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ORIGINAL PAPER

Extracellular production of beta-amylase by a halophilic isolate, *Halobacillus* sp. LY9

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Abstract A moderately halophilic strain LY9 with high amylolytic activity was isolated from soil sample obtained from Yuncheng, China. Biochemical and physiological characterization along with 16S rRNA sequence analysis placed the isolate in the genus *Halobacillus*. Amylase production started from the post-exponential phase of bacterial growth and reached a maximum level during the early-stationary phase. The isolate LY9 was found to secrete the amylase, the production of which depended on the salinity of the growth medium. Maximum amylase production was observed in the presence of 10% KCl or 10% NaCl. Maltose was the main product of soluble starch hydrolysis, indicating a β-amylase activity. The enzyme showed optimal activity at 60°C, pH 8.0, and 10–12.5% of NaCl. It was highly active over broad temperature (50–70°C), NaCl concentration (5.0–20.0%), and pH (4.0–12.0) ranges, indicating its thermoactive and alkali-stable nature. However, activity dropped off dramatically at low NaCl concentrations, showing the amylase was halophilic. Ca^{2+} was found to stimulate the β-amylase activity, whereas ethylenediaminetetraacetic acid (EDTA), phenylarsine oxide (PAO), and diethyl pyrocarbonate (DEPC) strongly inhibited the enzyme, indicating it probably was a metalloenzyme with cysteine and histidine residues located in its active site. Moreover, the enzyme exhibited remarkable stability towards sodium dodecyl sulfate(SDS) and Triton X-100. This is the first report of β-amylase production from moderate halophiles. The present study indicates that the extracellular β-amylase of *Halobacillus* sp. LY9 may have considerable potential for industrial application owing to its properties.

Keywords Moderate halophile · Amylase production · *Halobacillus* · β-Amylase

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Introduction

As an important group of hydrolytic enzymes, amylases have been studied extensively because of their potential application in the biotechnological-based food, detergent, paper, and pharmaceutical industries [25, 29]. Amylases represent one of the three largest groups of industrial enzymes and account for approximately 25% of the total enzyme sales worldwide [18]. However, most industrial process are carried out under harsh physicochemical conditions, which may not be definitively adjusted to the optimal points required for the activity of the available enzymes; thus, it would be of great importance to develop enzymes that exhibit optimal activities at various ranges of salt concentration, pH, and temperature.

Moderate halophiles are a group of halophilic microorganisms able to grow optimally in media containing 3–15% NaCl [30]. Besides their important role in the ecology of hypersaline environments, these prokaryotes have received considerable interest because they are a good source for halophilic enzymes such as amylases, proteases, lipase, and nucleases of potential commercial value, which are not only salt-tolerant, but also may be active at high temperature and pH values [7, 13, 21]. β-Amylase is an exo-type enzyme that hydrolyzes the α-1,4-glucosidic linkages and successively liberates β-maltose from the non-reducing end

of starch, glycogen, and malto-oligosaccharides. Although α-amylases, endo-type enzymes, are widely distributed in various kinds of organisms, including some moderate halophiles [6, 9, 10, 16, 22], β-amylases are produced only by plants and some Gram-positive spore-forming bacteria such as *Bacillus* sp. KYJ963 [8], *Bacillus circulans* [24], and *Clostridium thermosulfurogenes* [23]. To date, there are no reports about β-amylase production from moderate halophiles. During the screening for halophilic bacteria producing extracellular hydrolyses from the salt lake of Yuncheng, we isolated and characterized a new moderate halophile (strain LY9) with high amylolytic activity and determined its enzyme production and activity optimum

Materials and methods

Bacterial isolation, media, and culture conditions

Soil samples were obtained from the salt lake of Yuncheng, China. Aliquots of the soil were added to a complex medium (CM) containing (g/l): casein peptone, 7.5; yeast extract, 10; sodium citrate, 3; $MgSO_4 \cdot 7H_2O$, 20; KCl, 2; $FeSO_4 \cdot 7H_2O$, 0.01; and NaCl, 120. The pH was adjusted to 8.0 prior to sterilization (121°C, 15 min). Incubation was carried out at 37°C under aerobic conditions. After 2 days of incubation, the enrichment cultures were spread on CM agar plates (2% agar, w/v). Pure cultures were isolated and screened for extracellular hydrolytic activity, and the isolate LY9 was chosen for further study because it appeared to be the best producer of extracellular amylase.

