

Purification and Characterization of a Cold-Adapted α -Amylase Produced by *Nocardiopsis* sp. 7326 Isolated from Prydz Bay, Antarctic

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Abstract

An actinomycete strain 7326 producing cold-adapted α -amylase was isolated from the deep sea sediment of Prydz Bay, Antarctic. It was identified as *Nocardiopsis* based on morphology, 16S rRNA gene sequence analysis, and physiological and biochemical characteristics. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and zymogram activity staining of purified amylase showed a single band equal to a molecular mass of about 55 kDa. The optimal activity temperature of *Nocardiopsis* sp. 7326 amylase was 35°C, and the activity decreased dramatically at temperatures above 45°C. The enzyme was stable between pH 5 and 10, and exhibited a maximal activity at pH 8.0. Ca^{2+} , Mn^{2+} , Mg^{2+} , Cu^{2+} , and Co^{2+} stimulated the activity of the enzyme significantly, and Rb^{2+} , Hg^{2+} , and EDTA inhibited the activity. The hydrolysates of soluble starch by the enzyme were mainly glucose, maltose, and maltotriose. This is the first report on the isolation and characterization of cold-adapted amylase from *Nocardiopsis* sp.

Keywords: cold-adapted α -amylase — *Nocardiopsis* — purification and characterization

Introduction

Microorganisms living in extreme environments, such as deep sea and Polar Regions, presumably have developed particular characteristics that allow them to thrive in such environments (Takami et al. 1997). For example, their cold-active or cold-adaptive enzymes have received a great deal of attention be-

cause they are essential in some fundamental scientific study areas and have the ability to withstand certain industrial reaction conditions (Deming 1998). Recent microbial studies of the deep sea have led to significant new discoveries of unusual microbial diversity, new species, and cold-adaptive enzymes (Feller 2003; Feller and Gerday 2003; Zeng et al. 2003, 2006), which were regarded as critical elements for the survival of the microorganisms in extreme environments (Chessa et al. 1999). Likewise, the high activity of cold-adaptive enzymes at low and moderate temperatures offers potential economic benefits (Russell 1998; Gerday et al. 2000; Cavicchioli et al. 2002).

Actinomycetes are one of the most investigated groups because they constitute a potential source of biotechnologically interesting substances (Lealem and Gashe 1994). *Nocardiopsis* strains are distributed ubiquitously in the environment (Kroppenstedt and Evtushenko 2002). They are frequently isolated from habitats with moderate to high salt concentrations such as saline soil or marine sediments (Evtushenko et al. 2000; Al-Zarban et al. 2002) and salterns (Chun et al. 2000). Here we first report the purification and characterization of a cold-adaptive α -amylase secreted by a *Nocardiopsis* strain that was isolated from deep sea sediment of Prydz Bay, Antarctic. The culture has been deposited in the Marine Culture Collection of China (<http://www.mccc.org.cn>) as *Nocardiopsis* sp. 7326.

Materials and Methods

Sample Collection. The deep sea sediment was collected via multicore sampler at a depth of 900 m of site PN5-6 (740°25'E, 66°55'S) during the 21st cruise of Chinese Antarctic Research (Nov. 2004–Mar. 2005). The sediment was transferred to sterile

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Falcon tubes in clean bench and kept aseptically at 4°C until usage.

Strain Isolation and Culture Conditions. The sediment was diluted with artificial seawater (ASW) containing 0.3% NaCl, 0.07% KCl, 0.53% MgSO₄·7H₂O, 1.08% MgCl₂·6H₂O, and 0.1% CaSO₄·7H₂O and the supernatants were grown on glycerol/asparagines (ISP-5) medium containing 0.5% starch under 10°C for 5 days. The single colony was isolated after being picked and cultured on ISP-5 agar medium repetitively and verified by microscope. The strains were preserved on ISP-2 medium (Pridham et al. 1956), supplemented with 1% (wt/vol) agar starch slants at 4°C.

