

Characterization of α -maltotetraohydrolase produced by *Pseudomonas* sp. MS300 isolated from the deepest site of Mariana trench

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Previously, we have reported the isolation of bacterium which produced exo- α -maltotetraohydrolases (G4-amylase) from the deepest site of the Challenger deep of the Mariana trench. In this manuscript, we report the identification of the amylase producer, MS300 strain as *Pseudomonas* sp. and the purification and characterization of G4-amylase produced by MS300. The strain MS300 produced two major and two minor α -maltotetraohydrolases (G4-amylase). The two major G4-amylases share the same molecular weight of 55,000 had different pI values of 5.0 and 4.7. The optimum temperature for activity of both major G4-amylases is 40°C and the optimum pH is 6.8 for one and 8.9 for the other. The activities of these G4-amylases were not affected by the hydrostatic pressure. Under high hydrostatic pressure, the MS300 produced more amylase than under atmospheric pressure. Strain MS300 may be active in the deep sea at a depth of 10,897m.

Key words : Mariana trench, Amylase, enzyme reaction under, high pressure, *Pseudomonas*

マリアナ海溝から分離したMS300株が生産する G4アミラーゼの精製と諸性質

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前報(JAMSTEC深海研究第14号)において、マリアナ海溝チャレンジャー海淵から、G4アミラーゼを生産する細菌MS300株を分離したことを報告した。今回はそのMS300株の性質を調べ、G4アミラーゼの精製および諸性質の検討を行った。その結果、MS300株はシュードモナス属の細菌であり、4つのG4アミラーゼを生産した。4種類のG4アミラーゼの中で生産量の多いAmylase AおよびAmylase Bを精製した。Amylase A, Bの等電点はそれぞれ5.0, 4.7であり、分子量は55,000であった。また、反応の至適pHおよび温度は6.8, 40°Cであった。これらの酵素を高圧環境下において反応させたところ、常圧下における反応と差が認められなかった。しかしながら、高圧環境下において、細胞あたりの酵素生産の向上が認められた。したがって、MS300株は深海底という高圧環境において積極的に酵素生産を行っているかと推察された。

キーワード：マリアナ海溝、アミラーゼ、高圧下の酵素反応、シュードモナス

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1. Introduction

There are various environments that support life on the earth. Some bacteria adapt its surroundings and produce useful enzymes such as amylases, proteases and cellulases. Most of microorganisms known at present were isolated from the continents that occupy only 30% of the earth. We expect that the novel microorganisms will be isolated from mud of the ocean which occupies another 70% of the earth. The deep-sea has an ecosystem different from that of the land, because there is no sunlight, low temperature and high hydrostatic pressure. Thus, we expect that the deep-sea will be a resource of novel enzyme producers.

We have collected the mud sample from Challenger Deep (11°21.111'N, 142°25.949'E) region of Mariana trench at a depth of 10,897 m by using unmanned submersible Kaiko, on March 2 1996. The Challenger Deep of Mariana trench is the deepest point on the earth. We have isolated various non-extremophiles and extremophiles such as alkaliphiles, thermophiles, barophiles and psychrophiles from these mud samples¹⁾²⁾. What kinds of enzymes would be produced by these bacteria? Our interest has been focused on amylase. Several hydrolysis profiles of amylases are known to be obtained with starch as substrate and this is one of the enzymes with uses in various industrial fields. In previous report, we isolate G4 amylase producer from this sea sediment³⁾. In this study, we identified this amylase producer to *Pseudomonas* sp. We also characterized the amylases and examined the effect of the hydrostatic pressure on the amylase production by the strain MS300.

2. Materials and methods

2.1 Bacterial strain and media

The isolation of the amylase producer was described in the previous paper¹⁾. Among all amylase producer, strain MS300 formed the largest halo around the colony. Strain MS300 was grown at 25°C in a medium containing 10 g of potato starch (Difco lab. USA), 10 g of Yeast extract (Difco lab. USA), 10 g of polypeptone (Wako Ltd. Japan) and 10 g of NaCl per liter (PYP medium) or Marine broth 2216 (Difco lab. USA). In the case of cultivation under high hydrostatic pressure, YP medium which contains 10 g of Yeast extract (Difco lab. USA), 10 g of polypeptone (Wako Ltd. Japan) and 10 g of NaCl per liter or Marine broth 2216 (Difco lab. USA) was used.

