Extremophiles (2005) 9:85–89
DOI 10.1007/s00792-004-0423-2

NOTE

**Tadamasa Fukushima · Toru Mizuki · Akinobu Echigo
Akira Inoue · Ron Usami**

Organic solvent tolerance of halophilic α-amylase from a Haloarchaeon, *Haloarcula* sp. strain S-1

Received: 5 July 2004 / Accepted: 27 August 2004 / Published online: 17 September 2004

Abstract A halophilic archaeon, *Haloarcula* sp. strain S-1, produced extracellular organic solvent-tolerant α-amylase. Molecular mass of the enzyme was estimated to be 70 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. This amylase exhibited maximal activity at 50°C in buffer containing 4.3 M NaCl, pH 7.0. Moreover, the enzyme was active and stable in various organic solvents (benzene, toluene, and chloroform, etc.). Activity was not detected at low ionic strengths, but it was detected in the presence of chloroform at low salt concentrations. On the other hand, no activity was detected in the presence of ethyl alcohol and acetone.

Keywords *Haloarcula* sp. strain S-1 · Halophilic α-amylase · Halophilic archaea · Organic solvent tolerance · Purification

Starch-degrading enzymes, such as amylases, are found widely among the three domains Eucarya, Bacteria, and Archaea, playing a central role in carbohydrate metabolism, and evolutionary relatedness of α-amylase from Archaea and Plantae was reported (Janecek et al. 1999). There is a great variety of enzymes implicated in the hydrolysis of starch, and these are widely used in industrial processes (Kadziola et al. 1998; Machius et al. 1995). Moreover, amylases have become one of the most valuable enzymes in biotechnology, especially in the food- and starch-processing industries (Vihinen and Mäntsälä 1989).

In halophilic archaea, amylase from *Natronococcus amylolyticus* was purified for the first time (Kanai et al. 1995; Kobayashi et al. 1992) and gene cloned (Kobayashi et al. 1994). In addition, amylase from *Haloferax mediterranei* was purified (Rodriguez-Valera et al. 1983; Perez-Pomares et al. 2003). Usually, halophilic enzymes are able to deal with high ionic strength in their environment and also need high ionic strength to maintain their function and structure (Dym et al. 1995). These enzymes function under extremely high salt concentrations, and they are stable under dry conditions. Archaea provide us with a ready source of proteins that may serve as model proteins that exist in high salt concentrations and dry environments (Danson and Hough 1997; Marhuenda-Egea and Bonete 2002).

Since halophilic enzymes were stable in dry conditions, halophilic archaea were isolated from many hydrocarbon-containing environments (Zviagintseva et al. 1995), and we also found organic solvent tolerance in some halophiles (Usami et al. 2003) (especially strong tolerance was observed in type strains of halophiles), we supposed that enzymes from halophilic archaea may have organic solvent tolerance.

Organic solvent-tolerant proteases, lipases, and cyclodextrin glucanotransferase were isolated from *Pseudomonas aeruginosa* PST-01, LST-03 and *Paenibacillus illinoisensis*, respectively (Ogino et al. 1999a, b; Doukyu et al. 2003). However, organic solvent-tolerant enzymes of the halophilic archaea have not been reported. In this study, we report about an amylase of the new isolate, *Haloarcula* sp. strain S-1, and about the organic solvent tolerance of the enzyme.

