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Novel cold-active amylase-producing bacterium from Chukchi Sea and its enzyme properties

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Running title: Cold-active amylase from Chukchi Sea

Abstract: A cold-active amylase was purified from *Alteromonas* sp. KS7913 isolated from the Chukchi Sea in the Arctic Ocean. After purification with use of ammonium sulfate precipitation, phenyl column chromatography, and size exclusion chromatography, 200.34 U mg^{-1} of purified amylase was obtained. The final yield was 3.4%, and the activity was 5.7-fold higher than that of the initial culture broth. KS7913 origin amylase showed a molecular weight of 70 kDa and optimal activity at 25°C, pH 7.0 in Tris-HCl buffer. The amylase was highly active, especially at 5°C, and maintained stability at basic conditions below 25°C. Copper and zinc ions inhibited enzyme activity, whereas manganese, barium, and calcium ions exhibited positive effects. This activity was maintained even in the presence of alcohol. The findings of this study supplement our understanding of cold-active amylases, and may have practical applications in low-temperature industries.

Keywords: the Arctic, *Alteromonas*, cold-adapted amylase, enzyme purification.

Introduction

Amylase is an enzyme that breaks down polysaccharides, particularly α-amylase, which randomly cuts the 1,4-D-glucosidic bonds between nearby glucose units to produce smaller polymers. Amylase is used in manufacturing industries for the bend sizing of textile fibers, baking, biomass conversion, molecular biology, food, and liquidation. α-amylase is found in plants, animals, and bacteria. Commercial amylase is mostly produced by bacteria because of its high production cost (Hassan *et al.* 2018). Amylase is a widely distributed enzyme produced by various bacteria (Peltier and Beckord 1945). The characteristics of the amylase produced by each strain can differ from one organism to another. Additionally, the structure and function of amylases can vary depending on the specific substrate they target (Konsula and Liakopoulou-Kyriakides 2004; Yazdanparast *et al.* 2005), such as starch, glycogen, or other polysaccharides. This diversity in amylase production and characteristics offers a range of possibilities for industrial applications because different strains may be more suitable for specific processes or environments. As a result, the aims of ongoing research are to identify new sources of amylase and optimizing the production and characteristics of existing strains to meet the needs of various industries. When enzymes are used in manufacturing, they are mainly required at medium temperatures for maximum efficiency, which contributes to increased production costs (Singh *et al*. 2016).

Cold-active amylases have been identified as valuable biocatalysts with high specific activity at low temperatures. This unique property of cold-active amylases offers economic and environmental benefits by reducing energy consumption during industrial processes that employ enzymes (Yao *et al*. 2019; Bhatia *et al*. 2021). For example, cold-active amylases can be used to process starch at lower temperatures, leading to reduced energy costs and environmental impacts. Therefore, the study of cold-active amylases has gained increasing attention because of their potential for various industrial applications, such as food and biofuel production. Therefore, amylase could be used industrially.

Microorganisms can adapt to various environments to survive and thrive, and this adaptation is closely linked to the enzymes and metabolites they produce. For example, certain microorganisms produce specific types of enzymes to survive in low-temperature, high-pressure, and high-salinity environments; these enzymes are structurally adjusted to function in these environments and optimized for activity. Therefore, the ability of microorganisms to adapt to their environment for survival and proliferation is closely related to the enzymes and metabolites they produce, which can be utilized in various industries and applications (Bukhari and Rehman

2015; Paul *et al.* 2021). Arctic bacteria are exposed to extremely low temperatures, which can influence the characteristics of the amylase they produce (Yao *et al*. 2019). Marine microorganisms, including *Alteromonas* sp., are capable of decomposing various organic materials and are adapted to low-temperature, high-pressure, and high-salinity environments (Ottoni *et al*. 2020; Qin *et al*. 2020, Vera-Villalobos *et al*. 2023). Therefore, we screened these bacteria from samples collected from the Arctic Ocean over the years.

Moreover, there is increasing scientific interest in the structure of cold-resistant enzymes and the mechanism of their thermal stability (Bhatia *et al.* 2021). Therefore, discovering new cold-active amylases from diverse sources is essential to characterizing their diverse structures and characteristics. While a few cold-active amylases have been discovered, the scope of these analyses remains limited. This study described the characteristics of a cold-active amylase purified from *Alteromonas* sp. KS7913 isolated from the Chukchi Sea in the Arctic Ocean.

Material and methods

Exploration for bacteria that produce amylase and subsequent identification. — Approximately 20 300 colonies were isolated from various samples (Bongo Net, box-core, dredge, ice-core, and multicore samples, and conductivity, temperature, depth (CTD) membranes collected from the Chukchi and the Beaufort Sea. The Arctic samples were incubated on various solid media: R2A (MB Cell), marine agar (MB) (2216, BD Difco), super ZoBell (SZB), ISP Medium 4 (BD Difco), and yeast extract-peptone-glucose (YPG) media (Table 1). Amylase-producing bacteria were isolated through growth on plates containing 1% [w v^{-1}] soluble starch (Junsei, Japan). The isolates inoculated on the substrate-added plates underwent incubation at 15°C for 3 days, and bacteria exhibiting extracellular amylase activity were chosen by flooding Lugol's solution (Peltier and Beckord 1945). In total, 162 strains were sorted based on the observation of the clear zones, and their sizes were measured and listed sequentially. Finally, the eight bacterial strains displaying the widest clear zones were chosen and assessed for amylase activity employing a liquid culture medium (Table 2). Identification of these eight bacterial strains was performed using the universal primers 27-F (AGAGTTTGATCCTGGCTCAG) and 1492-R (GGTTACCTTGTTACGACTT) by Macrogen (Macrogen Online Sequencing Order System). The contigs of each strain were assembled and used to perform BLAST with rRNA databases. The assembled contigs were deposited to NCBI GenBank. The 16S rRNA contig sequences of the strains were used to construct the phylogenetic tree with MEGA 11.0 software (Tamura *et al.*