Salt tolerance assays were performed by growing the isolate LY9 in CM broth plus various concentrations of NaCl (0–20%, w/v) at 37°C. Bacterium growth was monitored at a temperature range of 10–60°C and over a culture medium pH range from 4.0 to 12.0.

Identification of the isolate LY9

Morphological, physiological, and biochemical characteristics of the isolate LY9 were studied either on CM agar plate or in CM broth with 12% of NaCl. Gram staining, motility, catalase and oxidase activities, nitrate reduction, hydrolysis of aesculin and Tween 80, Voges–Proskauer, indole production, and methyl red tests were performed as recommended by Smibert and Krieg [26]. Utilization of various carbohydrates and acid production were determined as described by Ventosa et al. [31].

To confirm the identity of the isolate LY9, genomic DNA extraction and purification were done as described preciously [19]. 16S rRNA gene was amplified using the universal primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACCTTGTTACGACTT-3′). The amplification was done by initial denaturizing at 95°C for 6 min followed by 35 cycles of 95°C for 30 s, 52°C for 30 s, 72°C for 30 s, and final extension at 72°C for 10 min. The polymerase chain reaction (PCR) product was purified and sequenced in both directions using an automated sequencer by SEQLAB Sequence Laboratories (Göttingen, Germany). The phylogenetic relationship of the isolate was determined by comparing the sequencing data with its closely related neighbor sequences retrieved from the GenBank database of the National Center for Biotechnology Information, via BLAST search.

Phylogenetic analysis was performed using the software package MEGA version 5.0 [11] after obtaining multiple alignments of data available from public databases by CLUSTAL W [28]. Pairwise evolutionary distances were computed using the correction method and clustering was performed using the neighbor-joining method [20]. Bootstrap analysis was used to evaluate the tree topology of the neighbor-joining data by performing 1,000 replicates [15].

Amylase activity assay

After cultivation of the isolate LY9 at 37°C for 2 days, cell-free supernatants were collected after centrifuging for 20 min at 10,000 rpm at 3°C and were analyzed immediately for amylase activity. One milliliter of reaction mixture consisting of 700 µl of 0.5% soluble starch (prepared in 50 mM glycine-NaOH, pH 8.0, and 10% NaCl) and 300 µl of suitably diluted enzyme solution was incubated for 20 min at 60°C. Amylase activity was determined by measuring released reducing sugars using the DNS (3,5-dinitrosalicylic acid) method [14]. An enzyme blank with DNS added before the addition of enzyme at 60°C was used as control. One unit of enzyme activity was defined as the amount of enzyme necessary to produce 1 µmol of reducing sugar under the assay conditions per minute.

Extracellular amylase production and effects of different salts

The kinetics of bacterial growth and amylase production were determined at different time intervals. The isolate LY9 was inoculated in CM broth and incubated with shaking at 37°C. Samples were withdrawn aseptically every 2 h and bacterial growth, along with enzyme activity, was measured by spectrophotometry (Shimadzu model UV-160A).

The effect of salts on amylase production was tested with different concentrations of KCl, NaCl, $NaNO_3$, Na_2SO_4, and sodium citrate, separately. After incubation of

LY9 at 37°C for 48 h, the culture broths were centrifuged and cell-free supernatant was used for amylase activity assay.

Effects of temperature, pH, and NaCl concentration on amylase activity

To determine the temperature optimum for the amylase activity, the assay was carried out at various temperatures from 30 to 90°C. The effect of pH on amylase activity was studied by incubating the reaction mixture with different pH values ranging from 4.0 to 12.0, in the following buffer systems: 0.1 M sodium acetate (pH 4.0–5.0); 0.1 M sodium phosphate (pH 6.0–7.0); 0.1 M Tris–HCl (pH 8.0–9.0); 0.1 M glycine-NaOH (pH 9.0–12.0). Also, amylase activity was assayed at 60°C with different NaCl concentrations (0–20%) in the reaction mixture.

Effects of various metal ions and chemical reagents on amylase activity

The effects of metal ions (Ca^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+}, Hg^{2+}, and Fe^{3+}) and chemical reagents [ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulfate(SDS), Triton X-100, β-mercaptoethanol, phenylmethylsulfonyl fluoride (PMSF), phenylarsine oxide (PAO), and diethyl pyrocarbonate (DEPC)] on the amylase activity were examined after the enzyme had been pre-incubated with them for 30 min at 50°C, separately, and then the residual activity was determined using the standard assay method as described above. The level of inhibition was expressed as percent activity remaining compared to a control without any additive.