The seed cultural medium (A) was ISP-5 medium supplemented with 1% (wt/vol) starch and ASW (pH 7.5). The optimal amylase-producing medium (B) contained 2.5% (wt/vol) starch, 1% glucose, 0.05% K₂HPO₄, and ASW (pH 8.0). The seed culture was prepared in Erlenmeyer flasks by inoculating a 2.0-ml spore suspension containing 3.6 to 4.0×10⁸ UFC ml⁻¹ into 50 ml of presterilized medium A and cultivating under agitation at 180 rpm for 48 h. Then 50 ml of the seed culture was added to 500 ml of medium B and was incubated at 20°C for 120 h. Samples (5 ml) were taken from each of three replicate flasks at 12-h intervals. The cell-free supernatant containing α -amylase was harvested by centrifugation at 4000 rpm for 15 min at 4°C and subjected to purification and characterization.

Identification of Strain 7326. The genome DNA was extracted from the strain and used as template for polymerase chain reaction (PCR) amplification of the 16S rRNA fragment according to the method described previously (Rainey et al. 1996). The primers used were Eubac27F: 5'-AGAGTTTGATCCTGG CTCAG-3' and Eubac1492R: 5'-GGTTACCTTG TTACGACTT-3' (DeLong 1992). The PCR product was cloned into pMD18-T (Takara) and sequenced by Shanghai Sangon Biological Engineering Technology & Services Company. The 16S rRNA gene sequence was checked for similarities to 16S rRNA sequences in the EMBL and RDPII (<http://rdp.cme.msu.edu>) database. The alignment and phylogenetic analysis of sequences were achieved via DNAMAN (Lynnon Biosoft). Tests of physiological and biochemical characteristics were carried out according to the methods described previously (Schipper et al. 2002).

Purification of Amylase. Amylase was purified at 4°C as follows. The cell-free supernatant was subjected to ammonium sulfate precipitation, and the protein precipitating at 60% saturation was resuspended in 20 mM Tris-HCl buffer (pH 8.0)

and dialyzed against the same buffer for 18 h. The prepurified protein was analyzed via anion-exchange chromatography on a DEAE Sepharose CL-6B column (5×30 cm) that had been preequilibrated with 20 mM Tris-HCl (pH 8.0) containing 5% glycerol. The protein was eluted with 20 mM Tris-HCl buffer (pH 8.0) containing 10 mM CaCl₂ in a linear gradient between 0 and 0.8 M NaCl. The active fractions were applied to a Sephadex G-75 column (1×45 cm) and eluted with 20 mM Tris-HCl buffer (pH 8.0). Finally, the resulting enzyme preparation was desalted and concentrated by dialysis and lyophilization.

The purified amylases were identified via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and zymogram activity staining. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250. For activity staining, the gel was suspended in 20% (vol/vol) isopropanol and incubated for 30 min, then transferred to 20 mM Tris-HCl buffer (pH 8.0) and incubated for 30 min. Amylolytic activity was detected by placing the renatured gel onto an agarose gel containing 0.8% soluble starch, and incubating at 35°C for 3 h. The transparent band on the amylase-containing agarose gel was examined after it was flooded with I₂-KI solution.

Assay of Enzyme Activity and Protein Concentration. Amylase activity was determined by detecting the amount of reducing sugars liberated. The reaction mixture (1 ml) contained 0.25 ml of 2% soluble starch, 0.25 ml of 0.4 M Tris-HCl buffer (pH 8.0), and 0.5 ml of enzyme. The reaction was terminated by addition of 2 ml of 3,5-dinitrosalicylic acid reagent after incubation at 35°C for 30 min (Sengupta et al. 2000). One unit of enzyme activity was defined as the amount of enzyme that released 1 μ mol of reducing sugar as glucose per minute under the assay conditions. The protein concentration was measured with bovine serum albumin as a standard (Lowry et al. 1951). All measurements in this experiment were made in triplicate.

Thin-Layer Chromatography (TLC) Analysis. The hydrolysis products of starch were submitted to TLC on silica gel plates. Enzymes were incubated with 1% of soluble starch in 10 mM Tris-HCl buffer (pH 8.0) at 35°C for 5 h. Aliquots (5 μ l) of the reaction mixtures were chromatographed on a silica gel (Merck) with chloroform-acetic acid-ddw (18:21:3, by vol), and the products were detected by spraying the gel with aniline-diphenylamine-phosphate followed by baking at 120°C for 30 min. Glucose (G1), maltose (G2), maltotriose (G3), and maltotetraose (G4) were the standards.

