Purification and characterization of a cold-adapted alpha-amylase produced by *Nocardiopsis* sp. 7326 isolated from Prydz Bay, Antarctic

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Abbreviated title:

Cold-adapted α-amylase from Antarctic *Nocardiopsis* sp. Strain 7326

Abstract

The endophytic actinomycete from the deep sea sediment of Prydz Bay, Antarctic, were screened according to their novel ability to produce cold-adapted α-amylase. The strain with the highest amylase activity was identified as *Nocardiopsis* species, which displays sigmoidal growth even at 0 °C. The α-amylase purified by ammonium sulphate precipitation and column chromatography on DEAE Sepharose CL-6B and Sephadex G-75 shows a molecular weight of about 55000 and a pI of 4.38. The enzyme is stable from pH 5 to 10 and has a maximal activity at pH 8.0. Compared with the α-amylase from mesophiles and thermophiles, the cold-adapted enzyme shows a high enzyme activity at lower temperatures and a high sensitivity at temperatures higher than 45 °C, and the activity decreases dramatically at temperatures above 55 °C. Ca^{2+}, Mn^{2+}, Mg^{2+}, Cu^{2+} and Co^{2+} show a significant effect on maintaining the structure and the activity of the enzyme. Rb^{2+}, Hg^{2+} and EDTA are its inhibitors. The products from the hydrolysis of soluble starch with the cold-adapted enzyme are mainly maltose and other oligosaccharides. This is also the first report of isolation of a cold-adapted amylase from *Nocardiopsis* species.

Key words *Cold-adapted α-amylase · Marine 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 at such an environment (Takami et al. 1997). For example, their cold-active or cold-adaptive enzymes have been received a great deal of attention due to their essentiality in some fundamental scientific study areas, and their abilities 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, cold-adaptive enzymes(Feller 2003; Feller and Gerday 2003), which was regarded as a critical element for the survival of the microorganisms in extreme environment (Chessa et al. 1999). Likewise, the high activity of adaptive enzymes at low and moderate temperatures offers potential economic benefits(Cavicchioli et al. 2002; Gerday et al. 2000; Russell 1998).

Actinomycetes are one of the most investigated groups since 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 (Al-Zarban et al. 2002; Evtushenko et al. 2000) and salterns (Chun et al. 2000).The aim of this study was to purify and characterize the amylase produced by strain 7326 in order to determine its potential for use in industrial applications.

The organism was isolated from deep sea sediment of Prydz Bay, Antarctic; and identified on the basis of by morphological features and molecular biological methods. The culture was deposited in the National Collection of Marine Biogenetic Resources, Chinese State Oceanic Administration as *Nocardiopsis* sp. 7326.

Materials and methods

Sample collection

The deep sea sediment was collected by multi-core sampler at the depth of 900 m of site PN5-6(740º25’E, 66º55’S) during the 21st cruise of Chinese Antarctic Research (Nov. 2004-Mar.

2005). The sediment was transferred to sterile falcon tubes in clean bench and kept aseptically at 4ºC till usage.

Strain isolation and culture conditions

The sediment was diluted with artificial sea water (ASW) containing 0.3% NaCl, 0.07% KCl, 0.53% $MgSO_4 \cdot 7H_2O$, 1.08% $MgCl_2 \cdot 6H_2O$, and 0.1% $CaSO_4 \cdot 7H_2O$ and the supernatants were grown at 10 ºC on glycerol/asparagines (ISP-5) medium containing 0.5% starch for 5 days, and then we spread the diluted cultural sample in the ISP5 agar plate medium at 10 ºC until the single *Nocardiopsis* bacterial clones were formed.

Stock cultures were maintained on medium ISP-2(Pridham et al. 1956), supplemented with 1% (w/v) agar starch slants at 4 °C. The seed cultural medium (A) was glycerol/asparagines (ISP-5) containing 1% (w/v) starch diluted with ASW, pH 7.5.The optimized amylase production medium (B) contained 2.5% (w/v) starch, 1% glucose, 0.05% K_2HPO_4 diluted with ASW, pH 8.0.