Product analysis of soluble starch hydrolysis induced by the β-amylase

The starch hydrolysis products were analyzed by high-performance liquid chromatography (HPLC) as described previously [17]. One milliliter of the crude enzyme solution was incubated at 60°C with 0.5% soluble starch in Tris–HCl buffer (100 mM, pH 8.0) containing 10% NaCl. After different time intervals (0.5, 1, 1.5, 2, 2.5, and 3 h), samples were withdrawn and hydrolysis was stopped by boiling them. After centrifugation at 12,000 rpm for 10 min, each sample was analyzed by HPLC analysis on a Waters μBondapack Amino Carbohydrate column (4.1 × 300 mm). Samples (25 μl) were injected and eluted with acetonitrile/water (70:30) 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 *Halobacillus* sp. LY9

On the basis of morphological observation and physiological and biochemical characteristics, the isolate LY9 is a Gram-positive, motile, rod-shaped, and aerobic spore-forming bacterium. Colonies are light yellow, uniformly round, circular, regular, and convex on CM plate. The isolate grew well in a wide range (0–20%) of salt concentrations. Optimal bacterial growth was observed at pH 10, 37–42°C, and 12% NaCl. H_2S production, methyl red, and Voges–Proskauer tests were negative, whereas nitrate reduction, oxidase and catalase, hydrolysis of Tween 80 and gelatin were positive. Acid is produced from maltose, D-fructose, sucrose, and glucose. Phylogenetic analysis based on 16S rDNA gene sequence comparisons revealed that isolate LY9 (GenBank accession number HQ589901) fell within the branch encompassing members of the genus *Halobacillus* and was most closely related to *Halobacillus trueperi* (99.0% 16S rDNA gene sequence similarity) (Fig. 1); however, unlike *Halobacillus trueperi*, the isolate

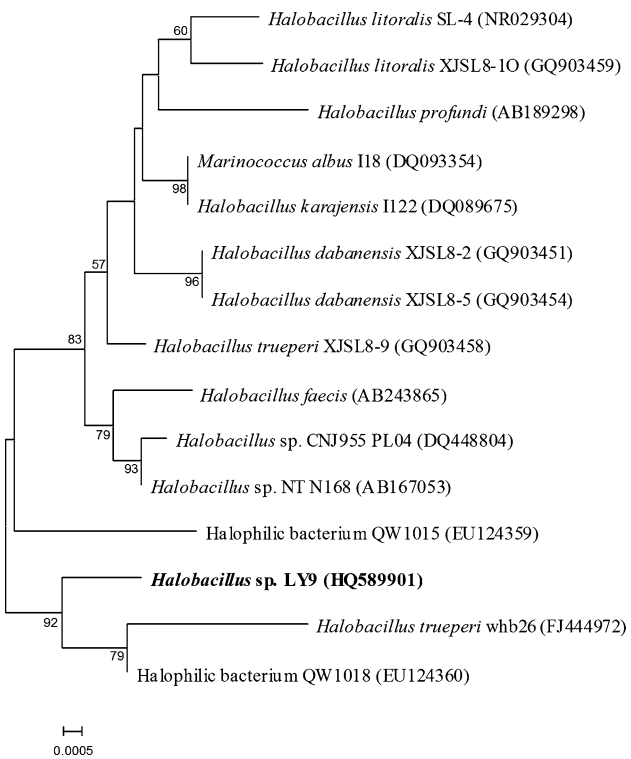

Fig. 1 Phylogenetic tree based on 16S rRNA sequences, showing the relationship of the isolate LY9 to other members of the genus *Halobacillus*. Accession numbers of the sequences used in this study are shown in *parentheses* after the strain designation. *Numbers at nodes* are percentage bootstrap values based on 1,000 replications; only values greater than 50% are shown. *Bar* 0.0005 substitutions per nucleotide position

hydrolyzed casein and starch, and these differing biochemical characteristics suggested that the bacteria were probably not the same strain [27]. Thus, the strain LY9 was tentatively named as *Halobacillus* sp. LY9.