Effects of pH, Metal Ions, EDTA, and Temperature on Activity. The optimal pH for amylase activity was determined at 35°C in 50 mM acetate–NaOH (pH 4.0 to 5.7), phosphate–NaOH buffer (pH 5.7 to 6.3), 3-(*N*-morpholino)propanesulfonic acid (MOPS)–NaOH (pH 6.5 to 7.8), Tris–HCl (pH 7.8 to 8.6), 2-(*N*-cyclohexylamino)ethane (CHES)–NaOH (pH 8.7 to 9.5), and glycine–NaOH (pH 9.5 to 11.0). All pH values were adjusted at room temperature, and the pH stability of the amylase was evaluated by determining the residue activities after a 24-h incubation in the aforementioned buffers at 4°C. The effects of metal ions and EDTA on amylase were examined by determining the activities after 30-min incubation at 35°C in 50 mM Tris–HCl (pH 8.0) buffers containing various metal ions at 5 mM.

The effects of temperature on amylolytic activity were determined by measuring the activity at different temperatures. Before thermostability studies, the enzyme was dialyzed extensively against 50 mM Tris–HCl (pH 8.0) containing 2 mM EDTA and then dialyzed twice against the same buffer without EDTA. Thermostability of the amylase was examined by measuring the residual activities of EDTA-treated enzyme fractions that were preincubated at 15°C, 25°C, 35°C, 45°C, and 55°C in 50 mM Tris–HCl (pH 8.0) containing 5 mM Ca^{2+} for different periods of time.

Nucleotide Sequence Accession Numbers. The nucleotide sequence of the 16S rRNA gene has been deposited in EMBL under accession no. AM111064.

Results and Discussion

Identification of Strain 7326. Strain 7326 can grow at 0°C, and the optimum and highest temperatures for

its growth were 20°C and 37°C, respectively. Its vegetative hyphae were long and slim (0.15 to 0.3 μm in width), well-developed, and fragmented under light microscopy (Figure 1). Short spore chains were borne on the aerial hyphae. Spores (dimensions 0.4 to 0.6 \times 0.6 to 1.0 μm) were rod-shaped, smooth-surfaced, and nonmotile. Strain 7326 could not grow on most of the actinomycete-special agar mediums without additional NaCl. Nevertheless, the colonies were well growing, aerobic, Gram-positive, coarsely wrinkled to folded, and white in color on these mediums containing 20 to 100 g/L of NaCl. Aerial filaments were well developed and abundant. Strain 7326 could utilize the following substrates as the sole source of carbon and energy: D-arabinose, D-fructose, D-galactose, glycerol, mannitol, D-mannose, raffinose, D-ribose, sorbitol, sucrose, D-trehalose, and D-xylose. It could utilize the L-alanine, L-asparagine, L-glutamic acid, L-histidine, L-phenylalanine, L-serine, and L-threonine as sole source of nitrogen. The sources of carbon and nitrogen that could not be utilized by strain 7326 included cellobiose, D-glucose, inositol, lactose, D-maltose, L-rhamnose, D-xylitol, L-cystine, L-methionine, L-glycine, and L-valine. The 16S rRNA gene analysis showed that strain 7326 was most closely related to the genus *Nocardiopsis* (Figure 2), with the highest levels of similarity (99.454%) to *Nocardiopsis* sp. 20088 [AY336519]. Thus we placed this strain in the genus *Nocardiopsis*, as *Nocardiopsis* sp. 7326. Data obtained from RDP II (Ribosomal Database Project) also suggested that strain 7326 was a member of the *Nocardiopsis* genus.

Purification of *Nocardiopsis* sp. 7326 Amylase. The purification procedure of the *Nocardiopsis* sp. 7326 extracellular amylase is summarized in Table 1. The amylase exhibited a specific activity of 548 U/

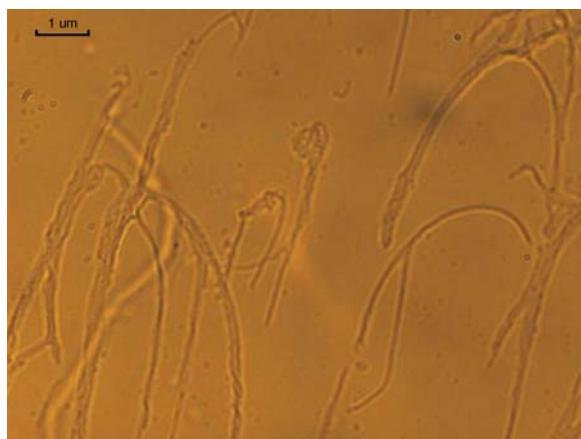


Figure 1. Light micrograph of *Nocardiopsis* sp. 7326 grown on ISP-5 plates for 15 days at 20°C.

