–5 M (Fig 5). As shown in Fig 5, there was little activity in the absence of added NaCl. The activity increased with salt concentration up to 2.5 M NaCl and then decreased again.

Effects of metal ions on enzyme activity

The effects of various metal ions on the activity of purified enzyme are shown in Table 3.

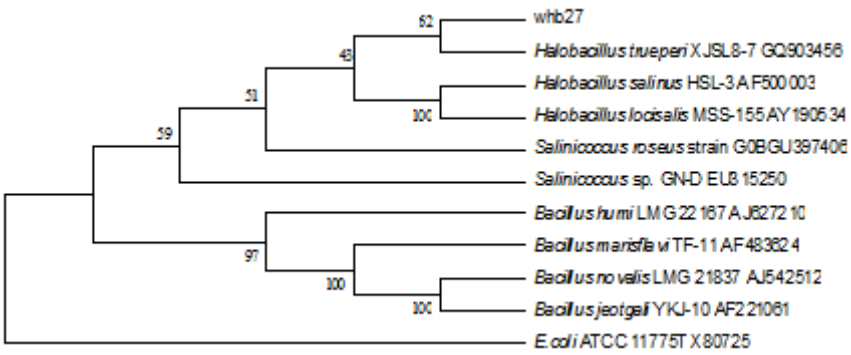

Fig. 1. Maximum parsimony tree showing the position of isolate whb27 among related species. The 16S rRNA gene sequence of *E. coli* ATCC 11775T was used as the outgroup. The numbers at branching points refer to bootstrap values based on 1000 replications

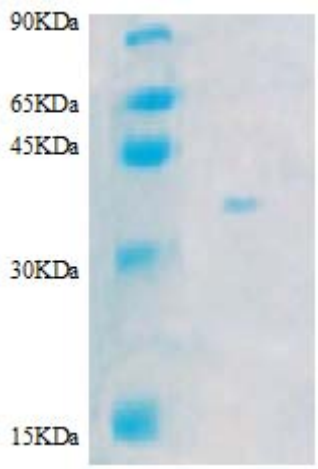

Fig. 2. SDS-PAGE analysis of the purified esterase from whb27. The assay was conducted as described in Materials and Methods section. Lane 1 was the protein molecular weight marker, while lane 2 represented the purified esterase

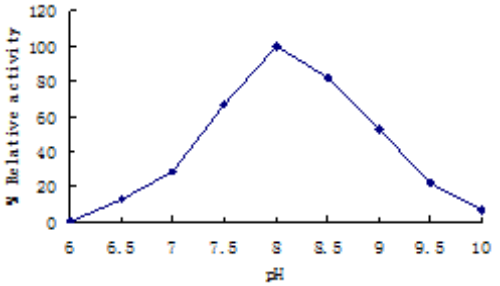

Fig. 3. Effects of pH on esterase activity. The activity was determined according to the standard assay with different pH. The relative activity of the enzyme at pH 8.0 was defined as 100%. Each value represents the mean ± S.E. of three independent experiments

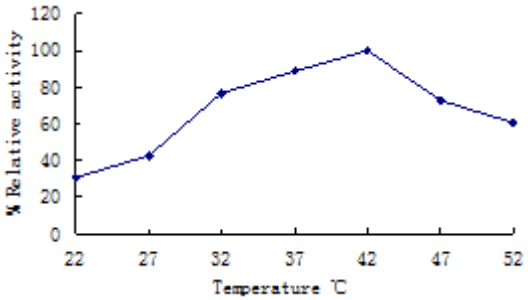

Fig. 4. Effects of temperature on esterase activity. Activity was determined according to the standard assay at temperatures from 22 to 52°C. The relative activity of the enzyme at 42°C was defined as 100%. Each value represents the mean ± S.E. of three independent experiments

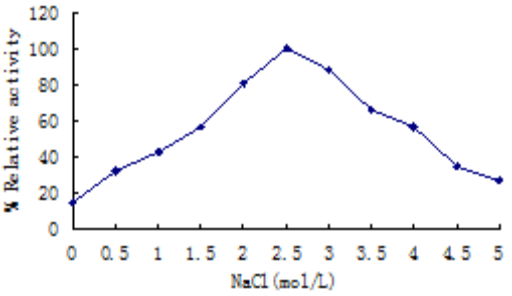

Fig. 5. Effects of temperature on esterase activity. The activity was determined according to the standard assay with concentrations of NaCl from 0 to 5 mol/L. The relative activity at 2.5M NaCl was defined as 100%. Each value represents the mean ± S.E. of three independent experiments

Esterase activity was stimulated in presence of calcium ions (Ca^{2+}) and other divalent cations, such as Mn^{2+} and Zn^{2+}, were also found to potentiate enzyme activity, but not to the extent of Ca^{2+}. Esterase activity was inhibited by Ba^{2+} and Fe^{2+} ions. Metal ions such as K^{+} and Mg^{2+} had almost no effect on esterase activity.

