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Characterization of Extracellular beta-amylase and Serine Metalloprotease from a Halophilic Isolate, *Halobacillus* sp. LY4

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A newly moderate halophile *Halobacillus* sp. LY4 producing extracellular amylase and protease was isolated and identified. Production of both enzymes was synchronized with bacterial growth and reached a maximum level during the stationary phase. The amylase and protease were purified to homogeneity with molecular masses of 62 and 39 kDa, respectively. Optimal amylase activity was observed at 60°C, pH 10.0 and 12.5% NaCl. It was strongly stimulated by Ca^{2+}, but inhibited by EDTA, DEPC and PAO, suggesting it was a metalloenzyme with histidine and cysteine residues located in its active site. Maltose was the main product of starch hydrolysis, indicating an β-amylase activity. The purified protease showed highest activity at 60°C, pH 9.0 and 10% NaCl. Complete inhibition by PMSF, DEPC, PAO and EDTA indicated it maybe a serine metalloprotease with histidine and cysteine residues essential for catalysis. Both enzymes were high active over broad temperature (40°C-80°C), NaCl concentration (0-20%) and pH (6.0-12.0) ranges, indicating their thermostable, halotolerant and alkali-stable nature. Also, they showed remarkable stability towards various surfactants. Results from the present study suggested the enzymes produced by *Halobacillus* sp. LY4 may have considerable potential for industrial application from the perspectives of their properties.

Key words: Moderate halophile, β-amylase; metalloprotease, purification and characterization, *Halobacillus*.

As important hydrolytic enzymes, amylases and proteases have been studied extensively because of their potential application in the biotechnological-based food, detergent, sugar and pharmaceutical industries[1]. They represent two of the three largest groups of industrial enzymes and account for approximately 85% of the total enzyme sales in the world[2]. Despite the fact that many different enzymes have been purified and characterized so far, and some have been used in biotechnological and industrial applications, the present known enzymes are not sufficient to meet most industrial demands[3]. Most industrial processes are carried out under harsh conditions, which cannot always be adjusted to the optimal values required for the activity and stability of the available enzymes. Therefore, it would be of great importance to have available enzymes showing optimal activities at extreme values of pH, temperature, and different concentrations of salts.

Moderately halophiles are a group of halophilic microorganisms able to grow optimally in media containing 3-15% NaCl [4]. Besides their important role in the ecology of hypersaline environments, these prokaryotes have received considerable interest because of their potential for use in biotechnology. Most enzymes produced by halophiles are usually not only salt-tolerant, but also may be active at high temperature and pH values[5-6]. There are many reports for extracellular amylase and protease production from halophiles, however, studies on their purification and characterization were scarce. During the screening

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for hydrolase-producing halophilic bacteria from salt lake of Yuncheng, a newly moderate halophile (strain LY4) with high amylolytic and proteolytic activities was isolated and identified. Also, purification and characterization of both enzymes was performed and characteristics of the purified enzymes were studied.

MATERIALS AND METHODS

Bacterial isolation, growth and enzyme production

The strain LY4 (CICC 10442) was isolated from saline soil in Yuncheng, China, and cultivated aerobically at 37°C in the complex medium (CM) with the following composition (g/l): casein peptone 7.5; yeast extract 10.0; sodium citrate 3.0; $MgSO_4 \cdot 7H_2O$ 20.0; KCl 2.0; $FeSO_4 \cdot 7H_2O$ 0.01; NaCl 120.0 and pH 8.0. The kinetics of bacterial growth and extracellular enzymes production were determined at different time intervals. Bacterial growth, along with enzyme activity, was measured by spectrometric method (Shimadzu model UV-160A).

Salt tolerance assays were performed by growing the isolate LY4 in CM broth plus various concentrations of NaCl (0 to 20%, w/v) at 37°C. Bacterium growth was monitored at a temperature range of 10°C-60°C and over a pH range from 2.0 to 12.0 of the culture medium.

Identification of the isolate LY4

Morphological, physiological and biochemical characteristics of the isolate LY4 were studied according to Spring *et al*.,[7]. 16S rRNA gene was amplified using the general bacterial primers 8F and 1492R. The PCR product was purified and sequenced in both directions using an automated sequencer by Seq Lab laboratory (Germany). The obtained 16S rRNA gene sequence was aligned with its closely related neighbor sequences retrieved from GenBank and a phylogenetic tree was constructed as described previously[8].