A salt sample was dissolved in 50 ml of a sterile medium (consisting of 10 g/l soluble starch, 1 g/l sodium glutamate, 3 g/l trisodium citrate, and 2 g/l KCl, 20 g/l $MgSO_4 \cdot 7H_2O$) at a concentration of 4.3 M and incubated at 50°C for 1 week without shaking. The culture was then spread on an agar plate of the same medium

Communicated by K. Horikoshi

T. Fukushima (✉) · T. Mizuki · A. Echigo · A. Inoue · R. Usami
Department of Applied Chemistry, Faculty of Engineering,
Toyo University, 2100 Kujirai, Kawagoe,
Saitama 350-8585, Japan
E-mail: bioeng@mail.cc.eng.toyo.ac.jp
Tel.: +81-492-391390
Fax: +81-492-311031

T. Fukushima · A. Echigo · A. Inoue · R. Usami
Bio-Nano Electronics Research Center, Toyo University,
2100 Kujirai, Kawagoe, Saitama 350-8585, Japan

containing 4.3 M NaCl. Thirty colonies were picked up at random and were tested for the amylase activity. One isolate that exhibited most high amylase activity was picked up from these isolates.

Optimal growth conditions of strain S-1 were investigated. NaCl and $MgSO_4 \cdot 7H_2O$ concentrations and pH of the medium were changed between 0.8 and 5.1 M, 0.05 and 0.5 M, 5.0 and 10.0, respectively. After shaking vigorously (140 rpm) at 37°C, growth was monitored by measuring absorbance at 660 nm. Temperature was changed between 25 and 60°C. The base of these media was JCM medium 168. 16S rRNA analysis of strain S-1 was performed. The 16S rRNA-encoding genes were amplified by PCR with the following forward and reverse primers: 5′-ATTCCGGTTGATCCTGCCGG (positions 6–25 in *Escherichia coli* numbering) and 5′-AGGAGGTGATCCAGCCGCAG (positions 1540–1521). The amplified 16S rRNAs were cloned into pCR2.1 T-vector (Invitrogen) and sequenced using the Big Dye Sequencing Kit, version 3.1 (Applied Biosystems), by the ABI 310 DNA sequencer (Applied Biosystems). Sequencing primers used were 1: 5′-ATTCCGGTTGATCCTGCCGG (positions 6–25 in *E. coli* numbering); 6: 5′-AGGAGGTGATCCAGCCGCAG (positions 1540–1521); 7: 5′-ATTGGGCCTAAAGCGTCCGTA (positions 563–585); −20: 5′-GGAAACAGCTATGACCATG (vector side's primer); and Rev: 5′-GTAAAACGACGGCCAGT (vector side's primer).

Strain S-1 was inoculated into 2 ml liquid medium (JCM modified medium 168 containing 4.3 M NaCl) and overlaid with 1 ml organic solvents (organic solvents used were *n*-decane, *n*-nonane, hexylether, isooctane, cyclooctane, and cyclohexane). After shaking (140 rpm) at 30°C, growth was estimated by measuring the OD 660 nm of 80 µl of cultures. Tolerance of bacteria, including archaea, to solvent has been estimated by the solvent parameter log P_{ow} (common logarithm of the partition coefficient of a given solvent in a mixture of *n*-octanol and water), which is an index of biological toxicity (Inoue and Horikoshi 1991). Lower values represent higher toxicity.

We isolated strain S-1, which produced organic solvent-tolerant extracellular amylase from a commercially available French solar salt (Sel Marin DE NOIRMOUTIER). Temperature range for growth extended from 30–60°C, with an optimal temperature at 45°C. Growth of strain S-1 occurred within a pH range of 6.0–9.0, at 45°C, with an optimal pH of 7.5. No growth was observed above pH 9.5. Growth was found between 2.5 and 5.1 M NaCl concentration and maximal growth occurred at 3.9 M NaCl. No growth was observed at 1.7 M NaCl. Optimal concentration of $MgSO_4 \cdot 7H_2O$ was 200 mM, and no growth was observed below 30 mM. Sequences of 16S rRNA gene of the strain S-1 belonged to the genus *Haloarcula*, and most closely related to *Har. hispanica* (Juez et al. 1986). Good growth of strain S-1 was observed in the presence of *n*-decane, *n*-nonane, and hexylether. No growth was observed in the presence of organic solvent with log P_{ow} value below 5.0.