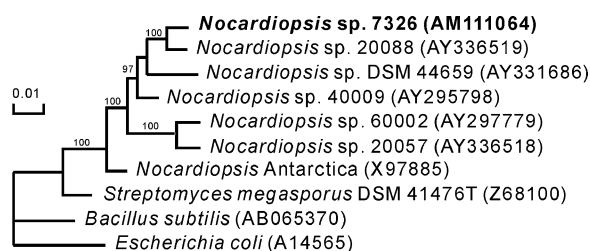


Figure 2. Phylogenetic analysis of *Nocardiopsis* sp. 7326. The tree was constructed by neighbor-joining method based on 16S rRNA sequences. The scale bar shows 0.01 substitutions per base position. Numbers refer to bootstrap values for each node out of a total of 100 replicate resamplings. The numbers in the bracket are the EMBL accession numbers of the 16S rRNA sequences of reference bacteria. *Streptomyces megasporus*, *Bacillus subtilis*, and *Escherichia coli* were used as the outgroup.

Table 1. Purification of *Nocardiosis* sp. 7326 amylase

Step	Total protein (mg)	Total activity (unit)	Specific activity (unit mg protein ⁻¹)	Yield (%)	Purification (-fold)
Cell-free extract	860	22,500	26.0	100	1.0
(NH ₄) ₂ SO ₄ fractionation	418	18,380	44.0	82.3	1.7
DEAE Sepharose CL-6B	45.5	9180	202	41.0	7.8
Sephadex G-75	4.0	2190	548	10.1	21.0

mg, corresponding to a purification factor of 21-fold and a total yield of 10.1%. The purified protein showed a single band equal to a molecular mass of about 55 kDa on SDS-PAGE and activity staining gel, confirming high enzyme purity (Figure 3).

TLC analysis. TLC was used to analyze the hydrolysis patterns of soluble starch digested by the purified amylase (Figure 4). The main hydrolysis products of soluble starch were mainly G1 (glucose), G2 (maltose), and G3 (maltotriose) and included a little G4 (maltotetraose). The main hydrolysis product from G2 was G1, mainly G1 and a little G2 from G3, and mainly G1 and a little G2 and G3 from G4. The concentration of the resulting sugars increased with increase of the incubation time. These hydrolysis patterns revealed that amylase from strain 7326 functioned as a typical amylase to hydrolyze the α -(1,4)-glycosidic linkage only.

Effects of Temperature and pH on Amylase Activity.

Nocardiosis sp. 7326 showed maximal enzymatic activity at 35°C, and maintained 38% of its highest activity at 0°C (Figure 5), which was in agreement

with the character of the cold-adaptive enzymes (Gerday et al. 2000; Cavicchioli et al. 2002; D'Amico et al. 2003; Feller 2003; Feller and Gerday 2003). The amylase retained more than 70% of the highest activity between 20°C and 45°C. Up to now, the optimum temperatures for enzymatic activity of α -amylases that were normally used in industry were ordinarily between 50°C and 55°C (Declerck et al. 2003). As shown in Figure 6, the amylase retained most of the activity after incubation at 15°C or 25°C for 60 min, while under its optimum activity temperature the amylase lost half of its activity after 40 min of incubation. The activity decreased to 18% after 30-min treatment at 55°C, reflecting the thermosensitivity of the enzyme. The special properties of higher catalytic efficiency at low temperature and more thermosensitivity than that of its mesophilic counterparts (Lee et al. 1994; Igarashi et al. 1998; Hagihara et al. 2001; Yang et al. 2004; Ballschmitter et al. 2006) are the typical characteristics of cold-adaptive amylase.