Extracellular enzymes purification

The isolate LY4 was cultivated in CM broth at 37°C with shaking for 42 h. After cultivation, the culture broth (200 ml) was centrifuged at 12000 rpm for 20 min. Various steps of enzyme purification were carried out at 4°C. Culture supernatant containing extracellular enzymes was treated with solid ammonium sulphate to 80% saturation with continuous overnight stirring. The precipitate collected was dissolved in 0.05 M Tris-HCl buffer containing 10% NaCl (pH 8.0), and dialyzed against the same buffer for 12 h with several changes to remove the salt. The enzyme preparation was then loaded on a Sephacryl S-100 gel filtration column (1.6 cm×60 cm) pre-equilibrated with the same buffer and eluted at a flow rate of 1.0 ml/min. Active fractions with amylase and protease activity were pooled and concentrated by ultrafiltration, respectively, and used as the purified enzymes for further characterization.

Polyacrylamide gel electrophoresis and zymograms

SDS-Polyacrylamide gel electrophoresis (12% acrylamide) was performed to determine the molecular masses of the purified amylase and protease according to the method of Laemmli[9]. Following running the gel, the proteins were stained with Coomassie Brilliant Blue R-250. Zymographic analysis for protease activity was performed in polyacrylamide slab gels containing SDS and gelatin (0.1%) as co-polymerized substrate, as described by Karbalaei-Heidari *et al*.,[3]. After electrophoresis, the gels were rinsed in 2.5% (v/v) Triton X-100 for 1 h to remove SDS and were incubated under optimal assay conditions (pH 9.0 and 60°C) for 20 min to perform the proteolytic activity. Finally, the gels were stained in a solution of 0.5% (w/v) amido black 10B. The activity band was observed as a clear colorless area depleted of gelatin in the gel against the blue background when destained in water-methanol-acetic acid (60:30:10) solution. Zymographic analysis for amylase activity was performed on non-denaturing electrophoresis slab gels (10% polyacrylamide) prepared with 10% of sucrose, as described by Cadenas and Engle[10]. After electrophoresis, the slab gel was laid on an agar sheet containing 0.3% soluble starch and 12.5% NaCl at pH 10.0 and left for 1 h at 60°C. The band of protein that was associated with amylase activity was seen as clear zones on a dark blue background on the replica agar sheet.

Properties of the purified amylase and protease

Amylase and protease activity assay

The amylase activity, with soluble starch as the substrate, was determined by measuring released reducing sugars using the DNS (3,5-dinitrosalicylic acid) method[11]. One unit (U) of

amylase activity was defined as the amount of enzyme necessary to produce 1 ìmol of reducing sugar per minute under the assay conditions. While protease activity, with casein as the substrate, was measured as described previously[3], with some modifications. Enzyme solution (500 µl) was added to 2 ml of 0.3% casein solution (pH 10.0), and incubated at 60°C for 5 min. The reaction was stopped by adding 2 ml of 10% trichloroacetic acid (TCA) and kept at room temperature. The supernatant was obtained by centrifugation. Blanks were prepared in which 2-ml TCA solution was added before incubation. To 1 ml of the supernatant, 1.5 ml of Na_2CO_3 solution (0.4 M) and 0.5 ml of Folin-Phenol reagent (Solarbio) were added and mixed thoroughly. The color developed after 20 min of incubation at 40°C was measured at 680 nm. One unit (U) of protease activity was defined as the amount of enzyme yielding 1 ìmol of tyrosine per minute under the assay conditions.

Effects of temperature, pH and salt on enzyme activity

To determine the optimal temperature for purified enzymes, the assay was determined under different temperatures from 30°C to 90°C. The effect of pH on their activity was studied in the 6.0-12.0 range, using the appropriate buffers at a concentration of 0.1 M (6.0-7.0, sodium phosphate; 8.0-9.0, Tris-HCl; 10.0-12.0, glycine-NaOH). Moreover, the activities of the purified enzymes were measured as above in enzyme reactions containing 0-20% NaCl under standard assay conditions.