The seed cultural was prepared in Erlenmeyer flasks, containing 50 ml of medium A, previously sterilized at 121°C, for 15 min, inoculated with 2.0 ml spore suspension containing 3.6-4.0×10^8UFC $ml^{-1}$and cultivated under agitation at 180 rpm, for 48 h. Fifty milliliters of the seed cultural were added to 500 ml of starch-urea medium B incubated at 20 °C, for 120 h. At 12 h intervals, samples (5 ml) were taken from each of three replicate flasks. The cells were harvested by centrifugation at 4000 rpm for 15 min, at 4 °C. The supernatant containing a-amylase was used as the starting material to perform purification, then the purified enzyme was used to evaluate enzymatic properties.

Preparation of genomic DNA and 16S rDNA analysis

The genome DNAs were extracted from the strains and used as templates for PCR amplification of the 16S rDNA fragments according to the methods described previously(Rainey et al. 1996). The Primers used were Eubac27F (5'-AGAGTTTGATCCTGGCTCAG-3') and Eubac1492R (5'-GGTTACCTTGTTACGACTT-3') (DeLong 1992). The PCR products were cloned into pMD18-T (Takara) and sequenced by Shanghai Sangon Biological Engineering Technology & Services Company. The 16S rDNA sequences determined were checked for similarities to DNA sequences

in the EMBL and RDPII (http://rdp.cme.msu.edu) database. The alignment and phylogenetic analysis of sequences were achieved by DNAMAN (Lynnon Biosoft).

Purification of amylase

The purification of amylase was achieved at 4 ºC as follows. The cell-free supernatant was subjected to ammonium sulfate precipitation, and the protein precipitating at saturation was resuspended in 20 mM Tris-HCl buffer (pH 8.0) and dialysed against the same buffer for 18 h. The pre-purified protein was applied to an anion exchange chromatography on DEAE Sepharose CL-6B column (5×30 cm) which was pre-equilibrated with 20 mM Tris-HCl (pH 8.0) containing 5% glycerol. The protein was eluted with 20mM Tris-HCl buffer (pH 8.0) containing 10mM $CaCl_2$ 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 20mM Tris-HCl buffer (pH 8.0). Subsequently, fractions containing amylolytic activity were desalted and applied to preparative SDS-PAGE (Model 491 Prep Cell, Bio-Rad, USA) and eluted with 20mM Tris-HCl buffer (pH 8.0). Finally, the resulting enzyme preparation was desalted and concentrated by dialysis and lyophilization.

The purified amylases were identified by SDS-PAGE and zymogram activity staining. After electrophoresis, the gel was stained by 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 20mM Tris-HCl buffer (pH 8.0) and incubated for 30 min. Amylolytic activity was detected by placing the re-natured gel onto and agarose gel containing 0.8% soluble starch. The incubation was done at 35 °C for 3 h, and examined for transparent bands on the amylase containing agarose gel, which flooded with I_2 -KI solution.

Assays of enzyme activities and protein concentration

Amylase activities were 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 which released 1μmol of reducing sugar

as glucose per min 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 plate. 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 μl) of the reaction mixtures were chromatographed on a silica gel (Merck) with chloroform-acetic acid-ddw (18:21:3, vol/vol), and the products were detected by spraying the gel with aniline-diphenylamine-phosphate followed by baking at 120 °C for 30min. Glucose (G1), maltose (G2), maltotriose (G3), maltotetraose (G4) were the standards.

Measurement of kinetic and thermodynamic parameters

The *K*m and Vmax values were determined by a double reciprocal Lineweaver-Burk plot. Steady-state kinetics were examined with variable concentrations of the substrate. The activation energies (E_a) of the enzymes were calculated according to the method previously reported (Feller et al. 1994). The thermodynamic activation parameters of the enzyme reactions were calculated by using equations as described previously(Lonhienne et al. 2000) .