2.2 Amylase assay

Amylase activity was measured by the amyloclastic method⁴⁾ or 2-dinitrosalitic acid reaction⁵⁾. Potato starch which was solubilized in 0.1 N NaOH solution was used as the substrate. The amylase reaction was performed to 0.6% potato starch as substrate for 20 min at 40°C, unless otherwise stated. One unit of enzyme was defined as the activity forming reducing sugar corresponding to 1 μ mol of glucose per 1 min in 2-dinitrosalitic acid reaction.

2.3 Protein content

Protein content was measured by the method of Lowry⁶⁾ or Bradford method⁷⁾, using bovine serum albumin as the standard.

2.4 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out on 15% polyacrylamide gel by the method described by Laemmli⁸⁾. Proteins were dissolved in 16 mM Tris-HCl buffer (pH 6.8), 1% (wt/vol) SDS, 2.5% (vol/vol) β -mercaptoethanol, 20% (wt/vol) sucrose and heat-treated at 100°C for 5 min. Protein in the gel was stained with Coomassie Brilliant Blue R-250. A zymogram was prepared by transferring the gel onto 1% agarose gel containing 0.1% soluble starch (Difco lab.) and 50 mM MOPS-Na (pH 7.0). Then the gels were incubated for 1 h at 30°C. Amylase activity was visualized by staining the agarose gel in a solution containing 0.2% (wt/vol) I_2 - 2% (wt/vol) KI⁹⁾.

2.5 Isoelectric equilibrium electrophoresis of protein

The isoelectric equilibrium electrophoresis was performed on a polyacrylamide gel column containing Ampholine of pH 3.5 to 9.5 as described by Catsimpoolas¹⁰⁾. Myoglobin-basic-band (pI-7.35), myoglobin-acidic band (pI-6.85), human carbonic anhydrase B (pI-6.55), bovine carbonic anhydrase B (pI-5.85), β -lactoglobulin A (pI-5.20), soybean tyrosine inhibitor (pI-4.55) and amyloglucosidase (pI-4.55) were used as the pI standard.

2.6 Thin layer chromatography of amylase product

After the enzymatic reaction with 0.2% potato starch solution, 1 μ l of the reaction mixture was spotted onto 0.2 mm silica gel 60 plate (Merck) and the plate was developed with 1-butanol: methanol: water (4: 2: 1). A so-

lution comprising a mixture of 0.1% glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose was used as the standard. The oligosaccharides were detected by spraying 1% diphenylamine dissolved in acetone containing 10% phosphoric acid to TLC plate, then heated that at 160°C for 10 min.

3. Results

3.1 Isolation of amylase producers from Mariana trench

The collected sea-mud was spread onto Marine broth plate containing 1% potato starch and incubated 4, 25 or 55°C. The amylase producers were detected as those which showed the formation of a halo around the colony.

We could isolated 39 amylase producers and, among these, the strain MS300 formed the largest halo. The strain MS300 was found to be an aerobic, Gram negative, motile, rod-shaped bacterium. Test for oxidase and denitrification were positive. We identified the MS300 as a member of species *Pseudomonas* on the basis of these data.

3.2 Purification of the amylase produced by *Pseudomonas* sp. MS300

MS300 was grown aerobically in the medium described in Material and Methods for 2 days at 25°C. After harvesting cells, ammonium sulfate was added to the culture supernatant at 40% of saturation and the mixture was held at 4°C for 16 h. The SDS-PAGE of culture supernatant

a)