Effects of various metal ions and chemical reagents on enzyme activity

The effects of different metal ions and chemical reagents [ethylenediaminetetraacetic acid (EDTA), SDS, Triton X-100, Tween 80, β-mercaptoethanol, phenylmethylsulfonyl fluoride (PMSF), phenylarsine oxide (PAO) and diethyl pyrocarbonate (DEPC)] on the activity of purified enzymes was examined after they had been pre-incubated with them for 30 min at 40°C, respectively, and the residual activity was determined by standard assay method. Activity in the absence of any additives was taken as 100%.

Determination of kinetic parameters

The kinetic parameters V_{max} and K_m of the purified amylase were determined by incubating the amylase with various concentrations of soluble starch ranging from 0.5% to 5.0% (w/v) under the standard conditions. While kinetic parameters of the protease were examined using various concentrations of casein ranging from 0.1% to 2.0% (w/v) under the standard conditions. V_{max} and K_m values were obtained from Lineweaver–Burk plot.

Analysis of hydrolysis products

The hydrolytic products of soluble starch were analyzed by high-performance liquid chromatography (HPLC). Amylase solution (1 ml) were incubated at 60°C with 0.5% soluble starch in Tris-HCl buffer (0.1 M, pH 10.0) containing 12.5% NaCl. After determined time intervals, samples were withdrawn and hydrolysis was stopped by boiling them. After centrifugation at 12000 rpm for 10 min, each sample was analyzed by HPLC analysis on a micro Bond pack Amino Carbohydrate column (4.1 mm×300 mm). Samples (25 µl) were injected and eluted with acetonitrile:water (70:30 ratio) at a flow rate of 1 ml/min. The hydrolyzed products were detected using a refractive index detector. Glucose, maltose, maltotriose and maltopentaose (Sigma) were used as standards.

RESULTS

Identification of the isolate LY4

Based on morphological, physiological and biochemical characteristics, the isolate LY4 is a Gram-positive, motile, rod shaped and aerobic spore-forming bacterium. Colonies are light yellow, uniformly round, circular and convex on CM agar plate. It grown well in a wide range (5-15%, w/v) of NaCl concentrations. No growth was found in the absence of NaCl. Optimum bacterial growth was observed at pH 8.0, 37°C-40°C and 10% NaCl. H_2S production, methyl red and hydrolysis of Tween-80 tests were negative, while Voges-Proskauer test, nitrate reduction, oxidase and catalase, gelatin hydrolysis were positive. Acid is produced from maltose, D-fructose, sucrose and glucose. Phylogenetic analysis based on 16S rRNA gene sequence comparisons revealed that islate LY4 (accession number in GenBank: HQ683723) fell within the branch encompassing members of the genus *Halobacillus* and was most closely related to *Halobacillus trueperi* (99.0% 16S rRNA gene sequence similarity) (Fig. 1). Thus, the strain was tentatively named as *Halobacillus* sp. LY4.

Kinetics of bacterial growth and enzymes production

As shown in Fig. 2. The lag phase of bacterial growth was short (2 h), and after 24 h the bacterial growth reached to the stationary phase. No amylase or protease activity was detected during the early-exponential growth phase. Amylase production started at the post-exponential phase (14 h), while protease was found to secrete at the mid- exponential phase (10 h). However, it was observed that both enzymes production reached a maximum level during the stationary growth phase, 30 h for amylase and 38 h for protease, respectively.

Enzymes purification and molecular weights determination

The partial purification of both enzymes was achieved by ammonium sulphate precipitation at 80% concentration. The β-amylase and protease could be well separated from the concentrated enzyme solutions by size exclusion chromatography on Sephacryl S-100. This procedure typically resulted in 24.5- and 22.3-fold purifications, with respective yields of 21.1% and 27.6% for β-amylase and protease. In addition, specific activity of the purified β-amylase and protease were 203.7 and 967.3 units/mg protein. Each enzyme preparation was tested for homogeneity by SDS-PAGE. Molecular masses of the β-amylase and protease were estimated at 62 and 39 kDa, respectively, by comparison with molecular mass standards (Fig. 3, lane 2 and 3). Meanwhile, zymogram activity staining also revealed the clear zones of proteolytic and amylolytic activity against the background for purified samples at corresponding positions on SDS-PAGE (Fig. 3, lane 4 and 5).