Amylase production and effects of different salts

Effects of different salts on the bacterial growth and amylase production of LY9 are shown in Table 1. The salinity of the culture medium strongly influenced the enzyme production. Optimal production occurred when 10% (w/v) NaCl or 10% KCl was added (about 78.0 U/ml). In addition, the culture was able to produce amylase in the presence of Na_2SO_4. No growth was found in the medium containing $NaNO_3$, whereas 5 and 10% of sodium citrate provided some bacterial growth and the amylase production.

Studies on the kinetics of bacterial growth and amylase production revealed that the lag phase of bacterial growth was short (2 h), and after 28 h the bacterial growth reached the stationary phase (Fig. 2). No amylolytic activity was detected during the early and mid-exponential growth phase. However, the amylase production started from the post-exponential phase and the maximum amylase was secreted in the early stationary phase of bacterial growth (32 h).

Table 1 Effect of various salts on β-amylase production by *Halobacillus* sp. LY9

Salt	Growth	Activity (U/ml)
None	+	5.0 ± 0.1
NaCl		
5%	+	57.2 ± 1.3
10%	+	77.8 ± 2.3
15%	+	39.3 ± 1.2
KCl		
5%	+	56.3 ± 0.3
10%	+	78.0 ± 0.6
15%	+	33.5 ± 1.1
Na_2SO_4		
5%	+	42.4 ± 1.1
10%	+	57.5 ± 0.9
15%	+	52.9 ± 0.5
$NaNO_3$		
(5, 10, 15%)	−	–
Sodium citrate		
5%	+	26.3 ± 0.7
10%	+	11.0 ± 0.2
15%	−	–

Culture supernatant collected at 48 h of growth was used for determining the amylase production in the presence of salts as described in "Materials and methods." pH of the medium was adjusted to 8.0. Values are expressed as the averages of three independent experiments ± standard deviations

Effect of temperature, pH, and NaCl concentrations on amylase activity

As shown in Fig. 3a. The enzyme kept excellent activity over a broad range of temperatures from 50 to 70°C (relative activity greater than 55%) with optimal activity at 60°C. However, activity dropped off quickly at 0 and 90°C, as more than 90% of activity was lost. The amylase showed activity over a wide pH range with optimal activity at pH 8.0. It was relatively stable in acidic or alkaline conditions with at least 50% of the activity retained at pH 4.0 and 12.0 (Fig. 3b).

The enzyme activity was also determined with different NaCl concentrations (0–20%) at 60°C and pH 8.0 (Fig. 3c). The highest activity was obtained at 10–12.5% NaCl. At higher (20%) salinities, more than 70% of the activity still remained. However, the enzyme activity decreased largely in the absence of NaCl with about 80% of the activity lost, indicating its halophilic nature.

Effects of various metal ions and chemical reagents on amylase activity

As shown in Table 2, the amylase activity was markedly stimulated by Ca^{2+}, but was inhibited by Zn^{2+}, Fe^{3+}, Cu^{2+}, and Hg^{2+}, with 12.6, 15.5, 28.3, and 49.2% of the activity being lost, respectively. However, the other metal ions tested did not affect or only slightly inhibited the enzyme activity. The effects of some known enzyme inhibitors revealed that DEPC, PAO, and EDTA strongly inactivated the amylase, but PMSF and β-mercaptoethanol had no significant effect on the amylase activity. Moreover,

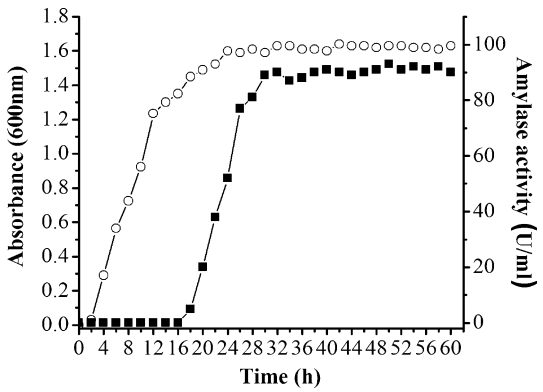

Fig. 2 Kinetics of bacterial growth (*open circles*) and extracellular β-amylase production (*closed squares*) in *Halobacillus* sp. LY9 in CM broth containing 12% (w/v) NaCl at 37°C. Results represent the means of three independent experiments