2021) and the Neighbor-Joining method (Saitou and Nei 1987) was used with bootstrapping of 1 000 replicates.

Bacterial cultivation and assessment of amylase activity. — The selected eight strains of bacteria were grown in liquid ZoBell medium containing soluble starch (1% [w v^{-1}]) as a substrate for three days at 15°C. Supernatants from the eight culture broths were obtained by centrifugation (9 000×*g*, 30 min), and their activities were assayed. Amylase activity was determined by combining 0.1 mL of each supernatant (enzyme solution) with 0.5 mL of soluble starch (1% [w v^{-1}]) in 50 mM Tris-HCl buffer (pH 7.0). Subsequently, incubation was carried out at 37°C for 30 min. The reaction was stopped by adding 0.6 mL of 3,5-dinitrosalicylic acid (DNS) reagent to the reaction mixture. The optical density at 540 nm (OD_{540}) was measured using a spectrophotometer (S-3100, Scinco, Korea) (Caf *et al*. 2014). The unit of activity per milligram (U mg^{-1}) was defined as the amount needed to release 1 µmol of reducing sugar in 1 min per mg of protein. Commercial amylase Amplify® Prime (Novozyme, USA) was used as a reference enzyme.

Purification of amylase enzyme. — The supernatant of the culture broth was obtained by centrifugation at 9 000×*g* for 30 min at 4°C (RC-5C Plus, Sorvall, USA) for protein purification. Desalting and buffer exchange were conducted with the supernatant and concentrated with a 10 kDa molecular weight cut-off membrane (Z615366, Sartorius, Germany). The concentrate was freeze-dried, and distilled water was added. The concentrated crude enzyme was loaded onto a phenyl-Sepharose column (HiTrap, GE Healthcare, USA). The enzyme was eluted at a flow rate of 1.0 mL min^{-1} with 1% [w v^{-1}] isopropanol and 20 mM potassium phosphate buffer (pH 7.0). The fractions displaying activity were combined and then introduced onto a Superdex 75 column (GE, USA) with potassium phosphate buffer (20 mM, pH 7.0). The concentration of the active fractions was achieved by using Vivaspin 20 (10 kDa cut-off) (GE, USA).

Gel electrophoresis and zymography. — Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and zymography were performed to confirm the molecular mass and activity of the starch-degrading enzyme. Samples were boiled at 100°C for 10 min and analyzed using an 8–16% SDS-PAGE gel (4561103, Bio-Rad, USA). The visualization of protein bands was achieved by staining the gels. For zymography, samples without heat treatment were used. The gel was washed with a 50 mM Tris buffer and placed on an agar plate containing 1% (w v^{-1}) soluble starch. The plate was subjected to incubation for 4 h at 15°C and treated with Lugol's solution staining. After 10 min, enzyme activity was detected by the absence of dyeing.

Effect of pH and temperature of purified amylase enzyme. — The pH effect on enzyme activity or stability was observed across the spectrum from 4.0 to 10.0. Activity was measured after the enzyme reaction solution worked for 30 min at 25°C. pH stability was determined after pre-incubating the purified enzyme with different buffer systems for 1 h at 25°C. In addition, 50 mM of sodium acetate and 50 mM of Tris-HCl were used to adjust the pH from 4.0 to 6.0 and from 6.0 to 10.0, respectively.

The enzymatic activity was investigated across temperatures of 5–85°C with intervals of 10°C, after incubating 5 µL (0.14 µg protein) enzyme, 50 µL soluble starch (1%, w v^{-1}), and 545 µL Tris-HCl buffer (pH 7.0) at each temperature for 30 min. The thermostability of the enzyme was assessed by pre-incubating the purified enzyme for 1 h at a specified temperature, and the enzyme reaction was performed at 25°C for 30 min. The remaining conditions were the same as those described initially. The enzyme activity was measured by determining the reducing sugar content using a 600 µL DNS solution added after the reaction, followed by boiling for 5 min and measuring the absorbance at 540 nm.

Effect of metal ions and alcohol tolerance of purified amylase enzyme. — The effect of the metal ions of Cl_2 and SO_4 salts on enzyme activity was investigated (Xian *et al*. 2015). The activity of the purified enzyme was measured in the presence of 1 mM metal ions ($BaCl_2$, $CoCl_2$, $MnCl_2$, $CuSO_4$, $CaCl_2$, $ZnSO_4$, Na_2SO_4, $MgCl_2$, or $FeSO_4$). The enzyme activity under standard conditions (enzyme reactions without additives) was considered 100%.