Determination of temperature, pH, metal ions and EDTA effects

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), MOPS-NaOH (pH 6.5 to 7.8), Tris-HCl (pH 7.8 to 8.6), CHES-NaOH (pH 8.7 to 9.5), Glycine-NaOH (pH 9.5 to 11.0) . All pHs were adjusted at room temperature. While the pH stability of the amylase was performed in the buffers mentioned above, and incubated at 4 °C for 24 h

The temperatures for maximal activities of amylase was determined by performing standard enzyme assays at different temperatures. The value obtained at 37 ºC was taken as 100%.

For stability studies at high temperatures, the enzyme was EDTA treated, which dialyzed extensively first against in 50 mM Tris-HCl (pH 8.0) containing 2 mM EDTA and then twice

against the same buffer without EDTA. Enzyme thermal inactivation studies were performed by incubating 1-ml gas chromatography tubes that contained 800 μl of purified enzyme in 50 mM Tris-HCl (pH 8.0) in the presence of 5 mM Ca^{2+} at 15 °C, 25 °C, 35 °C, 45 °C and 55 °C respectively. After various incubation periods, samples were withdrawn and tested for residual amylase activity under the standard assay conditions for each sample.

The effects of metal ions and EDTA on amylase were surveyed by determining the activities after 30 min incubation at 35 ºC in 50mM Tris-HCl (pH8.0) buffers containing 5mM various metal ions.

Nucleotide sequence accession numbers

The nucleotide sequences of 16S rRNA gene has been deposited in EMBL under accession no. AM111064.

Results and discussion

Identification of strain 7326

Strain 7326 was Gram-positive, which can grow at 0 °C, and the optimum and highest temperatures for its growth were 20 °C and 37 °C respectively. From the light micrograph (Fig .1)we can see that the strain`s vegetative hyphae were long, slim (0.15-0.3 μm in width), well-developed and fragmented. Short spore chains were borne on the aerial hyphae. Spores (dimensions 0.4-0.6×0.6-1.0 μm) were rod-shade, smooth-surfaced and non-motile. And the identification by bacterial 16S rDNA sequence analysis showed that strain 7326 was most closely related to the genus *Nocardiopsis* (Fig. 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 (Ribosomal Database Project) also suggested that strain 7326 is a member of the *Nocardiopsis* genus.

Fig. 1

Fig. 2.

Purification of amylase produced by *Nocardiopsis* sp. 7326

The purification procedure of the *Nocardiopsis* sp. 7326 extracellular amylase was summarized in Table 1. The amylase exhibited a specific activity of 540 U/mg, corresponding to a purification factor of 20.7-fold and a total yield of 8.4%. The elution pattern of the sample after DEAE Sepharose CL-6B chromatography is shown in Fig.3. The purified protein was eluted as a single peak and showed a single band equal to a molecular mass of about 55 kDa on SDS-PAGE, confirming high enzyme purity (Fig. 4).

Table 1.

Fig.3.

Fig. 4.

TLC analysis

TLC was used to analyze the hydrolysis patterns of soluble starch digested by the purified amylase (Fig. 5). It is clear that the main hydrolysis products were mainly G1 (glucose) and G2 (maltose), G3 (maltotriose), and included a little G4 (maltotetraose). The main hydrolysis product form G2 was G1; and was mainly G1 and a little G2 from G3; and was mainly G1 and included a little G2 and G3 besides from G4. The concentration of the resulted sugars increases with the increase of the incubation time. These hydrolysis patterns reveal that amylase from strain 7326 functions as a typical amylase to hydrolyze α-(1,4)-glycosidic linkage only.

Fig. 5.