Properties of the purified amylase

As calculated from the Lineweaver–Burk plot (data not shown), K_m and V_{max} values of the amylase were 3.51 mg/ml and 1.31 mg/ml/min, respectively. The enzyme hydrolyzed soluble starch to form maltose as main product (Fig. 4). This product was readily apparent even during the early stages of the reaction and increased in concentration along with the time course of the reaction. Trace amounts of longer oligosaccharides (maltotetraose and maltopentaose) and glucose were also produced. Meanwhile, it was found not to hydrolyze maltose or α-cyclodextrin (data not

Table 1. Effects of various metal ions and chemical reagents on the activity of purified amylase and protease

Substances	Final concentration (mM)	[a] Residual activity (%) of amylase	[a] Residual activity (%) of protease
Control	-	100	100
Ca^{2+}	4	180.1	218.2
Zn^{2+}	4	88.4	99.0
Fe^{2+}	4	99.1	98.9
Fe^{3+}	4	94.5	97.6
Cu^{2+}	4	100	150.1
Mn^{2+}	4	98.1	100
Hg^{2+}	4	53.8	60.1
Mg^{2+}	4	99.1	99.8
EDTA	10	8.2	0
SDS	10	80.1	78.1
β-Mercaptoethanol	10	98.3	95.1
Triton X-100	10	98.1	97.2
Tween-80	10	97.8	98.0
PMSF	10	99.1	0
DEPCPAO	10 10	00	00

[a] Residual activity was determined as described in "Materials and Methods" and expressed as the percentage of the control value (without any additives). The data represent the mean values of three experiments.

shown). Hence, the enzyme may preferentially cleave at the α-1,4-linkage adjacent to non-reducing ends, releasing successive maltose units, indicating an β-amylase activity.

The β-amylase showed excellent activity over broad temperature range (50°C-80°C) and pH range (6.0-12.0) with optimal activity at 60°C and pH 10.0 (Fig. 5a and 5b). However, activity dropped

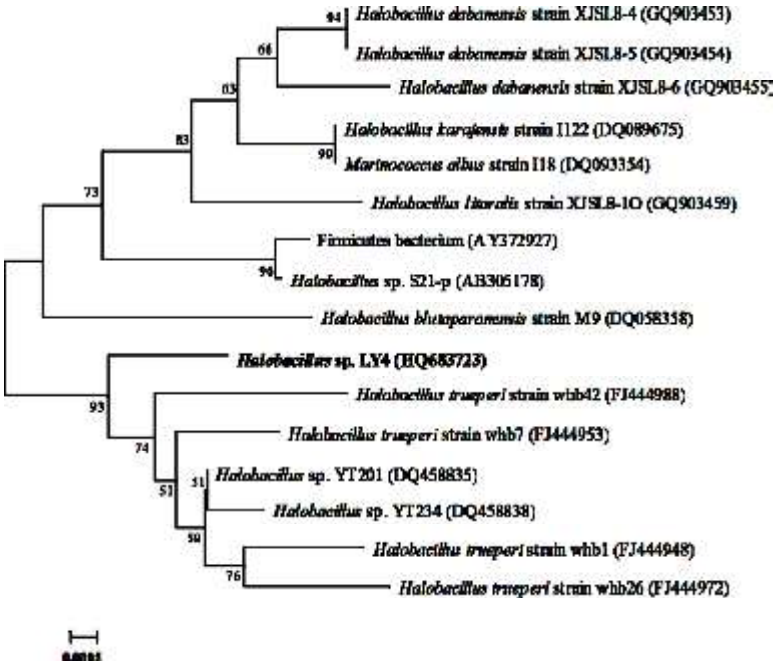

Fig. 1. Phylogenetic tree based on 16S rRNA sequences, showing the relationship of the isolate LY4 to other members of the genus *Halobacillus*. Accession numbers of the sequences used in this study are shown in parentheses after the strain designation. Numbers at nodes are percentage bootstrap values based on 1,000 replications; only values greater than 50% are shown. Bar 0.0005 substitutions per nucleotide position.