To confirm the effect of alcohols, including methanol, ethanol, n-butanol, and isoamyl alcohol, enzyme activity was checked in the presence of 25 and 90% concentrations of each alcohol (Dey and Banerjee 2015). After adding the enzyme to each concentration of the alcohol solution and setting it at room temperature for 1 h, the ability to disaggregate the soluble starch substrate was measured.

Statistical analysis. — Student's t-test was used to determine the significant differences in the data. Statistical significance was set at $p < 0.05$.

Results

Identification and selection of enzyme-producing bacteria. — The results of 16S rRNA identification of the eight strains with high amylase activity among the 162 screened strains are presented in Table 2 and Fig. 1. As a result of measuring the enzyme activity of the eight strains by liquid culture, KS7913's enzyme activity was the largest at 35.13 U mg^{-1}

(Fig. 2). *Alteromonas* sp. KS7913 strain showed higher starch-degrading activity than *Pseudoalteromonas* strains.

Purification and characterization of enzyme from KS7913. — After growing KS7913 cells, the cell culture broth was concentrated using a membrane. During the concentration process, a slight loss occurred; however, the specific activity of the enzyme attained after the phenyl-Sepharose column increased to 52.80 U mg^{-1}. Finally, the enzyme was purified using Superdex 75, obtaining an enzyme with a specific activity of 200.34 U mg^{-1}, 5.70-fold purification, and 3.4% yield. The detailed results of each purification step are summarized in Table 3. The enzyme activity was analyzed by zymography and the molecular mass was approximately 70 kDa (Fig. 3).

Effect of pH and temperature on amylase activity and stability. — The optimal pH is presented in Fig. 4. KS7913 amylase showed the highest activity at pH 7.0. The activity decreased toward an acidic state. When amylase activity was tested under acidic conditions using sodium acetate buffer, a significant decrease was observed. The enzyme was inactivated below pH 4.0. The stability of the enzyme was maintained over 80% in weakly acidic to alkaline conditions. Below pH 5.0, stability decreased by more than 20% in the same buffer.

When the effect of temperature on amylase of KS7913 was examined, the highest activity was achieved at 25°C (Fig. 5), and the activity gradually decreased at 35°C or higher temperatures. The enzyme showed 68% and 82% of its maximum activity at 5°C and 15°C, respectively. *Alteromonas* sp. KS7913 exhibited remarkably high activity levels at low temperature such as 5°C, corresponding to 68% of the maximal activity. The commercial enzyme, Amplify®, showed the highest activity at 75°C and the activity was approximately twice that of amylase from KS7913 at 25°C. However, at cold temperatures such as 5°C, the enzyme activity was severely reduced to 10% of the maximum activity. Compared with KS7913 amylase, Amplify® showed double the level of activity (271.6 U mg^{-1} and 132.5 U mg^{-1}) at 15°C; however, the activity rapidly decreased at 5°C (63.39 U mg^{-1} and 112.3 U mg^{-1}). For temperature stability, the activity was maintained at 80% or more from 5°C to 25°C. Only 40% of the maximum activity remained at 35°C and the decline continued at 45°C.

Effect of metal ions on the activity of amylase. — Copper and zinc ions were found to inhibit enzyme activity. The enzyme activity decreased by 74% in the presence of copper ions and by 21% in the presence of zinc ions (Fig. 6). In contrast, the amylase activity increased upon exposure to other ions. In particular, amylase activity in the presence of manganese,

barium, and calcium ions was strengthened 1.75 times more than sole KS7913 amylase condition.

Alcohol tolerance of amylase. — When KS7913 amylase was treated with 25% and 90% alcohol for 1 h, the activity, except for ethanol, was greatly reduced at a concentration of 90%. At a concentration of 25%, high activity was observed in methanol and ethanol; however little activity was observed in isoamyl alcohol and butanol (Fig. 7).

Discussion

Various amylase-producing bacteria were discovered in the Arctic Ocean, and the cold-active amylase was investigated. Compared with other amylases, the maximum activity of our KS7913 amylase was higher than that of cold-active α-amylase from *Pseudoalteromonas* sp. 2–3 (51.7 U mg^{-1}; Sanchez *et al*. 2019) or from *Bacillus* sp. dsh19-1 (16.4 U mg^{-1}; Dou *et al*. 2018); however, it was lower than that of cold-active amylase from *Zunongwangia profunda* (284.9 U mg^{-1}; Qin *et al*. 2014).

KS7913 amylase showed optimal activity at pH 7.0 and the enzyme was greatly stable within pH 6.0–10.0 for 1 h. However, its activity decreased in acidic conditions, indicating that the sensitivity of the enzyme toward low pH was high. Similar findings were also reported in previous studies (Kuddus *et al*. 2012; Roohi *et al*. 2013; Xie *et al*. 2014; Rathour *et al*. 2020). Therefore, this result suggests that KS7913 amylase could be more effective in cold-alkali fields such as the food industry.