Faction	Volume (ml)	protein (mg)	activity (u)	spec. activity (u/mg)
Culture supernatant	1800	286	1.6×10^5	550
(NH ₄) ₂ SO ₄ precipitation	152	176	9.1×10^4	439
DEAE-toyopearl-I	132	110	9.1×10^4	824
DEAE-toyopearl-II	8	26	2.8×10^4	1074
HPLC (PROTEIN-PAK300 x 2)				
F-1 (amylase A)	1.2	0.30	463	1544
F-2 (amylase B)	1.6	0.31	550	1775

b)

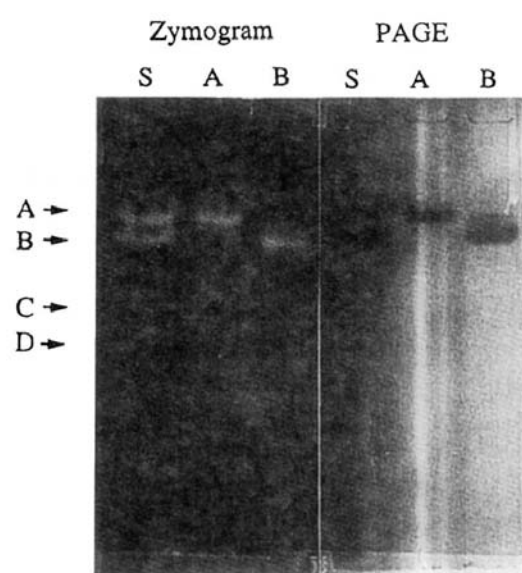


Fig. 1 Purification of amylase produced by MS300. Panel a shows purification steps of the amylase. Panel b shows PAGE and zymogram of each amylase fraction. Electrophoresis and staining were performed as described in Materials and Methods. Lanes : S, culture supernatant ; A, amylase A ; B, amylase B. Arrows indicate amylase activity bands in the culture supernatant.

showed only one prominent protein band and its size was estimated as 55 kDa (data not shown). However, a zymogram prepared using the culture supernatant showed four bands of amylase activity of which two were major amylases (A, B) and the other two were minor amylases (C, D) (Fig.1). The pI of major amylase A and B indicated 5.0 and 4.7, respectively. The preparation was dialyzed against 10 mM Tris-HCl buffer (pH 7.5) at 4°C and applied to DEAE-Toyopeal column (ID 2.5 x 6 cm, 30 ml) equilibrated with 10 mM Tris-HCl (pH 7.5). The amylases were eluted with a linear gradient of 0 to 0.1 M NaCl in the same buffer. The fractions containing amylase activity were collected. These fractions were applied to a DEAE-Toyopeal column (ID 1.7 x 7.4 cm, 13 ml) equilibrated with 10 mM Tris-HCl (pH 8.5). The amylases were eluted with a stepwise gradient of NaCl concentration of 40, 80 and 120 mM. The fractions containing amylase activity were collected. Then amylase-containing fraction was applied to 2 x PROTEIN-PAK300 column (MILLIPORE Co.) and eluted with 25 mM MOPS-NaOH buffer (pH 7.0). The purity of each fraction was checked by means of a zymogram. We purified the two major amylases on an extent such that a single band was obtained by SDS-PAGE in each instance. We determined the N-terminal amino acid sequences of two major amylases and both were found to be ADLAGKSPAGVRYHGGDEII. The N-terminal amino acid sequences of two minor amylases were same as that of major amylases.

3.3 The products of amylases from potato starch

Amylase A produced maltotetraose from potato starch as the main initial product (Fig.2A). Amylase B also produced maltotetraose from potato starch (Fig. 2B). Both amylases showed no reaction with α -, β -, γ -cyclodextrin (data not shown). The maltotetraoses produced by these amylases were the α -anomer as indicated their mutarotation (data not shown)

3.4 Effects of temperature and pH on the activity and stability of the amylases

The optimum temperature for activity of amylase A and B was 50°C in each instance and these enzymes were inactivated by 10 min-incubation at 60°C (pH 7.0) (Fig. 3). The enzymatic activity was stable for 30 min at temperatures below 40°C at pH 7.0 in the case of both (Fig.3).