Cells were grown in 4.3 M NaCl, at 37°C, pH 7.0. Medium contained 1.0% soluble starch. Cells were harvested by centrifugation at 6,000 *g* for 30 min, and the supernatant was used as crude enzyme preparation. The supernatant from 200 ml culture was loaded into a Millipore Centriprep centrifugal filter unit (molecular weight, 50,000) and 12 times centrifugation was performed at 700 *g* for 1 h. The sample was then applied to a TOYOPEARL Phenyl-650 (Tosoh) column and eluted using a linear gradient from 23.7–5.9% ammonium sulfate containing 4.3 M NaCl. Gel filtration on a Sephadex G-100 column was performed. Protein concentration was determined by the Bradford method (Bradford 1976). The sample was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The enzyme was finally purified to homogeneity by applying it to a Sephadex G-100 gel filtration column (Table 1).

Single and symmetrical peak of protein coincided with the peak of amylase activity. The final preparation had a specific activity of 297 U per mg of protein and gave a single protein band on SDS-PAGE. Molecular mass of the purified amylase was estimated to be about 70 kDa by SDS-PAGE. Molecular mass estimated by the gel-filtration purification step was 68 kDa. These results indicated that amylase had its activity in a monomeric form. The protein maker used was Precision Plus Protein Standards (Bio-Rad).

Amylase activity was routinely assayed by the iodine-binding assay in 50 mM Tris-HCl buffer containing 0.2% starch, pH 7.0, at 37°C, and the reaction was terminated by adding 0.2 N HCl. Color was developed by the addition of iodine solution (0.02% I_2, 0.2% KI solution), and the remaining starch was determined spectrophotometrically at 700 nm. One unit of activity was determined as the amount of protein that hydrolyzed 1 mg of starch in 1 min (Haseltine et al. 1996).

Fifty microliters enzyme solution and 300 µl substrate (containing 0.2% starch and 4.3 M NaCl, pH 7.0) were mixed in a test tube (screw-capped), with or without 100 µl organic solvents (*n*-decane, *n*-nonane,

Table 1 Purification steps of amylase from *Haloarcula* sp. strain S-1

Purification step	Volume (ml)	Activity (U/mg)	Protein conc. (mg/ml)	Yield (%)
Medium supernatant	200	8.7	0.524	100
Centriprep	10	20.7	0.668	63
Phenyl C-650 toyopearl	4	392	0.013	23
Sephadex G-100	4	297	0.007	17

n-octane, cyclohexane, xylene, styrene, toluene, benzene, or chloroform). It was then incubated with shaking at 37°C for 2 h after mixing by Vortex Mixer, and activity was estimated by measuring OD 700 nm. Inhibitory effects of each organic solvent were less than 20%. Activity of 100% remained in the absence of organic solvents. This amylase was stable and active in the presence of various organic solvents (*n*-decane, *n*-nonane, *n*-octane, xylene, styrene, toluene, benzene, and chloroform). *Haloarcula* sp. S-1 was tolerant to hexylether (log $P_{ow} = 5.1$). However, amylase purified from this strain was tolerant to chloroform (log $P_{ow} = 1.9$). These facts indicate that organic solvent tolerance of the purified enzyme is higher than that of the halophilic archaea (Usami et al. 2003).