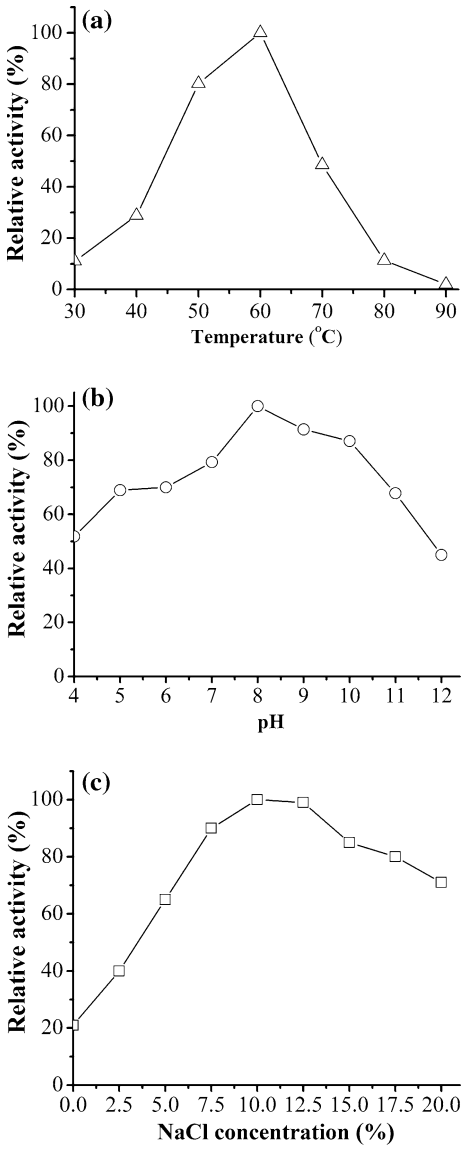

Fig. 3 Effect of temperature (**a**), pH (**b**), and NaCl concentration (**c**) on the activity of β-amylase from *Halobacillus* sp. LY9. The relative activity was defined as the percentage of activity detected with respect to the maximum enzyme activity. Values are averages of three independent experiments. See "Materials and methods" for further details

the amylase retained more than 90% of its activity when incubated with SDS and Triton X-100.

HPLC analysis of hydrolysis products of amylase

The amylase from LY9 hydrolyzed soluble starch to form maltose as the 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. Moreover, trace amounts of longer oligosaccharides (maltotetraose and maltopentaose) and glucose were also produced. Hence, the enzyme may preferentially cleave the α-1,4-linkage adjacent to non-reducing ends, releasing successive maltose units, which indicated a β-amylase activity.

Table 2 Effect of various metal ions and chemical reagents on β-amylase activity

Substances	Final concentration (mM)	Residual activity (%)
Control		100
Ca^{2+}	2	143.1 ± 1.1
Zn^{2+}	2	87.4 ± 0.5
Fe^{2+}	2	98.1 ± 1.2
Fe^{3+}	2	84.5 ± 0.5
Cu^{2+}	2	71.7 ± 1.1
Mn^{2+}	2	99.1 ± 1.0
Hg^{2+}	2	50.8 ± 1.2
Mg^{2+}	2	99.1 ± 1.1
EDTA	2	16.2 ± 0.1
SDS	2	91.1 ± 1.3
β-Mercaptoethanol	2	95.1 ± 1.1
Triton X-100	2	90.7 ± 1.0
PMSF	2	98.1 ± 0.5
DEPC	2	0
PAO	2	0

Residual activity was determined as described in "Materials and methods," and expressed as the percentage of the control value (without any additive). Values are expressed as the averages of three independent experiments ± standard deviations

Discussion

In recent years, although halophilic microorganisms have received increasing attention, most studies have been performed on extreme halophiles. However, the ability of the moderately halophilic bacteria to grow and produce enzymes over a wide range of salinities make them very attractive for research and for screening of novel enzymes with unusual properties. In this study, some moderate halophilic bacteria were isolated from salt lake of Yuncheng, China. Among these, the isolate LY9 was selected for further studies because it appeared to be the best producer of extracellular amylase. As determined by 16S rDNA sequence analysis, the strain LY9 was closely related to the species of the genus *Halobacillus* (Fig. 1); however, physiological and biochemical characteristics obtained for LY9 are different from other *Halobacillus* strains previously described [27], and thus we tentatively named the isolate as *Halobacillus* sp. LY9. Further studies and especially DNA–DNA hybridization data will be necessary to determine whether it constitutes a new bacterial species.