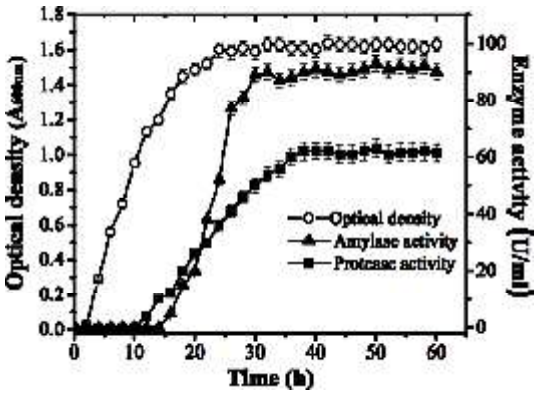

Fig. 2. Kinetics of bacterial growth and extracellular β-amylase and protease production of *Halobacillus* sp. LY4 in CM broth containing 12% (w/v) NaCl at 37°C. Results represent the means of three separate experiments, and deviation bars indicated

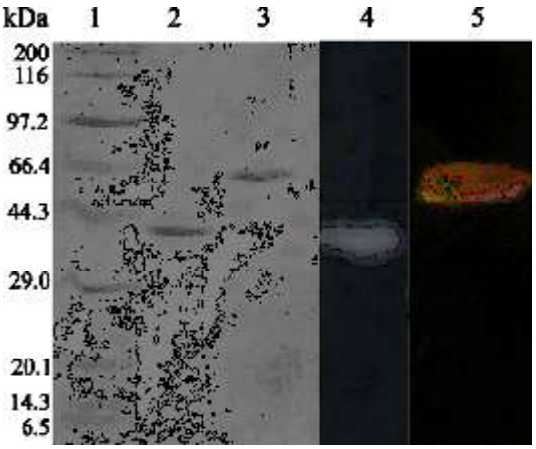

Fig. 3. SDS-PAGE and zymographic analysis of the purified β-amylase and protease from *Halobacillus* sp. LY4. Lane 1: molecular mass markers; lane 2 and 4: protease; lane 3 and 5: β-amylase.

off quickly at 30°C and 90°C with more than 80% of activity lost. Amylase activity was determined with various NaCl concentrations (0-20%) (Fig. 5c). Maximum activity was observed in the presence of 12.5% NaCl. When increasing the salinity (20%), 70% of the activity still retained. In contrast to other halophilic enzymes, the amylase did not lose its activity without NaCl, indicating its halotolerant nature.

As shown in Table 1, the β-amylase activity was markedly stimulated by Ca^{2+}, but greatly inhibited by Hg^{2+} with 46.2% of the activity lost. However, other metal ions did not affect or slightly inhibit the enzyme. The effects of some known enzyme inhibitors revealed DEPC, PAO and EDTA strongly inactivated the amylase, but PMSF and β-mercaptoethanol had no effect on the amylase activity. Moreover, more than 80% of the activity retained when incubated with SDS, Triton X-100 and Tween-80.

Properties of the purified protease

Kinetic analysis of the protease was conducted using casein as the substrate, and K_m and V_{max} values were determined to be 4.31 mg/ml and 1.05 mg/ml/min, respectively. The enzyme exhibited maximum activity at 60°C, and was high active over a broad temperature range (30°C-80°C). However, more than 80% of the activity was lost at 90°C (Fig. 5a). Optimal pH was found to be at pH 9.0, and the enzyme retained 70% of the activity in alkaline condition (pH 12.0) (Fig. 5b). As shown in Fig. 5c, in absence of NaCl, the enzyme did not

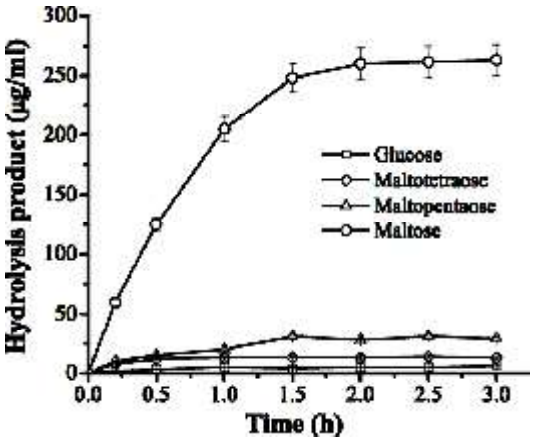

Fig. 4. Hydrolysis products analysis of the β-amylase using soluble starch as substrate, after different incubation times. Data are the average of three experiments, and deviation bars indicated