To examine stability of the amylase to organic solvents, 50 µl enzyme solution containing 4.3 M NaCl and 100 µl organic solvent were mixed into a 2-ml microcentrifuge tube with screw cap and mixed with the Vortex Mixer. It was then incubated at 25°C for 1 h with shaking and remaining activity was measured. More than 70% activity was observed in the presence of *n*-decane, *n*-nonane, *n*-octane, xylene, styrene, toluene, benzene, and chloroform. Activity of 45% was observed in the presence of cyclohexane. Cyclohexane is one of the cycloalkanes. Therefore, stability and activity of amylase were tested in the presence of cyclooctane (log $P_{ow} = 4.5$), which is another cycloalkane. Remaining activity was not detected in the presence of cyclooctane. Inhibitory effect of cyclooctane on amylase stability was stronger than that of cyclohexane. Cycloalkane (cyclohexane and cyclooctane) has both equatorial hydrogen and axial hydrogen, and aromatic hydrocarbon has only equatorial hydrogen. Since the enzyme activity was not inhibited by aromatic hydrocarbon (benzene, etc.), equatorial hydrogen may not inhibit amylase activity. On the other hand, axial hydrogen may have inhibitory effect on enzyme activity. Moreover, more than 80% activity and stability were observed in the presence of normal-hexane and normal-octane. As described above, cycloalkane influenced halophilic amylase to a greater degree than the other organic solvents.

Protease of *P. aeruginosa* PST-01 (Ogino et al. 1999b) is a hydrophilic organic solvent-tolerant enzyme. Therefore, we investigated hydrophilic organic solvent tolerance of halophilic amylase from *Haloarcula* sp. S-1. Hydrophilic organic solvents tested were 1-butyl alcohol, ethyl alcohol, 2-propyl alcohol, DMSO, methyl alcohol, and acetone. Amylase activity was inhibited by hydrophilic organic solvents. Among them, no activity was detected in the presence of ethyl alcohol and acetone.

Activities of organic solvent-tolerant lipase and cyclodextrin glucanotransferase from *P. aeruginosa* LST-03 (Ogino et al. 1999a) and *P. illinoisensis* (Doukyu et al. 2003), respectively, were slightly inhibited by cyclohexane. On the other hand, activity of cyclodextrin glucanotransferase from *P. illinoisensis* decreased, after 12 h of incubation with chloroform, to about 50% of initial level. More than 80% of the activity was observed when halophilic α-amylase was exposed to chloroform for 24 h at 25°C. Cyclodextrin glucanotransferase was slightly inhibited in the presence of hydrophilic organic solvents (ethyl alcohol, 2-propyl alcohol, and methyl alcohol), while activity of halophilic amylase was inhibited by hydrophilic organic solvents. As described above, there may be differences in structure between halophilic enzyme and organic solvent-tolerant enzymes from bacteria.

The major reaction products of this amylase were maltose and maltotriose, and the minor product was glucose in the absence or presence of chloroform. Reaction products did not change in the presence or absence of chloroform. Binding domain(s) and active site(s) of the enzyme may be stabilized in the presence of chloroform.

For pH studies in the absence or presence of chloroform, different buffers were used: 0.2 M citric acid/phosphate for pHs from 4–6, 0.2 M Tris-HCl buffers for pHs from 6–8, and 0.2 M glycine buffers for pHs from 8–10. All of them contained 4.3 M NaCl. The pH was checked after each reaction and showed no changes with respect to the initial values. For each pH, starch concentration was 0.2%. Results are shown in Fig. 1. In the absence of chloroform, amylase activity was highest at pH between 6.0 and 7.0, but in the presence of chloroform, it was highest at pH 6.0. To examine pH stability of amylase, the enzyme was incubated in buffers of various pH values for 30 min at 25°C, and the remaining activity was determined. Amylase was stable in pH range of 6.0–8.0 in the absence or presence of chloroform.

Temperature dependency of amylase was tested in 0.2 M Tris-HCl buffer, pH 7.0, containing 4.3 M NaCl, at various temperatures. For each temperature, starch