Effect of temperature and pH on amylase activity

Examination of the effect of temperature on the amylolytic activity of the purified amylase (Fig. 6) showed that the maximal enzymatic activity was obtained at 35 °C, and maintains 38% of its highest activity at 0 °C, which is in agreement with the character of the cold-adaptive enzyme

(Cavicchioli et al. 2002; D'Amico et al. 2003; Feller 2003; Feller and Gerday 2003; Gerday et al. 2000). The amylase remained relatively high activity between 20˚C-45 °C. Up to now, the optimum temperatures for enzymatic activity of normally used industrial α-amylase were ordinarily between 50 °C -55 °C (Declerck et al. 2003). As shown in Figure 7 the amylase can retain most of its activity after incubated at 15 °C or 25 °C for 60 min; while under its optimum temperature the amylase lost half of its activity after 40 minutes` incubation; but the amylase activity decreased to 18% after 30 min treatment at 55 °C, which reflects the little heat-unstable of the enzyme. The special properties of higher catalytic efficiency at low temperature and greater thermosensitivity than its mesophilic counterparts (Ballschmiter et al. 2006; Hagihara et al. 2001; Igarashi et al. 1998; Lee et al. 1994; Yang et al. 2004) are the typical characteristics of cold-adaptive amylase.

Figure 8A shows the pH stability of amylase with soluble starch as a substrate. The enzyme was stable between pH 5 and 10 at the indicated pH range when incubated at 4 °C for 24 h, but its activity decrease at acidic and alkaline pH values. Figure 8B shows the optimal pH for the amylase reaction with soluble starch as a substrate. The optimal pH was found to be around 8.0, while the optimal pH of a thermostable α-amylase from *Nocardiopsis* sp. endophyte was pH 5.0 (Stamford et al. 2001).

Fig. 6

Fig. 7

Fig. 8

Effect of metal ions and EDTA on amylase activity

The effect of metal ions on the activity of the α-amylase shows that the enzyme was activated by Ca^{2+}, Mn^{2+}, Mg^{2+}, Cu^{2+} ,Co^{2+}, However, it was negatively affected by Rb^{2+}, Hg^{2+} and EDTA (Table 2). As many previous reports have described, most amylases are inhibited with the presence of Ni^{2+}, Cd^{2+}, Cu^{2+}, Ag^{+}, Pb^{2+}, Fe^{2+}, Zn^{2+}, like, a-amylase from *Bacillus* sp. strain KSM-

1378(Cordeiro et al. 2002) and *Bacillus firmus*(Igarashi et al. 1988) was strongly inhibited by Ni^{2+}, Cd^{2+}, Zn^{2+}, and Hg^{2+}; while a-amylase from *Thermus* sp. was strongly inhibited by the addition of Cu^{2+} and Fe^{2+} ions(Shen et al. 1998), and a-amylase from *B. subtilis*, *B. amyloliquefaciens* I and *B. amyloliquefaciens* II strongly inhibited by Zn^{2+}, Ag^{+}, Cu^{2+} , Fe^{2+}(Elif and Velittin 2000). However, the amylase from strain 7326 showed no sensitivity to 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 needs further research.

Table 2

Discussion

Nocardiopsis sp. strain 7326 was isolated from a soil sample collected about 900 m below the water surface of Prydz Bay, Antarctic and was identified as a *Nocardiopsis* sp. exhibiting maximum similarity at the 16S rRNA level with *Nocardiopsis* sp. 20088. Strain 7326 was a psychrotroph, exhibiting sigmoidal growth even at 0 °C. Otherwise, the strain also show high closely related to *Nocardiopsis* Antarctica, but it was reported to be performed identification without further studied for enzymes production(Rainey et al. 1996). Therefore, it is necessary to further a study on enzymes from a psychrophilic *Nocardiopsis*.