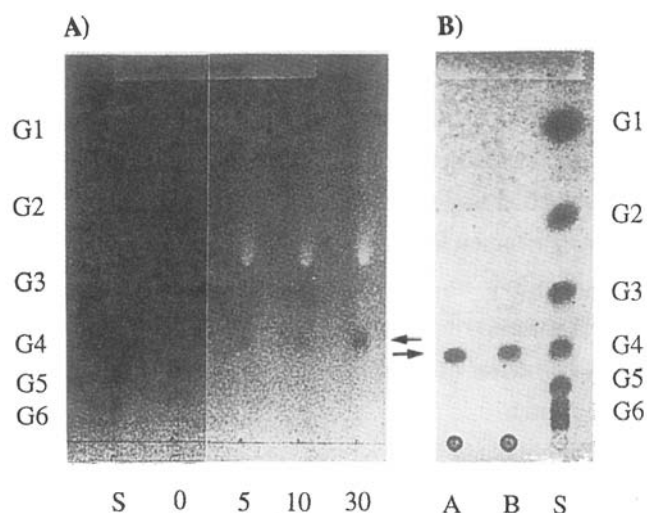


Fig. 2 TLC analyses of amylases product from potato starch. Panel A shows amylase A product from potato starch at various reaction times. The reaction mixture contained 50 mM MOPS-NaOH buffer (pH 7.0), 0.1% potato starch, 10 U/ml of amylase A. The reaction was performed at 40°C. Samples (1 μ l) were drawn after various periods of time and applied to TLC analysis. Panel B shows amylase A and B product from potato starch. The reaction mixture same as described above except for changing potato starch concentration from 0.1% to 1.0%. Arrow indicates the product of amylase A or amylase B. Lanes: S, standard of oligosaccharides from G1 (glucose) to G6 (maltohexaose); 0, 0 min; 5, 5 min; 10, 10 min; 30, 30 min.

In the presence of 1 mM of CaCl_2 , the activity at 60°C was about 1.3-fold (amylase A) and about 1.7-fold (amylase B) higher than that in the absence of calcium ions. The activity of each was lost at 60°C regardless of the absence or presence of 1 mM CaCl_2 .

The optimum pH for activity at 40°C was pH 6.3 for amylase A and pH 7.8 for amylase (Fig. 4A). Amylase A was stable over the range of pH 7.0 to 8.0 and amylase B was stable over the range of pH 7.0 to 9.0 upon incubation at 40°C for 30 min (Fig. 4B).

3.5 Effect of metal ions on the activity

The purified enzymes were treated in the starch solution (0.1%) with 1 mM metal chloride. As shown in Table 1, both amylases A and B were inhibited by Zn^{2+} , Cu^{2+} and Hg^{2+} , showing 1-3%, 2-4% and 1-3% of the activity of control, respectively. Upon addition of Mn^{2+} , Fe^{2+} and

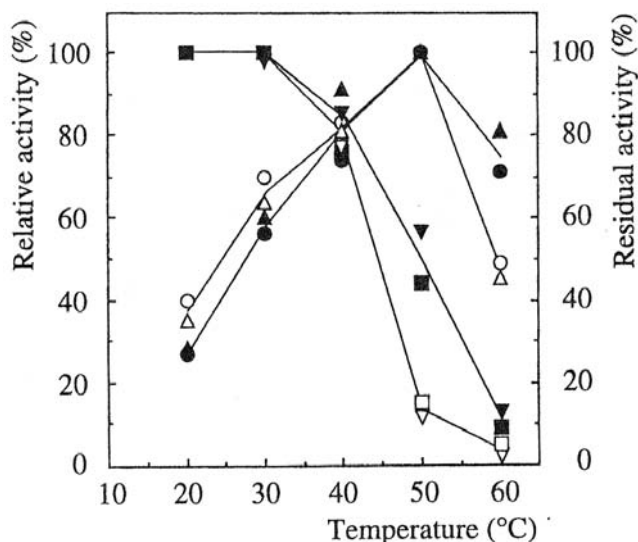


Fig. 3 Effects of temperature on the amylase A and B. Relative activity of amylase A (●, ○) and amylase B (▲, △) at each temperature in the presence or absence of 5 mM CaCl_2 were calculated from activity at 50 °C as 100%. Residual activity of amylase A (▼, ▽) and amylase B (■, □) in the presence or absence of 5 mM CaCl_2 were measured after incubation at each temperature for 30 min.