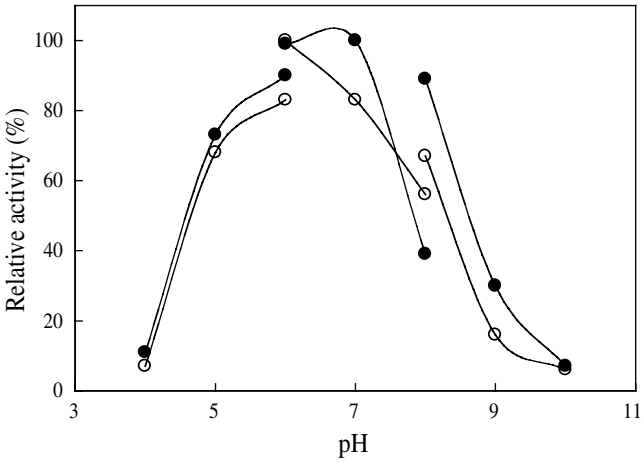

Fig. 1 Effect of pH on amylase activity in the absence (*filled circle*) or presence (*open circle*) of chloroform. Amylase was incubated for 30 min in buffers of various pH values containing 4.3 M NaCl in the absence or presence of chloroform, and activity was measured. Citrate–phosphate buffer (0.1 M) was used for pH values between 4 and 6, Tris-HCl buffer (0.1 M) for pH values between 6 and 8, and glycine–NaOH buffer (0.1 M) for pH values between 8 and 10

concentration was 0.2%. Stability of amylase at different temperatures was determined by incubating an enzyme sample in 50 mM Tris-HCl (pH 7.0) buffer containing 4.3 M NaCl for 30 min at various temperatures, and the remaining activity was measured. Activity of 50% was observed at 70°C. Activity of 100% remained at 37°C in the presence of 4.3 M NaCl and 0.2% starch. This enzyme was comparatively stable at high temperatures.

Effect of NaCl concentration in the absence or presence of chloroform on enzyme activity was tested by measuring the activity at 37°C in 50 mM Tris-HCl, pH 7.0. Various concentrations of NaCl were tested. For each NaCl concentration, starch concentration was 0.2%. To examine the stability of the enzyme to NaCl, amylase was incubated at 25°C and pH 7.0 for 30 min at various NaCl concentrations, and the remaining activity was measured. Optimal NaCl concentration in the absence of chloroform was 4.3 M. No activity was detected below 1.7 M NaCl. In the presence of chloroform, optimal NaCl concentration was 4.3 M, and activity was not detected below 0.9 M. Data are shown in Fig. 2. Enzyme activity was higher at low salt concentrations in the presence of chloroform. Amylase was stable under NaCl concentration between 1.8 and 4.8 M in the absence or presence of chloroform.

The enzyme sample and EDTA solution were added into test tube containing 50 mM Tris-HCl buffer (pH 7.0) and 4.3 M NaCl, with or without chloroform. Final EDTA concentration was changed from 5 to 15 mM. Amylase activity was inhibited in the presence of EDTA (5–15 mM), with or without chloroform. Metal ions tested were Ca^{2+}, Cs^{+}, Rb^{+}, Fe^{2+}, Fe^{3+}, Cu^{2+}, Co^{2+}, Mn^{2+}, Zn^{2+}, Ni^{2+}, Mg^{2+}, and Al^{2+} (each 20 mM). These ions and enzyme sample were mixed in test tubes, and enzyme activity was assayed in the presence or absence of chloroform. Amylase activity was inhibited by most of the metal ions tested except Ca^{2+}, Cs^{+}, Co^{2+}, Mn^{2+}, and Mg^{2+} in the presence or absence of chloroform.