We have purified and characterized a novel cold-adapted α-amylase from *Nocardiopsis* sp. 7326. Although many cold-active or cold-adapted amylases have been reported from microbial origins, to our knowledge, this is the first report on purification and characterization of a cold-adapted α-amylase from the genus *Nocardiopsis*. The hydrolysis patterns of soluble starch digested by the purified amylase is clear that the main hydrolysis products were mainly G1 (glucose) and G2 (maltose), G3 (maltotriose), and included few G4 (maltotetraose). The most desirable property of the amylase is that its optimum temperature for enzymatic activity is 35 °C, which is lower than that of any other amylase reported previously, except the psychrophilic α-amylase from *Pseudoalteromonas haloplanktis*, which shown optimum activity at 25 °C (Feller et al. 1999). This unique property of amylase from strain 7326 and its stability at alkaline pH permit its biotechnological application potential to be exploited: in the detergent industry as detergent

additive, removing the amylaceous stains efficiently at room temperature with much convenience; in the textile industry as a desizing agent in textile processing, without the necessity to use toxic reagents and cutting down the processes of heating and cooling so that to save considerable amount of time and energy; and in the cosmetic and dentifrice industry as additive, having high activity at the temperature of human body. In addition, the cold-adapted amylase would have a broad application in many other fields, such as clinical, medical, and analytical chemistries, as well as their wide spread application in starch sacccharification and in the food, fermentation, paper, brewing and distilling industries. Furthermore, the sensitivity of the amylase to Zn^{2+}, Ni^{2+} and Fe^{2+} and activation by Cd^{2+} and Cu^{2+} indicate special structure of the amylase and intrigue basic research about the mechanism.

Acknowledgement

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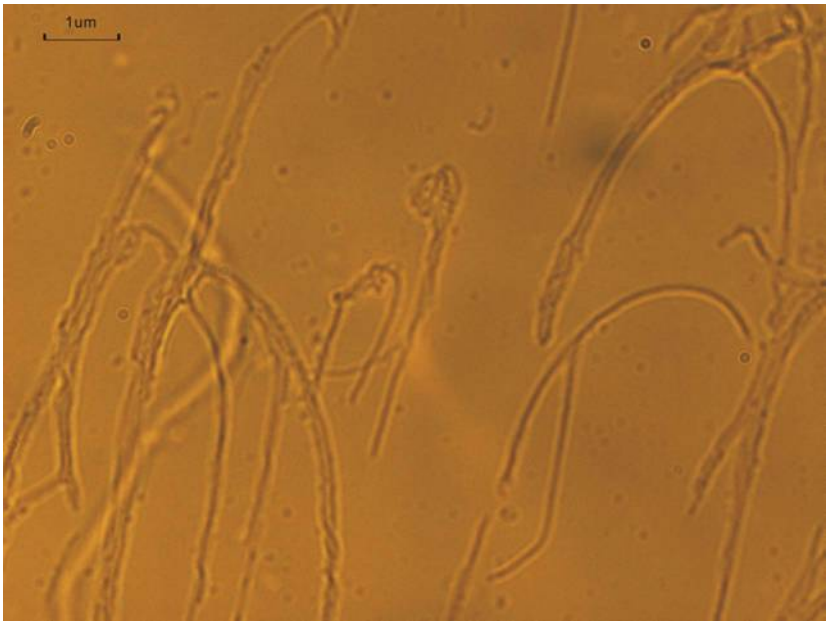

Fig.1 Light micrograph of *Nocardiopsis* sp. 7326 grown on ISP5 plates for 15 days at 20˚C.