Al^{3+} each of the amylases was inhibited by 81-90% as compared to the control. None of the metal ion tested in this study selectively inhibited only one of these enzymes.

3.5 Effect of hydrostatic pressure on the growth and amylase production of MS300

Strain MS300 was incubated at 4°C under various hydrostatic pressure for 2 weeks, and the number of colony forming unit (CFU) was found to increase at pressures less than 75 MPa, starting with initial CFU of 4×10^5 /ml, and the amylase activity was detected in the culture supernatant at pressures below 100 MPa (Fig. 5).

Optimum growth occurred at 25 MPa and the number of viable cells was about twice that observed at 0.1 MPa. The CFU at 100 MPa could not be accurately determined because of cell aggregation. The initial amylase activity was not detected because the cells were washed before inoculation of the medium. The amount of amylase activity in the supernatant decreased under high hydrostatic pressure. The amylase activity at 75 MPa was 73% of that at 0.1 MPa, although CFU at 75 MPa was 6% of that at 0.1 MPa. Amylase activity was also detected at 100 MPa in spite of cell aggregation.

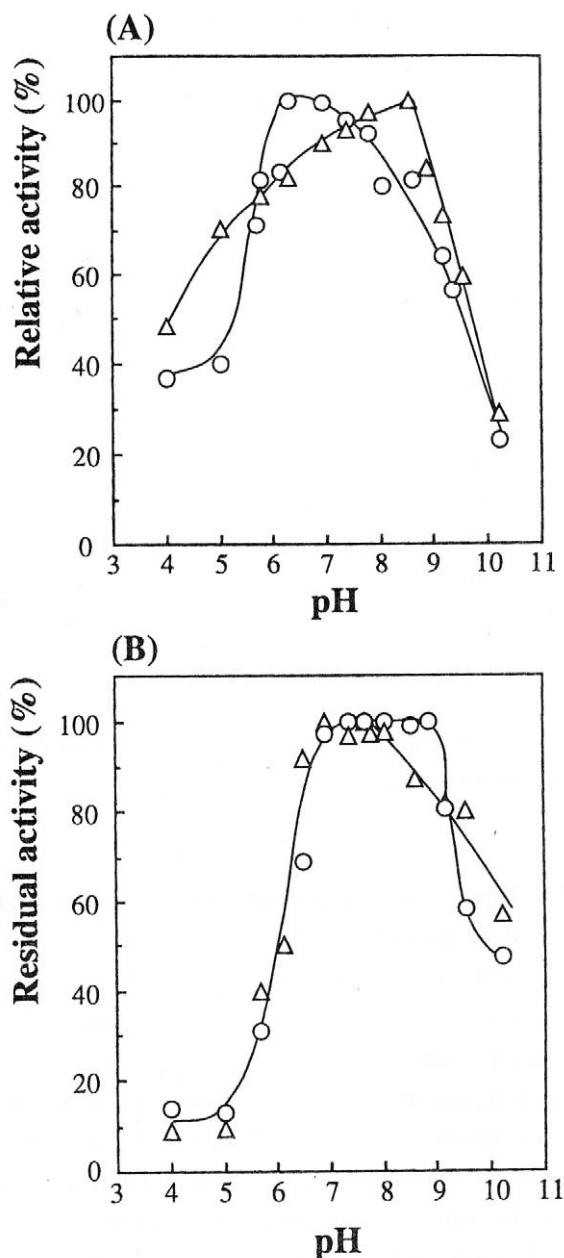


Fig. 4 Effects of pH on the amylase A and B. Panel A and panel B show relative activity and residual activity of amylase A (○) and amylase B (△) at various pH. Relative activity was calculated from maximum activity as 100%. Residual activity were measured after incubation at various pH for 30 min. Reaction mixtures were buffrized with 50 mM acetate/ NaOH biffer (pH 4.0-5.7), phosphate/ NaOH buffer (pH 5.7-6.3), MOPS/ NaOH buffer (pH 6.5-7.8), Tris/ HCl buffer (pH 7.8-8.6), CHES/ NaOH (pH 8.7-9.5) and glycine/ NaOH/ NaCl Buffer.