Compared to other Antarctic bacterial strains producing amylases, *Arthrobacter* sp. and *Carnobacterium iners* showed a decrease in activity to less than one-third at 5°C (Ottoni *et al*. 2020), which is contrary to KS7913 amylase. Particularly, *Alteromonas* sp., the strain investigated in this study, shares similarities with *Pseudoalteromonas* sp. 2–3, an Antarctic bacterium. Both strains exhibited optimal temperature characteristics at approximately 20°C. However, the amylases produced by *Pseudoalteromonas* sp. 2–3 showed a 60% decrease in activity at 10°C (Sanchez *et al*. 2019). Even compared to other strains producing cold-active amylases, such as *Bacillus* sp. dsh19-1, *Zunongwangia profunda* and *Microbacterium foliorum* GA2, they showed less than 40%, 50%, and 10% of maximal activity at 5°C, respectively (Dou *et al*. 2018; Qin *et al*. 2014; Kuddus *et al*. 2012). Therefore, according to revealed studies, KS7913 amylase demonstrates the highest relative activity at 5°C and 15°C. This result suggests that the amylase from *Alteromonas* sp. KS7913 possesses a strong cold-adaptive characteristic.

The effect of metal ions on amylase activity varies depending on the microbial source. In *Nocardiopsis* sp. 7326, amylase was activated in the presence of Ca^{2+}, Mn^{2+}, Mg^{2+}, Cu^{2+}, and Co^{2+} (Zhang and Zeng 2008). The amylase from *Shewanella* sp. ISTPL2 was inhibited by the addition of Mn^{2+}, Cd^{2+}, Zn^{2+}, Na^{2+}, and Co^{2+}; however, Cu^{2+} was required for efficient activity (Rathour *et al*. 2020). In *Salinispora arenicola* CNP193, Ca^{2+}, Na^{+}, and K^{+} significantly increased the α-amylase activity, whereas other metal ions, such as Hg^{+}, Cu^{2+}, Zn^{2+}, Pb^{2+}, and Fe^{3+}, inhibited the enzyme (Liu *et al*. 2019). The activities of the cold-active amylases from *Pseudoalteromonas* sp. 2–3 (Sanchez *et al*. 2019), *Bacillus* sp. dsh19-1 (Dou *et al*. 2018), and *Zunongwangia profunda* (Qin *et al*. 2014) increase in the presence of Ca^{2+}, consistent with the results of this study. Most known-amylases are metalloenzymes that require Ca^{2+} for activity, stability, and structural integrity (Gupta *et al*. 2003; Ghorbel *et al*. 2009; Chen *et al*. 2015; Dou *et al*. 2018). Inhibition of activity in Cu^{2+} and Zn^{2+} was also reported by Qin *et al*. (2014). Amylases appear to be affected differently by metal ions depending on their structure (Zhang and Zeng 2008). This suggests that the structure of the amylase produced by KS7913 differs from that of other bacterial amylases.

For alcohol tolerance results, the enzyme maintained its activity at a concentration higher than the theoretical ethanol production concentration of 23% during fermentation. Therefore, it can efficiently hydrolyze carbohydrate substrates and simultaneously undergo alcohol fermentation, making it suitable for simultaneous saccharification and fermentation processes. Additionally, they exhibit resistance to organic solvents and alcohols. This strain has been found to exhibit superior resistance to methanol compared to other strains (Pandey *et al*. 2018). This indicates the advantage of using the enzyme for organic solvent-based reactions, which increases the solubility of non-polar substrates, inhibits water-dependent side reactions, and eliminates microbial contamination in reaction mixtures (Anbu *et al*. 2020).

Conclusions

This study revealed that *Alteromonas* sp. KS7913, obtained from the Arctic sample, maintained exceptionally high activity levels at a low temperature of 5°C, surpassing the activity levels of the other amylase-producing Arctic and Antarctic bacterial strains mentioned earlier. These results suggest that the amylase produced by the KS7913 strain has a strong low-temperature activity. This amylase meets the conditions required for industrial use, including high activity at low temperatures and stability in various environments and substances. Therefore, the amylase produced by KS7913 can be expected to have structural characteristics that differ from

those of amylases and has industrial potential. However, productivity improvements need to be investigated through gene manipulation and mass culture using gene-recombination strains.

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Table 1.

Compositions of media used for bacteria cultivation.

Media	Composition (g L^{-1})		Media	Composition (g L^{-1})	
SZB	Glucose	20	MB	Peptone	5
	Peptone	5		Yeast extract	1
	Yeast extract	1		Ferric citrate	0.1
	75% seawater	up to 1 L		Sodium chloride	19.45
				Magnesium chloride	8.8
YPG	Glucose	10		Sodium sulfate	3.24
	Peptone	5		Calcium chloride	1.8
	Yeast extract	5		Potassium chloride	0.55
	Seawater	up to 1 L		Sodium bicarbonate	0.16
				Potassium bromide	0.08
R2A	Proteose peptone	0.5		Strontium chloride	0.034
	Casamino acids	0.5		Boric acid	0.022
	Yeast extract	0.5		Sodium silicate	0.004
	Dextrose	0.5		Sodium fluoride	0.0024
	Soluble starch	0.5		Ammonium nitrate	0.0016
	Dipotassium phosphate	0.3		Disodium phosphate	0.008
	Magnesium sulfate	0.05		Distilled water	up to 1 L
	Sodium pyruvate	0.3			
	80% seawater	up to 1 L	ZB	Peptone	5
				Yeast extract	1
ISP4	Soluble starch	10		75% seawater	up to 1 L
	Dipotassium phosphate	1			
	Magnesium sulfate	1			
	Sodium chloride	1			
	Ammonium sulfate	2			
	Calcium carbonate	2			
	Ferrous sulfate	0.001			
	Manganous chloride	0.001			
	Zinc sulfate	0.001			
	70% seawater	up to 1 L			

SZB: super ZoBell; YPG: yeast extract-peptone-glucose

Table 2.