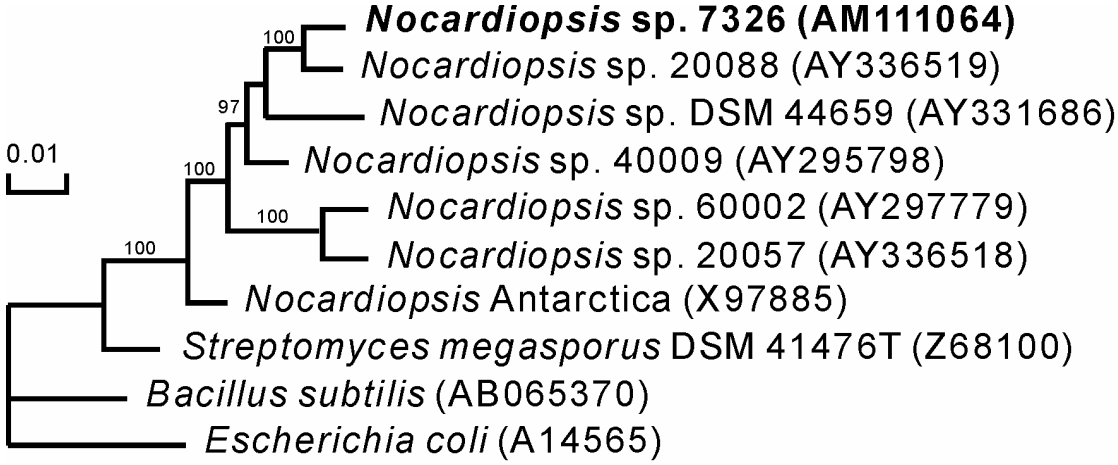

Fig. 2. Neighbour-joining tree, base on 16S rDNA sequences, showing the phylogenetic relationship of strain 7326 with recognized members of the genus *Nocardiopsis* and other genus. The numbers are the EMBL accession numbers of the 16S rDNA sequences of various bacteria. The tree was constructed by neighbor-joining method. 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. *Streptomyces megasporus*, *Bacillus subtilis* and *Escherichia coli* were used as the outgroup.

Table 1. Purification of α-amylase from *Nocardiopsis* sp. 7326

Step	Total protein (mg)	Total activity (unit)	Specific activity (unit mg protein^{-1})	Yield (%)	Purification (-fold)
Cell-free extract	860	22500	26.0	100	1.0
$(NH_4)_2SO_4$ fractionation	418	18380	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
preparative SDS-PAGE	3.5	1890	540	8.4	20.7

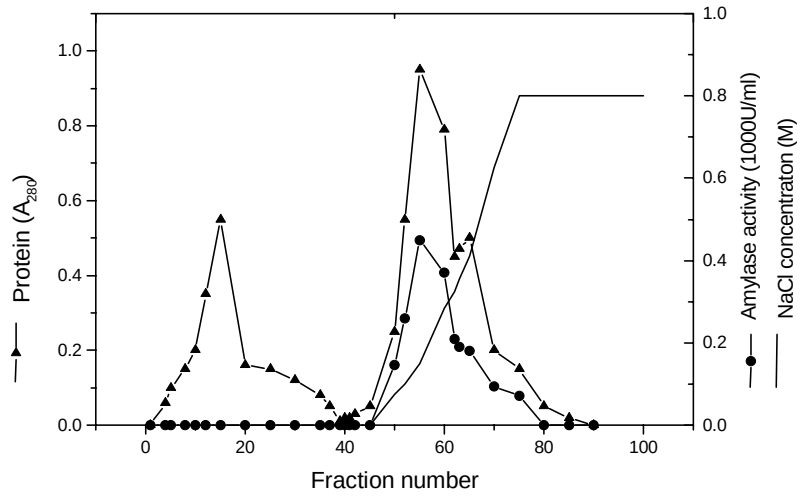

Fig.3. Elution profiles of the amylase on DEAE Sepharose CL-6B. The DEAE Sepharose CL-6B column(5cm×30cm) preequillibrated with 50 mM Tris-HCl eluting buffer (pH 8.0) was eluted with a linear gradient of 0-0.8 M NaCl in 50 mM Tris-HCl buffer at a flow rate of 65ml/h. Fractions 45-80 have been used for further purification.