Effects of chemicals on enzyme stability

As shown in Table 4, the enzyme was not significantly inhibited by the specific metalloenzyme inhibitor EDTA, but was strongly inhibited with the serine enzyme inhibitor phenylmethylsulfonyl fluoride (PMSF). The anionic detergent SDS showed a slight inhibitory effect on esterase activity.

Next, we examined the effects of organic solvents on esterase activity. As shown in Table 4, the enzyme was quite stable in the presence of methanol, ethanol, glycerol, and propylene. However, decreased enzyme activity was observed in the presence of DMSO, glycol, and acetone, while PEG4000 and PEG6000 enhanced the enzyme activity slightly.

DISCUSSION

The relationship between growth and enzyme secretion has been studied in halophilic organisms in some published works. Like most lipolytic emzymes found from other halophilic bacteria[17,18], esterase from strain whb27 was secreted at the end of the exponential phase of growth and reached its maximum level in late

stationary phase after 96 h of incubation. This indicates that esterase production was independent of the growth phase of the organism. Most of lipolytic enzymes were identified from the Metagenomic library[19,20] or isolated by constructing a strain genomic DNA library followed by screening for lipolytic enzymes[21]. In this study, we identified a moderate halophilic strain, and the esterase was purified using a rather direct, less expensive approach. The study provides evidence that marine resource is a valuable source for finding novel esterase.

The molecular masses for different halophilic esterases/lipases have been reported ranging from 15-35 kDa; 35 and 28 kDa respectively, found from a marine *Bacillus*[21]; 20 and 15 kDa, respectively from those in a salt-tolerant *Bacillus* species[17]; and 35 kDa for lipase EM3L4, identified from the metagenomic library of deep-sea sediment[19]. The molecular weight of the purified esterase was found to be 35 kDa as analyzed on SDS-PAGE similar as the lipase from the marine *Bacillus*[21].

The optimum pH (8.0) and temperature of the purified esterase was similar to other halophilic enzymes[11,17]. Many esterases/lipases were found to be optimally active at 35–45°C [11, 22, 23]. The optimal temperature (42°C) for the esterase from *H. trueperi* whb27 was in the same range. However, the temperature tolerance of this enzyme was a little lower than that of the esterase from *Bacillus* sp. BSE01 [17].

The inhibitory effects of the heavy metal ions Ba^{2+} and Fe^{2+} on the whb27 esterase were similar to their effect on the esterase from *Bacillus* sp. BSE01 [17]. As has been well documented, Ca^{2+} plays an important role in influencing the structure and function of the enzyme, and some lipases have been reported to be stimulated by Ca^{2+} [19, 24]. The enzyme from whb27 is similar with the lipase from *Staphylococcus aureus*[24]; Ca^{2+} activates the enzymatic activity.

EDTA and other chelators usually affect the enzymatic activity of esterases. However, our study confirmed that EDTA only slightly affects the activity of esterase from whb27; similar results have been reported by other investigators[17]. SDS as a detergent usually affects the esterase activity[19, 25]. It is very interesting that the esterase from whb27 is still active in the presence of SDS, suggesting that the enzyme can be used in some harsh conditions.

Although many halophilic enzymes require the presence of NaCl concentrations in the range of 1–4 M for optimum activity and stability[26-28]. Most of the esterases identified from marine sources *are not* shown any salt tolerance[29-31]. However, the esterase from Whb27 displayed the highest activity at 2.5 M NaCl, and the enzyme was active over a wide range of salt concentrations. Our study confirmed that very little esterase activity was detected in the absence of salt, indicating the halophilic nature of the enzyme; salt appears to be a prerequisite for enzyme production. The esterase activity of whb27 was re-established by adding an appropriate concentration of NaCl (data not shown). This property is quite useful, since the enzyme purification steps can be run without salt using classical chromatographic methods, and enzyme activity can be recovered in the presence of salt.

Another feature of our esterase is its activity and stability in the presence of organic solvents. There are some esterases displaying activity in the presence of organic solvents. However, their salt tolerance is quite poor[25,32,33]. And some salt tolerant esterases are not shown organic solvent resistance[34]. The stability and activity of enzymes in organic solvents depend not only on the properties of the organic solvents, but also on the nature of the enzyme[35]. Stability against SDS and organic solvents indicates that strong hydrophobic interactions may make up the stable core of the enzyme[32].

Taken together, total feature of the purified esterase is its stability in the presence of organic solvents and salt. Thus, the extracellular halophilic esterase isolated from strain whb27 can be used in several processes where high salt concentrations and hydrophobic organic solvents are present. More study is needed to address the molecular and structural properties of the halophilic esterases, to elucidate the structural basis of its salt adaptation.

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