Information on the eight strains showing high amylase activity from the Arctic Ocean.

Sample No.	Sampling year and location	Latitude (N) and longitude (W)	Closest match	Identification	Accession No.
KS7913	2012, Chukchi Sea	76°08'70.25" 174°56'87.61"	*Alteromonas naphthalenivorans* SN2	*Alteromonas* sp. KS7913	PP937156
KS16195	2012, Chukchi Sea	76°08'70.25" 174°56'87.61"	*Pseudoalteromonas agarivorans* DSM 14585	*Pseudoalteromonas* sp. KS16195	PP937157
KS16398	2012, Chukchi Sea	77°32'00.9" 161°46'05.8"	*Pseudoalteromonas tetraodonis* GFC KMM 458	*Pseudoalteromonas* sp. KS16398	PP935320
KS16483	2012, Chukchi Sea	77°45'00.0" 165°22'05.0"	*Pseudoalteromonas elyakovii* KMM 162	*Pseudoalteromonas* sp. KS16483	PP935319
KS16627	2012, Chukchi Sea	77°02'07.42" 173°18'10.12"	*Pseudoalteromonas carrageenovora NBRC 12985*	*Pseudoalteromonas* sp. KS16627	PP937155
KS17030	2012, Chukchi Sea	77°32'00.9" 161°46'5.80"	*Pseudoalteromonas tetraodonis* GFC KMM 458	*Pseudoalteromonas* sp. KS17030	PP935318
KS17134	2012, Chukchi Sea	76°11'41.75" 173°31'95.17"	*Pseudoalteromonas agarivorans* DSM 14585	*Pseudoalteromonas* sp. KS17134	PP937154
KS27803	2014, Beaufort Sea	69°42'06.81" 137°32'31.91"	*Pseudoalteromonas agarivorans* DSM 14585	*Pseudoalteromonas* sp. KS27803	PP937158

Table 3.

Purification summary for KS7913 amylase.

Stage	Total activity	Total protein content (mg)	Specific activity (U mg^{-1})	Fold	Yield (%)
Cell culture broth	340.1	9.677	35.13	1.000	100.0
10 kDa cut-off	86.77	4.213	20.61	0.587	25.51
Phenyl-Sepharose column	13.72	0.290	52.80	1.503	4.030
Superdex 75	11.45	0.057	200.3	5.702	3.370