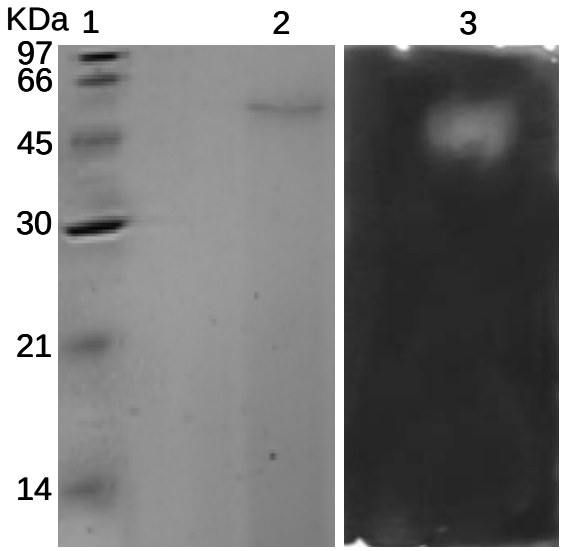

Fig. 4. SDS-PAGE and activity staining analysis of purified amylase

Lane 1, molecular mass standard; Lane 2, purified enzyme eluting from preparative SDS-PAGE (5μg protein); Lane 3, activity staining for the purified amylase protein

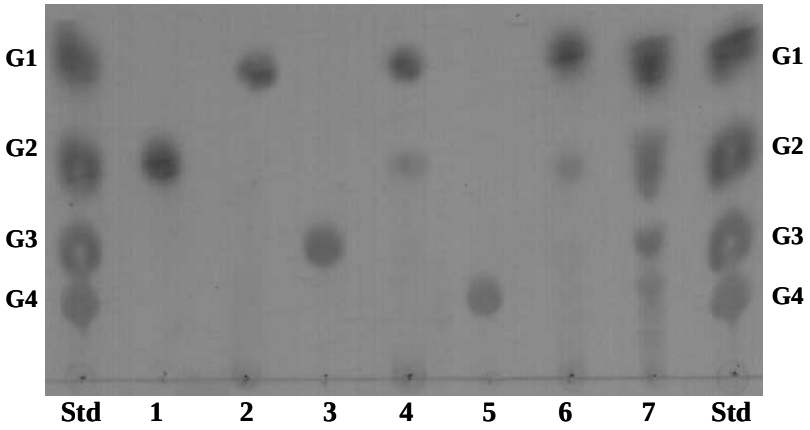

Fig.5. TLC analysis of hydrolysis products of soluble starch digested by amylases from psychrotrophs isolated.

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.