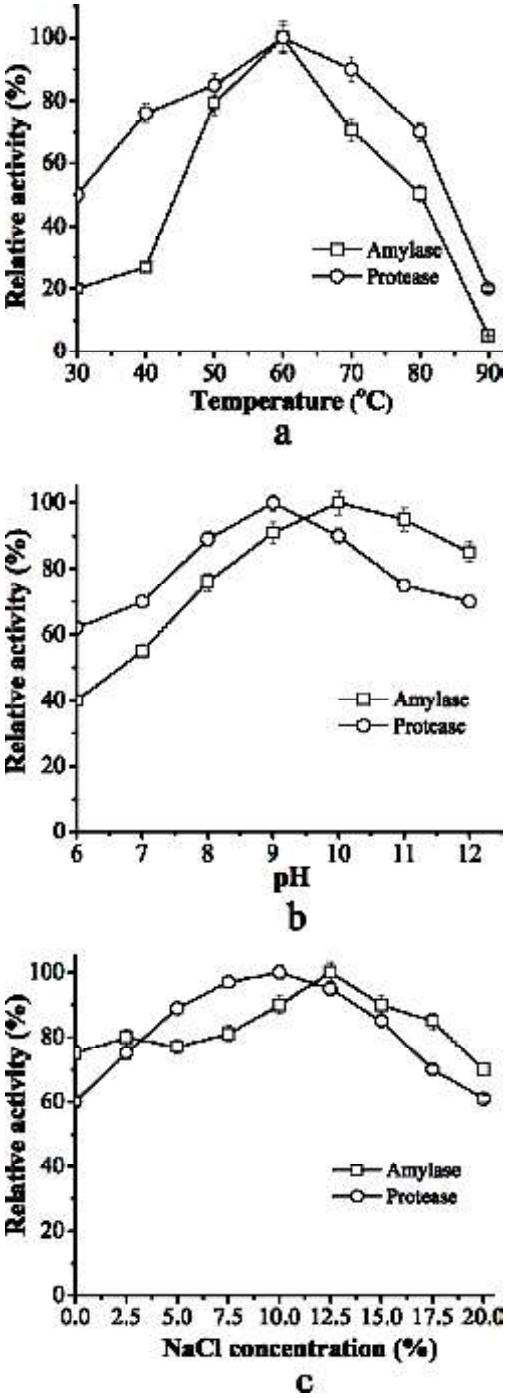

Fig. 5. Effect of temperature (a), pH (b) and NaCl concentration (c) on the activity of the purified β-amylase and protease. The relative activity was defined as the percentage of activity detected with respect to the maximum enzyme activity. Values are averages of three independent experiments, and deviation bars indicated. See "Materials and Methods" for further details

lose its activity. Over the wide range of NaCl concentration, maximal protease activity was observed with 10% NaCl, and 70% of the maximum activity retained under high salinity condition (20% NaCl).

The presence of Ca^{2+} and Cu^{2+} was found to enhance the protease activity (218.2% and 150.1% of the original activity, respectively). However, it was inhibited by Hg^{2+} and retained 60.1% of its original activity. Complete inhibition of the protease was shown by EDTA, PMSF, DEPC and PAO, but β-mercaptoethanol had no effect on the enzyme activity. Among the surfactants tested, more than 78% of the activity retained in the presence of SDS, Triton X-100 and Tween-80 (Table 1).

DISCUSSION

In recent years, the ability of the moderately halophilic bacteria to grow over a very wide range of salinities makes them very attractive for screening of novel enzymes with unusual properties. In this investigation, some moderately halophilic bacteria were isolated from salt lake of Yuncheng, China. Among these, the isolate LY4 was selected for further studies because it appeared to be the best producer of extracellular amylase and protease. As determined by 16S rRNA sequence analysis, strain LY4 was closely related to the species of the genus *Halobacillus* (Fig. 1). As we all know, protease can hydrolyze other proteins such as amylase. Therefore, reports for both enzymes produced at the same time have not been published. However, as shown in Fig. 2, it was observed that the production of both enzymes reached a maximum level during the stationary growth phase of LY4. Thus, we concluded that the β-amylase was probably not the substrate of the protease.