Figure 7 shows the pH stability and optimal pH of *Nocardiosis* sp. 7326 amylase with soluble starch as a substrate. The enzyme was stable between pH 5 and 10 after 24 h of incubation at 4°C. The optimal pH for enzymatic activity was found to be around 8.0, and the amylase retained

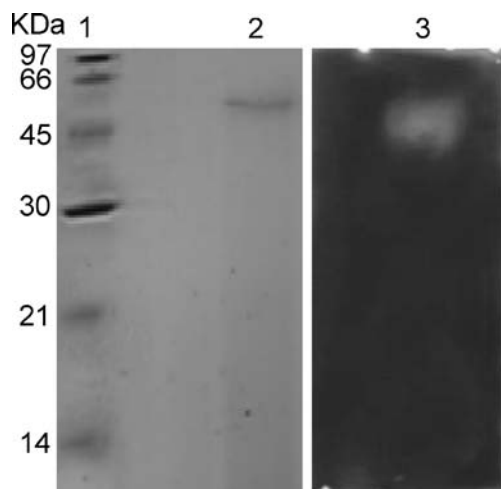


Figure 3. SDS-PAGE and activity staining analysis of purified amylase. Lane 1, molecular mass standard; lane 2, purified enzyme eluting from Sephadex G-75 (5 μ g of protein); lane 3, activity staining of purified enzyme.

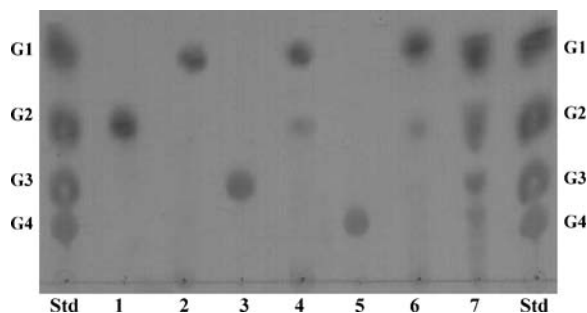


Figure 4. TLC analysis of hydrolysis products of soluble starch digested by *Nocardiosis* sp. 7326 amylase. Lane 1, maltose (G2); lane 2, product generated from G2; lane 3, maltotriose (G3); lane 4, products generated from G3; lane 5, maltotetraose (G4); lane 6, products generated from G4; lane 7, products generated from soluble starch; Std, standard marker.

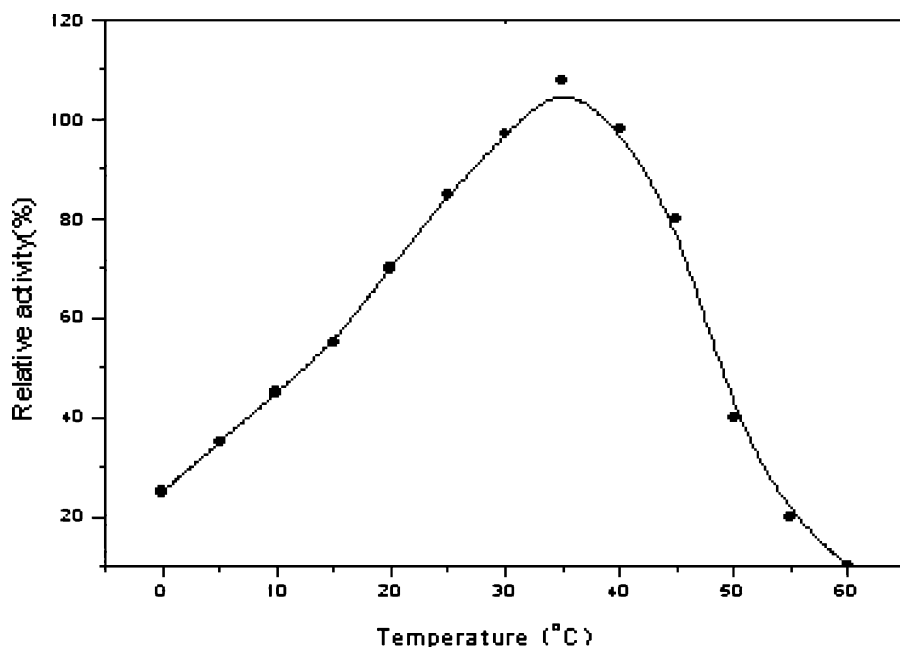


Figure 5. Effect of temperature on the activity of *Nocardiosis* sp. 7326 amylase. The value obtained at 37°C was taken as 100%.

more than 60% of the highest activity at pH 7 to 9. As a comparison, the optimal pH of a thermostable α -amylase from *Nocardiosis* sp. endophyte was pH 5.0 (Stamford et al. 2001).