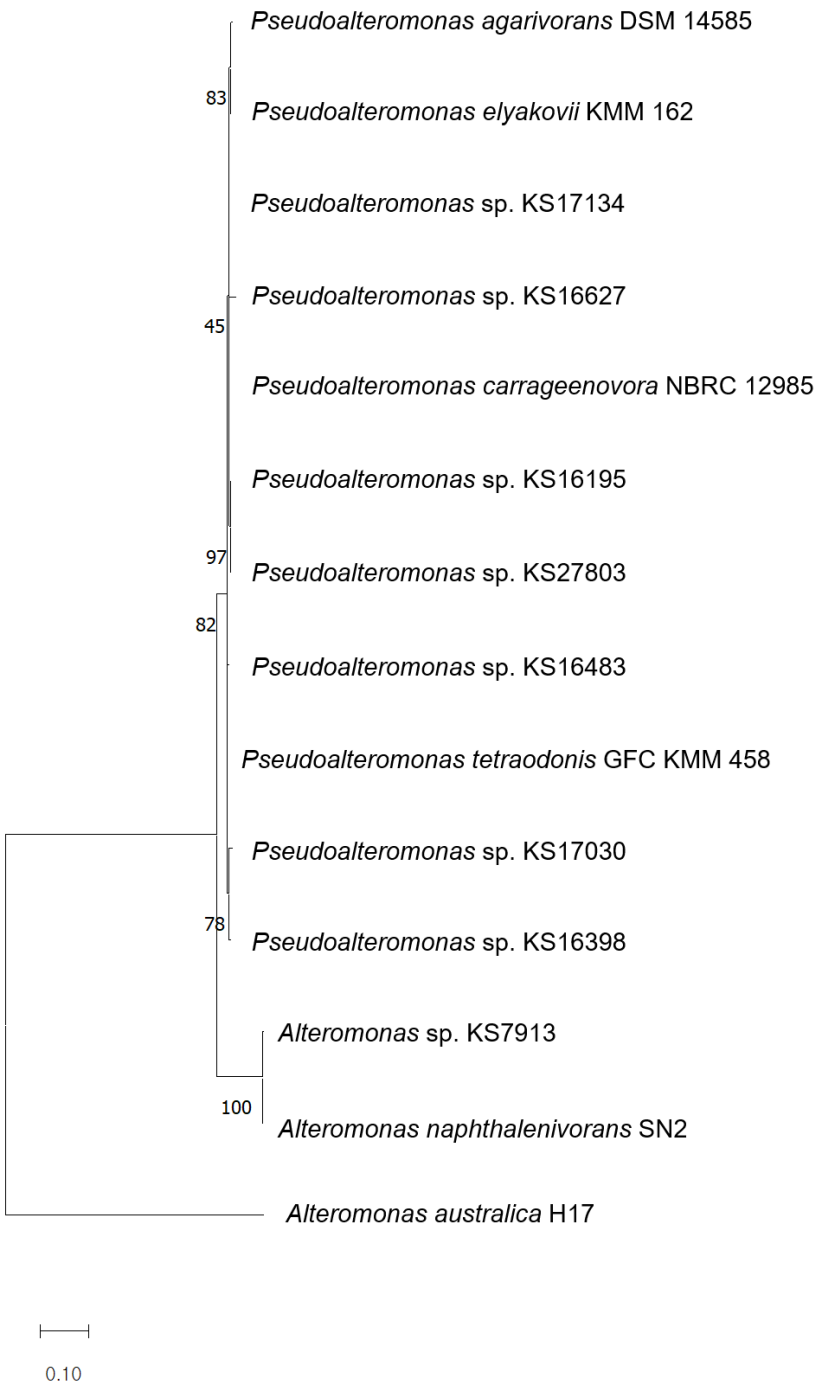

Fig. 1. Phylogenetic tree of amylase-producing strains from the Arctic Ocean. Neighbor-joining method based on 16S rRNA sequence of the isolates with bootstrapping of 1 000 replicates. The phylogenetic tree is constructed with MEGA 11.0 software. The evolutionary distance is represented with the lengths of the branches and it was computed using the Jukes-Cantor method (Jukes and Cantor 1969).

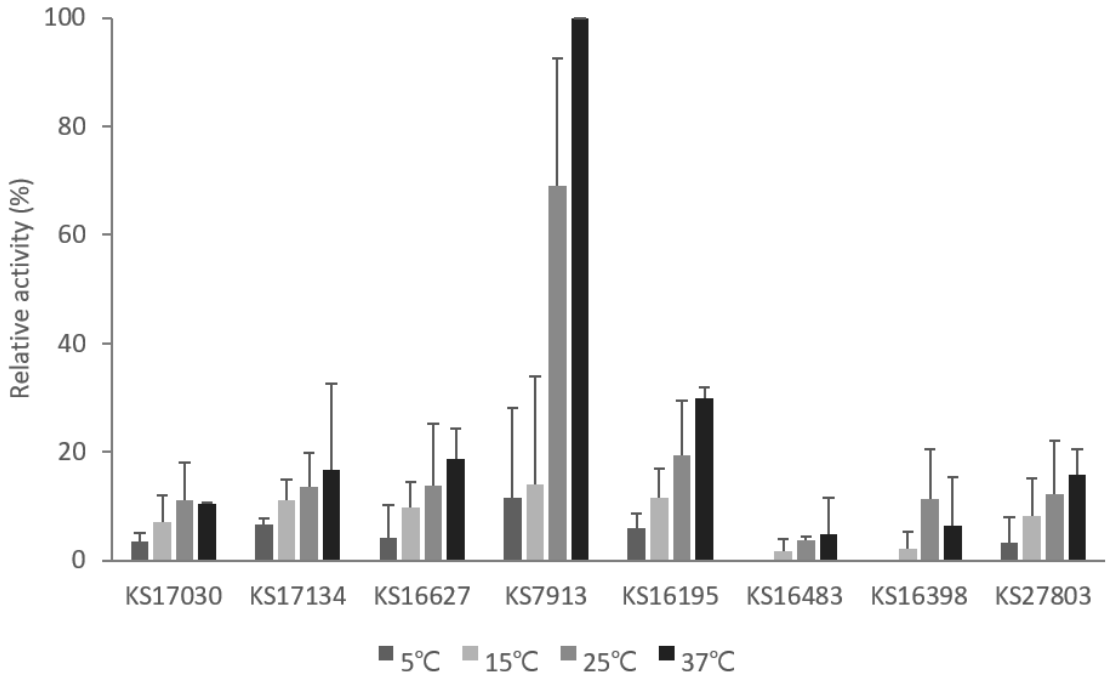

Fig. 2. Amylase activity measured for each cell culture broth. The assay was conducted at temperatures ranging from 5°C to 37°C. Data are presented as mean and standard deviation of duplicate experiments.

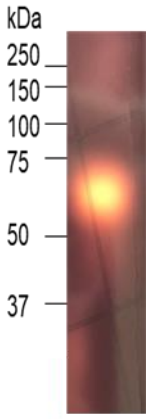

Fig. 3. Activity confirmation of amylase from *Alteromonas* sp. KS7913 by zymography analysis.