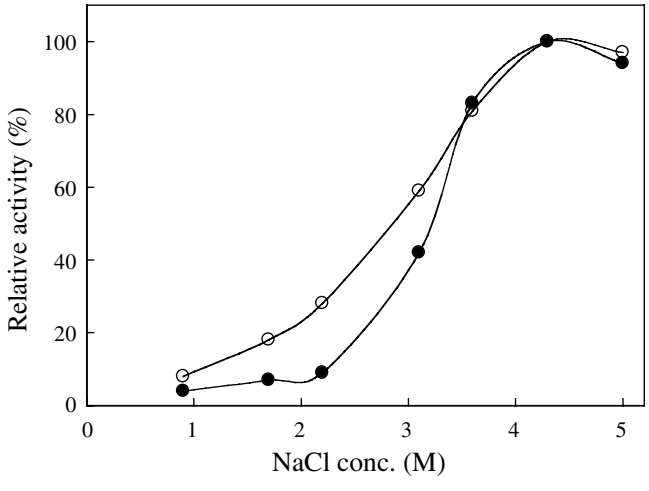

Fig. 2 Effect of NaCl concentration on amylase activity in the absence (*filled circle*) or presence (*open circle*) of chloroform. Amylase activity was measured in various buffers containing 0.9–5.0 M NaCl and 0.2% starch in the absence or presence of chloroform

At low NaCl concentrations, activity in the presence of chloroform was higher than that without chloroform. Normally, structure of halophilic enzymes was not stable in low salt concentrations because of ionic charge (Mardern et al. 2000). Moreover, when EDTA (5–15 mM) was added, the enzyme activity was higher in the presence of chloroform than without chloroform. Thus, chloroform may play a similar role as Na^{+} and metal ions lost by EDTA to stabilize the enzyme.

Organic solvent tolerance of other extracellular halophilic enzymes (α-amylase and serine protease) from the *H. mediterranei* JCM 8866^{T} (Perez-Pomares et al. 2003) and *Natrialba asiatica* 172P1 JCM 9576^{T} (Kamekura and Dyall-Smith 1995; Kamekura and Seno 1990) was tested. *H. mediterranei* was inoculated in 2 ml medium (consisting of 10 g/l soluble starch, 1 g/l sodium glutamate, 3 g/l trisodium citrate, 2 g/l KCl, 20 g/l $MgSO_4{\cdot}7H_2O$ and 200 g/l NaCl). After shaking (140 rpm) at 37°C, supernatant was taken, and amylase activity was tested. Amylase from *H. mediterranei* was incubated in buffer containing 4.3 M NaCl, 0.2% starch, and various organic solvents. This amylase had organic solvent tolerance (up to chloroform) as well as amylase of the strain S-1. *N. asiatica* 172P1 was cultivated in 2 ml of JCM medium 168 (http://www.jcm.riken.go.jp) at 37°C. Supernatant was used for measurement of protease activity. Serine protease from *N. asiatica* 172P1 was incubated in buffers containing 3.4 M NaCl and 0.05% azocasein at 37°C for 1 h in the presence of organic solvents. Activity was detected by measuring loose tylosin (OD 335 nm). This protease also had organic solvent tolerance.

As described above, extracellular halophilic enzymes may have organic solvent tolerance. Consequently, halophilic enzymes could be used in industrial processes containing high salt concentrations and hydrophobic organic solvents.

Acknowledgements Part of this study has been supported by a grant for the 21st Century's Center of Excellence Programs organized by the Ministry of Education, Culture, Sports, Science and Technology, Japan, since 2003.

References

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of proteins utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Danson MJ, Hough DW (1997) The structural basis of halophilicity. Comp Biochem Physiol 3:307–312

Doukyu N, Kuwahara H, Aono R (2003) Isolation of *Paenibacillus illinoisensis* that produces cyclodextrin glucanotransferase resistant to organic solvents. Biosci Biotechnol Biochem 67:334–340

Dym O, Mevarech M, Sussman JL (1995) Structural features that stabilize halophilic malate dehydrogenase from archaebacterium. Science 267:1344–1346

Haseltine C, Rolfsmeier M, Blum P (1996) The glucose effect and regulation of the α-amylase synthesis in the hyperthermophilic archaeon *Sulfolobus solfataricus*. J Bacteriol 4:945–950

Inoue A, Horikoshi K (1991) Estimation of solvent-tolerance of bacteria by solvent parameter log P. J Ferment Bioeng 71:194–196