The starch hydrolysis pattern produced by the amylase demonstrated maltose was the main end product, indicating an β-amylase activity (Fig. 4). This is the first report about β-amylase production from halophiles so far, which was different from other β-amylases produced by *Halomonas meridiana*[12] and *Nesterenkonia* sp. strain F[13]. Molecular weight of the β-amylase was determined to be 62 kDa (Fig. 3, lane 2). The value was slightly higher than the β-amylase reported from *Bacillus* sp. KYJ 963 [17], but much smaller than β-amylase produced by *Clostridium thermosulphurogenes*[14]. Other similar molecular masses for different halophilic β-amylases have also been reported: 58 kDa for amylase from *Haloferax mediterranei*[15] and 62 kDa for amylase II from *Chromohalobacter* sp. TVSP 101[16].

The β-amylase can be classified as a moderately thermoactive enzyme because of its optimal activity at 60°C (Fig. 5a). Moreover, it is rather thermostable, showing relatively high activity (residual activity>55%) at 80ºC. These characteristics made the enzyme obviously different from other β-amylases produced by non-extremophilic microorganisms, because most of them were neither active nor stable at temperatures above 65°C [14,17]. Studies to determine pH optimum revealed that the β-amylase was high active over broad range (6.0-12.0) with optimal activity at pH 10.0 (Fig. 5b). Even at pH 12.0, 85% of the activity retained, suggesting the alkalitolerant nature of the enzyme. In addition, the β-amylase was high active over a broad range of salt concentrations (0-20%) (Fig. 5c). This type of extreme halotolerance has also been reported in other amylases from halophiles[16-19]. Complete inhibition by DEPC, a histidine modifier[20] and PAO, a cysteine modifier[21], revealed that histidine and cysteine residues were essential for the amylase catalysis, which has not been observed in other amylases. The β-amylase was stimulated by Ca^{2+}, but strongly inhibited by EDTA, a metal ions cheletor, suggesting it was a metalloenzyme and Ca^{2+} dependent.

Analysis of the purified protease showed its molecular weight was 39 kDa. This value was close to that reported for other moderately halophilic proteases[3, 22, 23]. It was a moderately thermoactive enzyme showing maximum activity at 60°C. Moreover, the protease was high active over a broad pH range (6.0-12.0) and had the optimal activity at pH 9.0, which is a typical characteristic of alkaline proteases[24]. Meanwhile, In contrast to the protease from the *Salinivibrio* sp. strain AF-2004 [22] and similar with metalloprotease CP1 from *Pseudoalteromonas* sp. strain CP76 [25]; this enzyme requires saline conditions to show maximum activity (12.5% NaCl). It was also high active without NaCl, which was different from the catalytic activity of other proteases from halophilic

microorganisms that normally live in a hypersaline environment falls off dramatically and irreversibly when the enzyme is exposed to lower salt concentrations[26]. The activity of the purified protease was greatly inhibited by EDTA, suggesting that it was a metalloprotease. Meanwhile, complete inhibition by PMSF (a serine protease inhibitor) revealed that probably this protease belongs to the subclass of serine metalloproteases. Similar behavior was also shown by other proteases that have been previously characterized from moderately halophilic bacteria[22, 23]. However, it was noted that DEPC and PAO could inactivate the protease, indicating cysteine and histidine residues have important role in its catalytic function, which has not been observed in other proteases from halophiles.

Moreover, both enzymes showed remarkable stability towards various surfactants, such as SDS, Triton X-100 and Tween 80, and may be useful in surfactant industries. To the best of our knowledge, reports for surfactant-stable, thermostable, halotolerant, and alkalitolerant β-amylases and proteases are scarce. Results from the present study indicated that these two important enzymes from *Halobacillus* sp. LY4 may have considerable potential for industrial application from the perspectives of their properties.

CONCLUSIONS

In the present study, a moderately halophilic bacterium (strain LY4) with high amylolytic and proteolytic activity was isolated from salt lake of Yuncheng, China. Biochemical and physiological characterization along with 16S rRNA sequence analysis placed the isolate in the genus *Halobacillus*. Purification and characterization of two surfactant and detergent stable, thermostable, halotolerant, and alkalitolerant enzymes (β-amylase and protease) are reported here. These enzymes may have tremendous applications in industries where higher salt concentration, surfactant and detergents inhibit enzymatic conversions.

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