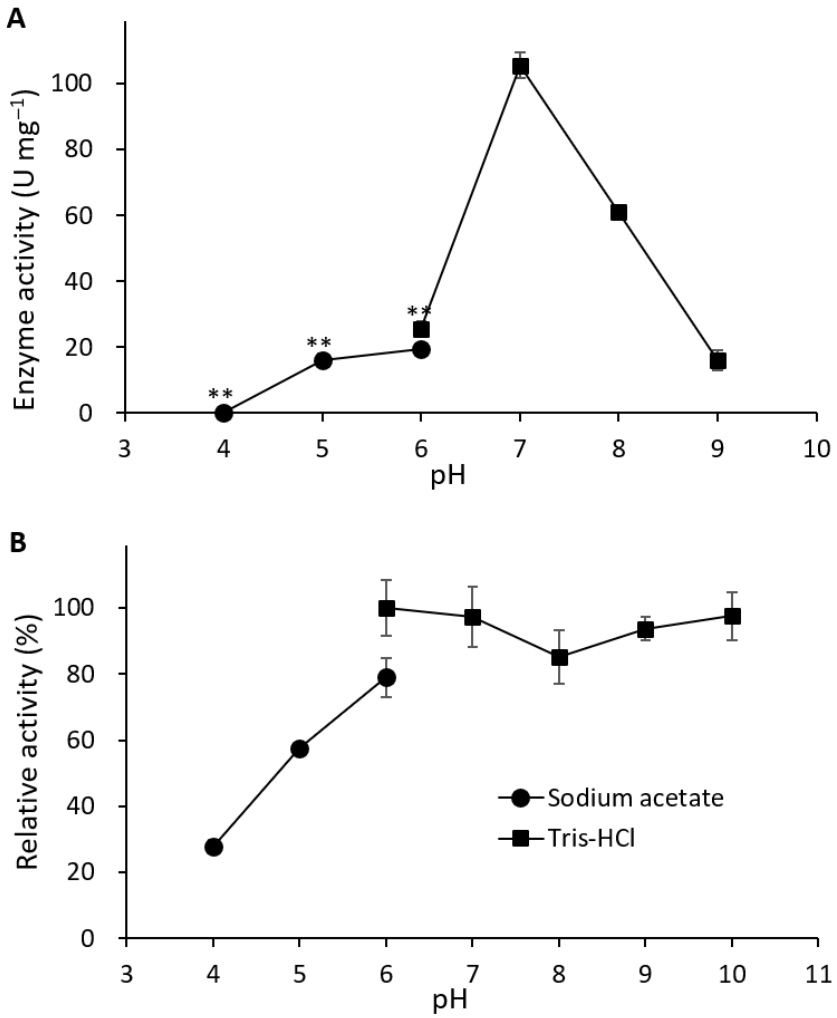

Fig. 4. Effect of pH on amylase activity. (**A**) Starch-degrading activity (U mg^{-1}) of α-amylase from KS7913 at pH 4.0 to 9.0. (**B**) pH stability of α-amylase from KS7913. To achieve the pH values, the following buffers were used: 50 mM sodium acetate buffer pH 4.0 to 6.0 (●) and 50 mM Tris-HCl buffer pH 6.0 to 10.0 (■). * $p < 0.05$ and ** $p < 0.01$ compared to the optimal pH (pH 7.0).