Effects of Metal Ions and EDTA on Amylase Activity.

The effects of metal ions on the activity of *Nocardiosis* sp. 7326 amylase showed that the enzyme was activated by Ca^{2+} , Mn^{2+} , Mg^{2+} , Cu^{2+} ,

and Co^{2+} . However, it was inhibited by Rb^{2+} , Hg^{2+} , and EDTA (Table 2). In previous reports, most amylases activity were inhibited in the presence of Ni^{2+} , Cd^{2+} , Cu^{2+} , Ag^+ , Pb^{2+} , Fe^{2+} , and Zn^{2+} . For example, the α -amylases from *Bacillus* sp. strain KSM-1378 (Cordeiro et al. 2002) and *Bacillus firmus* (Igarashi et al. 1998) were strongly inhibited by Ni^{2+} , Cd^{2+} , Zn^{2+} , and Hg^{2+} ; the α -

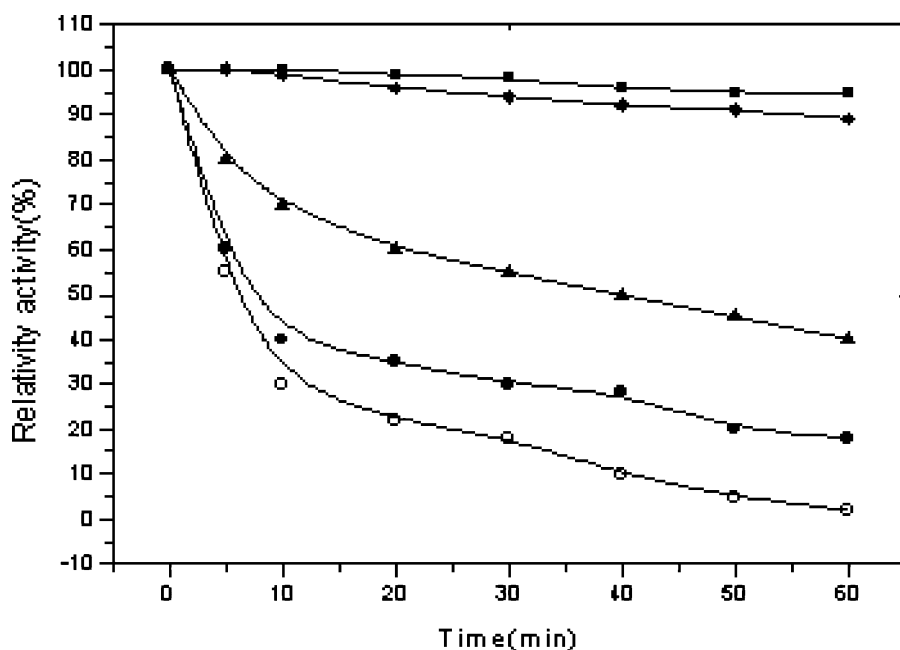


Figure 6. Effect of temperature on the stability of *Nocardiosis* sp. 7326 amylase. Amylolytic activity was determined after incubation at 15°C (■), 25°C (◆), 35°C (▲), 45°C (●), 55°C (○) for a period of time.