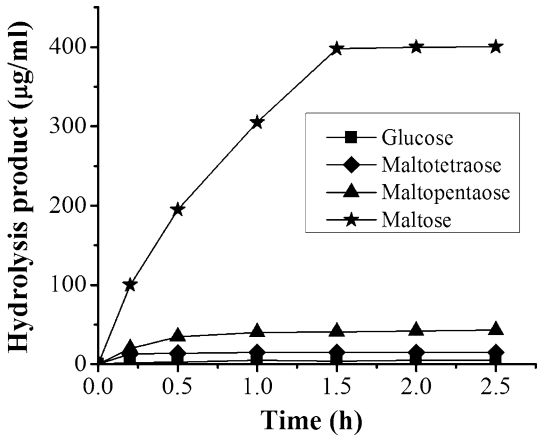

Fig. 4 Product analysis of the hydrolysis of soluble starch induced by extracellular β-amylase, after different incubation times. Data represent the means of three independent experiments

Amylase production of strain LY9 was strongly influenced by the salinity of the culture medium. These results clearly indicated the halophilic nature of the isolate for which the presence of salt appears to be a prerequisite for enzyme production. There was a good correlation between the optimal salt concentration for growth and amylase production; similar behaviors were reported for other moderately halophilic bacteria with the capability of producing extracellular amylases [2, 6]. The strain showed maximum amylase production in the presence of 10% KCl or 10% NaCl. This behavior was not exclusive for this amylase, which was also observed in other extracellular enzymes produced by halophiles, such as protease from *Salinivibrio* sp. strain AF-2004 [3] and lipase from *Salinivibrio* sp. strain SA-2 [4]. The amylase was secreted at the end of the exponential phase of bacterial growth, and reached a plateau during the early stationary phase, whereas in *Halobacillus* sp. strain MA-2, amylolytic activity was exhibited at the mid-exponential growth phase [2].

The starch hydrolysis pattern produced by the amylase from LY9 demonstrated that maltose was the main end product, indicating a β-amylase activity (Fig. 4). This is the first report about β-amylase production from moderate halophiles so far, and is obviously different from those concerning α-amylases from *Halomonas meridian* [2] and *Nesterenkonia* sp. strain F [22]. The β-amylase can be classified as a moderately thermoactive enzyme because of its optimal activity at 60°C (Fig. 3a). Moreover, it was rather thermostable, showing relatively high activity (residual activity greater than 55%) at 70°C. These characteristics made the β-amylase obviously different from those described previously, which were neither active nor stable at temperatures above 65°C [23]. The enzyme was highly active over a broad pH range with a maximum activity at pH 8.0 (Fig. 3b). Even at pH 12.0, more than 50% of the activity was retained, suggesting its alkali-tolerant nature similar to the amylases from other halophiles [2, 9]. Moreover, the amylase showed optimal activity at 10–12.5% NaCl. However, like other halophilic enzymes, its activity fell off dramatically when the enzyme was exposed to lower salt concentrations [1]. Therefore, it needs saline conditions to show maximum activity. The biochemical properties of the β-amylase, such as temperature, pH, and NaCl concentration profile, indicated that it may be an interesting candidate for application in biotechnological processes, such as the treatment of foodstuff wastewater with starch and high salt content.

Complete inhibition of the β-amylase by DEPC, a histidine modifier [32], and PAO, a cysteine modifier [12], were observed, indicating that histidine and cysteine residues at the active site were essential for the enzyme catalysis. The activity was stimulated by Ca^{2+}, but strongly inhibited by EDTA (a metal ion chelator), providing evidence that the β-amylase was a metalloenzyme and Ca^{2+}-dependent. PMSF and β-mercaptoethanol had no significant effect on the enzyme activity. This means that serine residues and disulfide bonds are not essential for the enzyme activity [22]. Similar findings have not been previously reported for other halophilic amylases. Moreover, the enzyme exhibited remarkable stability towards surfactants, such as SDS and Triton X-100, and may be useful in surfactant industries [5].

To the best of our knowledge, surfactant-stable, thermoactive, halophilic, and alkali-tolerant β-amylases have not been reported so far. The present study indicates that *Halobacillus* sp. LY9 may be a potential source of β-amylase production and the enzyme produced under high salinity may be of commercial value.

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