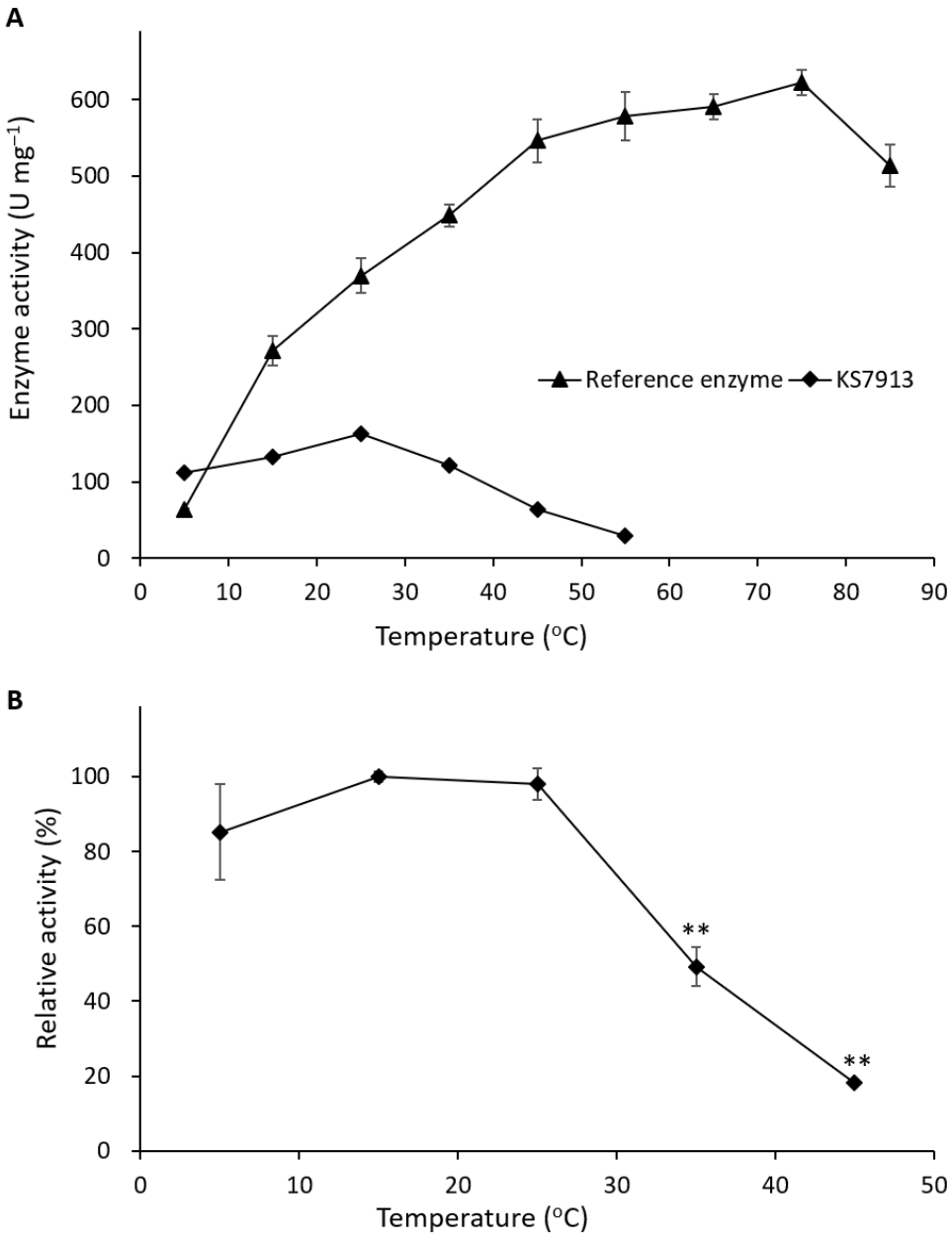

Fig 5. Effect of temperature on amylase activity. (**A**) Starch-degrading activities (U mg^{-1}) of α-amylase from KS7913 (♦) and the commercially available enzyme (▲) at temperatures ranging from 5°C to 85°C. (**B**) Thermostability of α-amylase from KS7913. * $p < 0.05$ and ** $p < 0.01$ compared to the optimal temperature (25°C).

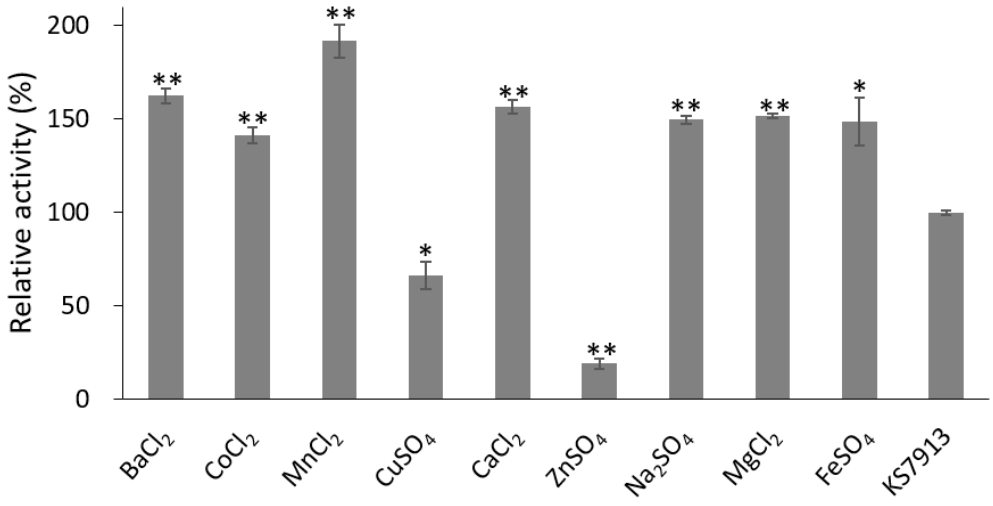

Fig. 6. Effect of metal ions on amylase activity. The enzyme activity under standard conditions (enzyme reactions without additives) is shown as the rightmost bar (KS7913). * $p < 0.05$ and ** $p < 0.01$ compared to the KS7913 amylase.

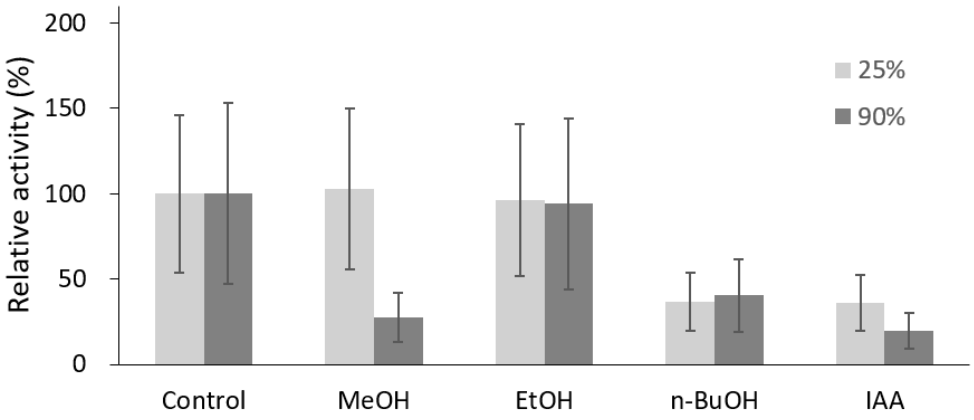

Fig. 7. Alcohol tolerance of α-amylase from KS7913. Enzyme reactions were performed in the presence of methanol, ethanol, n-butanol, and isoamyl alcohol at 25% or 90% concentration.