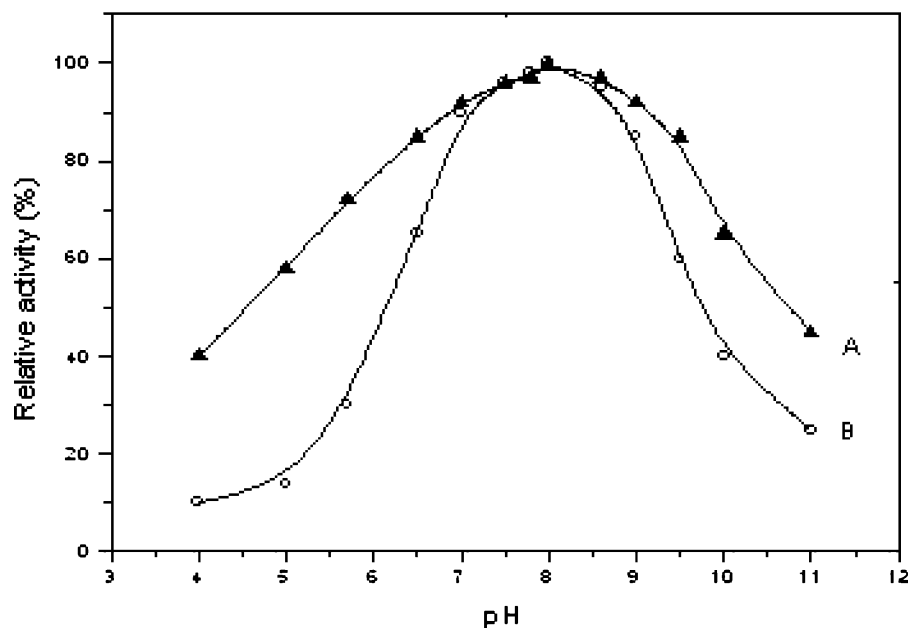


Figure 7. Effect of pH on the stability (▲) and activity (○) of *Nocardiosis* sp. 7326 amylase. Reaction mixtures were buffered with 50 mM acetate–NaOH (pH 4.0, pH 4.5), phosphate–NaOH buffer (pH 5.7), MOPS–NaOH (pH 6.5, pH 7.0, pH 7.5), Tris–HCl (pH 7.8, pH 8.0, pH 8.6), CHES–NaOH (pH 9.0, pH 9.5), glycine–NaOH (pH 10.0, pH 11.0).

amylase from *Thermus* sp. was strongly inhibited by Cu^{2+} and Fe^{2+} (Shen et al. 1998); and the α -amylases from *B. subtilis*, *B. amyloliquefaciens* I, and *B. amyloliquefaciens* II were strongly inhibited by Zn^{2+} , Ag^+ , Cu^{2+} , and Fe^{2+} (Elif and Velittin 2000). However, the activity of *Nocardiosis* sp. 7326 amylase was not affected by Zn^{2+} , Ni^{2+} , and Fe^{2+} , and was even activated by Cd^{2+} and Cu^{2+} . The unusual property might be related to the special structure of the amylase, and the mechanism requires further research.

Conclusion

Many cold-active or cold-adapted amylases have been reported from microbial origins (Feller et al.

1999; Groudieva et al. 2004; D'Amico et al. 2006; Siddiqui et al. 2006). To our knowledge, this is the first study on purification and characterization of a cold-adapted α -amylase from the genus *Nocardiosis*. On the other hand, only one α -amylase from *Nocardiosis* sp. had been studied (Stamford et al. 2001) up to now, which showed the highest activity at 70°C and pH 5.0. It exhibited thermostable properties as indicated by retention of 100% of residual activity at 70°C and 50% of residual activity at 90°C for 10 min. These features have potential for industrial applications. In this study, the most desirable properties of the *Nocardiosis* sp. 7326 amylase were its high activity at low temperature and stability at alkaline pH, which also permitted its biotechnological application in various industries. For example, it could be applied as a detergent additive, as a desizing agent in textile processing, and in the food industry.

The *Nocardiosis* sp. 7326 was a psychrotroph exhibiting sigmoidal growth even at 0°C. Based on the 16S rRNA sequence analysis, it was closely related to *Nocardiosis antarctica*, which was not studied for enzymatic activity (Rainey et al. 1996). Further, the different characteristics toward metal ions, such as the sensitivity to Zn^{2+} , Ni^{2+} , and Fe^{2+} and the activation by Cd^{2+} and Cu^{2+} , indicated a special structure of *Nocardiosis* sp. 7326 amylase. These results would make intriguing further research on gene expression and regulation and the protein structure in psychrophilic *Nocardiosis*.

Table 2. Effects of metal ions and EDTA on *Nocardiosis* sp. 7326 amylase activity

Metal ions	Relative activity (%)	Metal ions	Relative activity (%)
None	100	Mg^{2+}	113
K^+	108	Fe^{2+}	107
Na^+	100	Hg^{2+}	72
Li^+	97	Co^{2+}	139
Ca^{2+}	115	Cu^{2+}	142
Cd^{2+}	109	Zn^{2+}	106
Rb^{2+}	67	EDTA	85
Mn^{2+}	143	EDTA+ Ca^{2+}	110
Ni^{2+}	102		

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