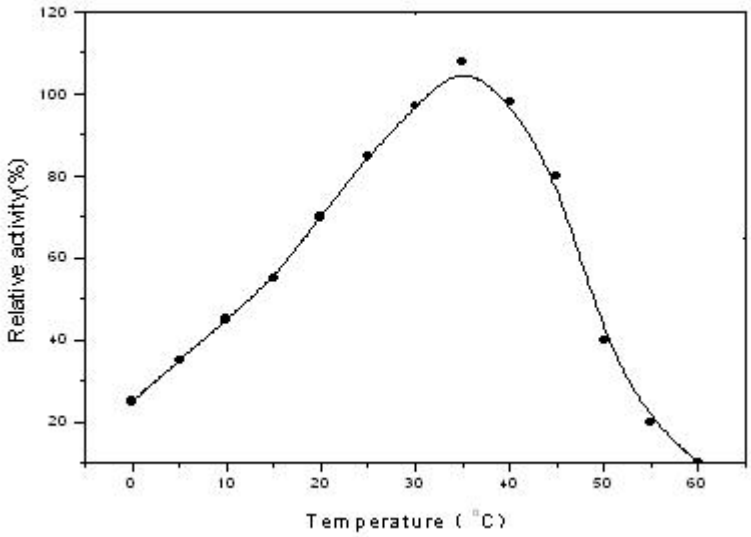

Fig. 6. Effect of temperature on the activity of *Nocardiopsis* sp. 7326 amylase

The value obtained at 37ºC was taken as 100%

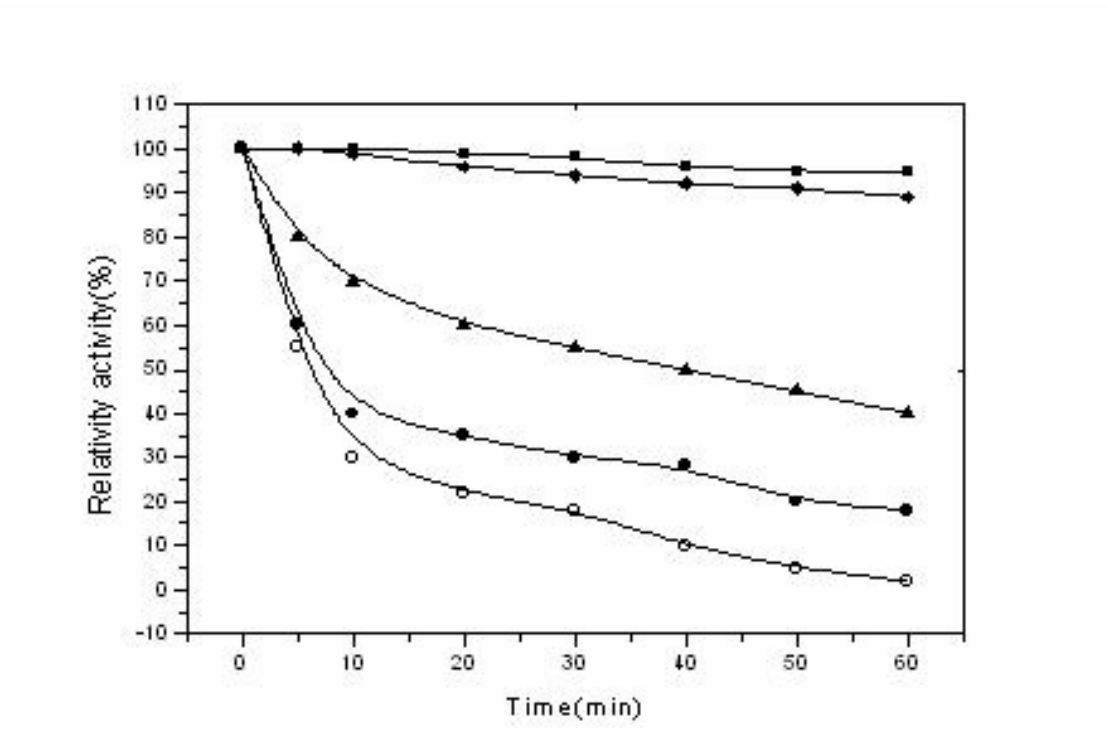

Fig. 7. Effect of temperature on the stability of *Nocardiopsis* sp. 7326 amylase

Amylolytic activity was determined after incubation at 15˚C (■), 25˚C (♦), 35˚C (▲), 45 ˚C (●), 55˚C (O) for a period of time.

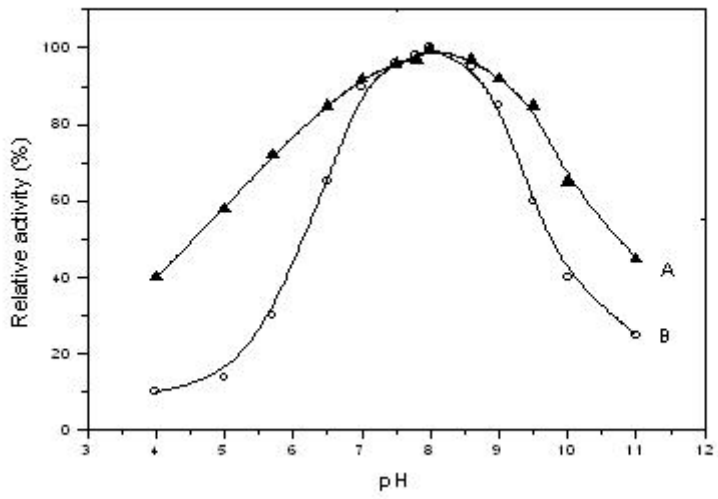

Fig. 8. Effect of pH on the stability (A) and activity (B) of *Nocardiopsis* sp. 7326 amylase

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)

Table 2 Effect of metal ions and EDTA on *Nocardiopsis* 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	Co^{2+}	139
Mn^{2+}	143	EDTA	85
Ni^{2+}	102	EDTA+ Ca^{2+}	110