Table 1. Effect of metal ions on amylases

Metal ions*	Relative activity (%)	
	Amylase A	Amylase B
Na ⁺	105	105
K ⁺	110	105
Ni ²⁺	41	38
Zn ²⁺	1	3
Co ²⁺	50	45
Fe ²⁺	90	85
Ca ²⁺	103	98
Zn ²⁺	2	4
Cu ²⁺	2	0
Mn ²⁺	89	81
Hg ²⁺	1	3
Mg ²⁺	98	91
Pb ²⁺	19	24
Al ²⁺	83	82
Fe ²⁺	23	13
none	100	100

* All metal ions were added as a chloride salt and at the concentration of 1 mM.

3.6 Higher amylase productivity under high hydrostatic pressure

MS300 cells grown to the stationary phase in YP medium were harvested and washed with same medium. Thereafter, the cells were suspended in fresh YP medium, and cell suspension was adjusted to an optical density of 2.0 at 660 nm (OD₆₆₀). The culture was incubated at 25°C under pressure conditions of 0, 30 and 60 MPa for 3 days. The amount of amylase produced by MS300 was 3.7 U/ml in 50 ml of the culture under 0.1 MPa. Much more amylase was produced under high pressure than under atmospheric pressure (Table 2). At 30 MPa and at 60 MPa, MS300 produced about 1.3 and 1.4 times as much amylase as that observed under 0.1 MPa. The OD₆₆₀ of these cultures did not change in the course of incubation.

4. Discussion

Takami et al first isolated various microorganisms from mud samples obtained from the Challenger Deep¹⁾. Some of them were amylase producers. In this study, we purified and characterized the two major amylases produced by strain MS300, which made the largest halo on the starch-containing medium among all of the Mariana isolates. MS300 was identified as a member of *Pseudomonas* spe-

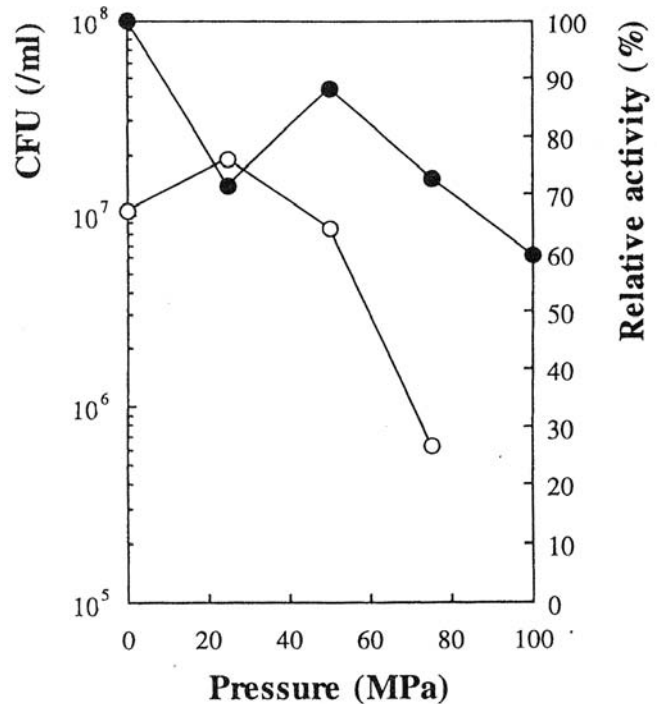


Fig. 5 Effects of pressure on the growth and amylase production of MS300. MS300 was incubated at 4°C under various pressures for 2 weeks. Colony forming unit (CFU, ○) was calculated from the colonies grown on the Marine broth plate after incubation. The CFU of before incubation was 4.0×10^4 /ml. Amylase activity (●) was measured by 2-dinitrosalicylic reaction described in Materials and Methods. Relative activity were calculated maximum activity (0.1 MPa) as 100%.