Janecek S, Leveque E, Belarbi A, Haye B (1999) Close evolutionary relatedness of α-amylase from archaea and plants. J Mol Evol 48:421–426

Juez G, Rodriguez-Valera F, Ventosa A, Kushner DJ (1986) *Haloarcula hispanica* spec. nov. and *Haloferax gibbonsii* spec. nov., two new species of extremely halophilic archaebacteria. Syst Appl Microbiol 8:75–79

Kadziola A, Søgaard M, Svensson B (1998) Molecular structure of a barley α-amylase-inhibitor complex: implications for starch binding and catalysis. J Mol Biol 278:205–217

Kamekura M, Dyall-Smith ML (1995) Taxonomy of the family Halobacteriaceae and the description of two genera *Halorubrobacterium* and *Natrialba*. J Gen Appl Microbiol 41:333–350

Kamekura M, Seno Y (1990) A halophilic extracellular protease from a halophilic archaebacterium strain 172 P1. Biochem Cell Biol 68:352–359

Kanai H, Kobayashi T, Aono R, Kudo T (1995) *Natronococcus amylolyticus* sp. nov., a haloalkaliphilic archaeon. Int J Syst Bacteriol 45:762–766

Kobayashi K, Kanai H, Hayashi T, Akiba T, Akaboshi R, Horikoshi K (1992) Haloalkaliphilic maltotriose-forming α-amylase from the archaebacterium *Natronococcus* sp. strain Ah-36. J Bacteriol 174:3439–3444

Kobayashi K, Kanai H, Aono R, Horikoshi K, Kudo T (1994) Cloning, expression, and nucleotide sequence of the α-amylase gene from the haloalkaliphilic archaeon *Natronococcus* sp. strain Ah-36. J Bacteriol 176:5131–5134

Machius M, Wiegand G, Huber R (1995) Crystal structure of calcium depleted *Bacillus lincheniformis* α-amylase at 2.2 Å resolution. J Mol Biol 246:545–559

Mardern D, Ebel C, Zaccai G (2000) Halophilic adaptation of enzymes. Extremophiles 4:91–98

Marhuenda-Egea FC, Bonete MJ (2002) Extreme halophilic enzymes in organic solvents. Curr Opin Biotechnol 13:385–389

Ogino H, Miyamoto K, Yasuda M, Ishimi K, Ishikawa H (1999a) Growth of organic solvent-tolerant *Pseudomonas aeruginosa* LST-03 in the presence of various organic solvents and production of lipolytic enzyme in the presence of cyclohexane. Biochem Eng J 4:1–6

Ogino H, Yamada M, Watanabe F, Ichinose H, Yasuda M, Ishikawa H (1999b) Peptide synthesis catalyzed by organic solvent-stable protease from *Pseudomonas aeruginosa* PST-01 in monophasic aqueous-organic solvent systems. J Biosci Bioeng 88:513–518

Perez-Pomares F, Bautista V, Ferrer J, Pire C, Marhuenda-Egea FC, Bonete MJ (2003) α-Amylase activity from the halophilic archaeon *Haloferax mediterranei*. Extremophiles 7:299–306

Rodriguez-Valera F, Juez G, Kushner DJ (1983) *Halobacterium mediterranei* spec. nov., a new carbohydrate-utilizing extreme halophile. Syst Appl Microbiol 4:369–387

Usami R, Fukushima T, Mizuki T, Inoue A, Yoshida Y, Horikoshi K (2003) Organic solvent tolerance of halophilic archaea. Biosci Biotechnol Biochem 67:1809–1812

Vihinen M, Mäntsälä P (1989) Microbial amylolytic enzymes. Crit Rev Biochem Mol Biol 24:329–418

Zviagintseva IS, Beliaev SS, Borzenkov IA, Kostrikina NA, Milekhina EI, Ivanov MV (1995) Halophilic archaebacteria from the Kalamkass oilfield. Mikrobiologiia 64:83–87