Table 2. Effect of hydrostatic pressure on amylase production.

Pressure (MPa)	Activity (U/ml)	OD ₆₆₀ *
0.1	3.6	2.0
30	4.5	2.1
60	4.8	1.9

* OD₆₆₀ of the culture after incubation

cies. This strain found to produce two major amylases (amylase A and B) and two minor amylases (Fig.1). Both major amylases generated maltotetraose from starch (Fig. 2) and did not react with α -, β - or γ -cyclodextrin. The maltotetraose produced by these amylases from potato starch was the α -anomer (data not shown). Therefore, both amylase A and B were identified as exo-a-

maltotetraohydase (G4-amylase). G4-amylases from *Pseudomonas stutzeri* strains have been reported¹¹). Robyt and Ackerman reported that *P. stutzeri* produced seven G4-amylases which differed in molecular mass and isoelectric point¹²). Sakano et al reported two G4-amylases which had the same molecular mass but different isoelectric point¹³). Those G4-amylase isozymes had almost the same properties. Strain MS300 found to produce four G4-amylases. The major amylase A and B had greater resistance to inhibition by metals than any other exo-maltotetraohydase from *Pseudomonas* (Table 1). The optimum pH for activity of amylase A and B differed. Furthermore, the pI, the optimum temperature and pH for activity of amylase A and B were different from those of the G4-amylases from *P. stutzeri* (Figs. 3, 4AB). Thus, our findings indicate the amylase A and B are the novel types of G4-amylases. The nucleotide sequence of the G4-amylase (*amy P*) from *P. stutzeri* MO-19 has been reported¹⁴). The first 20 amino acids of the N-terminal sequence of amylase A and B showed 80% similarity with that of G4-amylase from *P. stutzeri* MO-19. However, other properties of this G4-amylase have not been reported.

The microbial diversity in the Mariana trench has been reported as described above, but activity of the microbes there is still unknown. Mariana isolate MS300 could grow and produced amylase at pressure below 75 MPa in Marine broth (Fig.5). Furthermore, this strain produced much more amylase under high pressure conditions (Table 2). Although MS300 cells aggregated at pressure below 100 MPa, amylase activity was detected in the culture supernatant and turbidity of culture started increasing when the culture was decompressed to 0.1 MPa (data not shown). MS300 was active at a very low level at pressures below 100 MPa. Marine broth provides growth conditions far different from in situ conditions of Mariana trench. Our findings suggest that MS300 may be active at the sea bottom of Mariana trench. Although we have not fully established ideal methods for isolation and cultivation of deep-sea microorganisms, the increase of CFU or turbidity has been monitored as an indicator of bacterial activity¹⁵). When the microorganisms grow at very low rate, it is difficult to apply such an indicator for estimation of bacterial growth. In the case of enzyme producers, not only the CFU but also the amount of enzyme activity present in the culture supernatant can be applied

as an indicator of bacteria activity. Thus, the enzyme producer considered to be one of good sample organisms for studying deep sea ecosystem.

Strain MS300 could grow and produce amylase under 75 MPa (Fig.5). This strain produced much larger amounts of amylase under high hydrostatic pressure than at atmospheric pressure (Table 2). The amylase activity in culture at 75 MPa was 73% of that at atmospheric pressure in spite of lower cell density (Fig. 5). The mechanism of responsible for increase in amylase production at high pressure remains unknown as yet. There are some reports that some deep-sea bacteria have a pressure-regulated operons¹⁶). High hydrostatic pressure influences phospholipid membrane fluidity¹⁷) and caused a change in phospholipid composition of membranes¹⁸). These changes may promote the secretion of enzymes.

The deep-sea environment is thought to support a very low level of biological activity and the variety of microorganisms is thought to be very limited. However, we have isolated the novel type G4-amylase producer from this extreme environment. We are convinced that the deep sea is a valuable source of new microbial resources. Also, we can expect the deep sea environment will provide many novel microbial biocatalysts.

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