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Isolation and Characterization of Extracellular Halophilic Ribonuclease from Halotolerant *Pseudomonas* species

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ABSTRACT

A halotolerant bacterium isolated from Thai fish sauce, obtained in Surathani province, was identified as a *Pseudomonas* sp. No. 3241. This strain showed halophilic ribonuclease activity. When casamino acids (CA) and yeast extract (YE) were used as the nitrogen source in a mini jar fermenter, a ratio concentration of CA to YE of 15:20 g/l in Sehgal and Gibbons Complex (SGC) medium, without NaCl, gave maximum growth and ribonuclease activity (18.18 U/ml). The ribonuclease enzyme was optimal at pH 10.0 and at the temperature of 50°C. It had marked halophilic enzyme properties that required an optimal NaCl concentration of 3 M. The ribonuclease was stable between pH 6.0 and 9.0 and at temperatures between 30 and 40°C.

Key words : halophilic, nuclease, ribonuclease, halotolerant, *Pseudomonas* sp.

INTRODUCTION

As halophilic means "salt loving" halophilic enzymes or bacteria by definition require NaCl for activity or growth. In contrast, halotolerant forms do not require NaCl for growth. Nucleases comprise of deoxyribonucleases (DNase) and ribonucleases (RNase). The latter are enzymes that catalyze RNA to 5'-ribonucleotides (5'-GMP, 5'-AMP, 5'-CMP and 5'-UMP) or to 2', 3'-nucleotides. DNase, on the other hand, catalyzes DNA to 5'-deoxyribonucleotides (5'-GMP, 5'-AMP, 5'-CMP and 5'-TMP) or 2', 3'-nucleotides. According to Kuninaka *et al*. (1961), nucleoside-5'-monophosphates, especially 5'-IMP and 5'-GMP, are flavoured, and there is a specific synergistic action as regard taste between monosodium glutamate (MSG) and nucleoside–5'-monophosphate. Kamekura and Onishi (1974) first reported a halophilic nuclease produced by the moderately halophilic bacterium, *Micrococcus varians* var. *halophilus.* This enzyme has marked halophilic properties, requiring an optimal level of 2.9 M NaCl. Furthermore, Onishi *et al*. (1983) reported a halophilic nuclease from a moderately halophilic *Bacillus* sp. N23-2, which was later identified by Ventosa (1991) as *Bacillus halophilus.* Maeda and Taga (1976a, b) also studied an extracellular deoxyribonuclease from *Vibrio* sp. strain No. 2, which was isolated from seawater, but they did not report any halophilic nuclease properties. These halophilic and marine bacteria nucleases showed both ribonuclease and deoxyribonuclease activities in the presence of salt (Kamekura and Onishi, 1974; Onishi *et* al., 1983; Maeda and Tage, 1976b). Since then, no further halophilic ribonucleases from halotolerant microorganisms have been reported. Concerning

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the production of fish source, the application of halophilic ribonuclease may be expected that the synergistic action between 5'-GMP and L-glutamate will give better taste (Kurihara 1987). This paper describes the strain identification, enzyme production and characterization of a crude halophilic ribonuclease from a halotolerant *Pseudomonas* sp. No. 3241.

MATERIALS AND METHODS

Organism preparation

Strain No. 3241, isolated from fermented fish sauce in Surathani province Thailand, was stored at 4°C on an agar slant of Sehgal and Gibbons Complex (SGC) medium (Sehgal and Gibbons, 1960), supplemented with 2.0 M NaCl.

Nuclease activity test on plate agar

Firstly, Bacto DNase activity was tested using deoxyribonucleic acid as a substrate in petri dishes. DNase test medium was supplemented with 1, 2 and 3 M of NaCl. Control cultures lacked NaCl. The strain was spotted onto test agar and then incubated at 37°C for 10 days. A positive reaction for the DNase test was indicated by the disappearance of methyl green around the colony, and the width of this clear zone was considered to be directly related to the amount of extracellular deoxyribonuclease produced. The enzyme production and growth ratio was defined as A/B, where A was the diameter of clear zone (cm), and B the diameter of the colony size (cm).

Ribonuclease production in shaken flasks

An inoculum was prepared by transferring one loop of the strain into SGC seed medium (pH 7.0) with 2.0 M NaCl. This was then agitated on a rotary shaker at 30°C, 250 rpm for 18 h. Later, 5 ml of this seed culture was inoculated into 50 ml SGC production medium, containing various concentrations of NaCl, in 250-ml Erlenmeyer flasks. Cultivation conditions were the same as that for the seed culture.

Ribonuclease production in mini jar fermenter

A volume of 1,350 ml of SGC production medium, supplemented with twice the nitrogen source without NaCl was placed into a 2-L mini jar fermenter (Biostat B, B. Braun Biotech International GmbH, Germany) and autoclaved at 121°C for 30 min. After being cooled, it was inoculated with 150 ml of SGC seed culture containing 2.0 M NaCl. Fermentation conditions were maintained at 30°C, 600 rpm-agitation and air supply at 1vvm. The pH of the broth was not controlled during this process. After cultivation and cooling, the culture broth was centrifuged and the supernatant was subjected to partial purification (see below).

Ribonuclease activity

Halophilic ribonuclease activity was measured by a modificaiton of the method used by Kamekura and Onishi (1974). The assay substrate contained 1 mg/ml ribonucleic acid (from *Torula* yeast), 1.8 mM NaCl, 0.04 mM Tris-HCl buffer (pH 8.0), and 0.01 mM $MgSO_4 .7H_2O$. This was mixed with 1 ml enzyme solution and incubated at 40°C for 2 h. Enzyme activity was terminated with 6 ml 99.9% cold ethanol for 20 min at 0°C. The precipitate was removed by centrifugation at 8,000 × g at 4°C for 20 min. The supernatant (1 ml) was diluted 5 times with distilled water and absorbance was measured at 260 nm. A blank measure was carried out using 1 ml of substrate solution alone, followed by incubation at 40°C for 2 h. This was then supplemented with 1 ml of enzyme solution followed immediately with 6 ml 99.9% cold ethanol. One unit of the ribonuclease activity was defined as the amount of enzyme catalyzing an increase of 1.0 in absorbance at 260 nm under the above conditions. Ribonuclease activity was calculated from the following equation :

Ribonuclease activity (U/ml, where S and B refer to sample and blank, respectively.)

$$= \frac{Abs(S) - Abs(B)}{Abs(B)} \times dilution$$

Identification of bacterial strain

Identification of the strain No. 3241 producing nuclease was based first on Gram staining, followed by physiological properties determined using standard methods and the API system.

Partial purification of crude halophilic ribonuclease

All steps were carried out at 5°C in a refrigerated room. The supernatant from the mini jar fermenter (see above) was mixed slowly with an equal volume of 99.9% cold ethanol at 0°C on ice. After standing overnight, the mixture was centrifuged at 12,000 × g for 20 min. The precipitate was dissolved in a minimum volume of 0.01M Tris-HCl buffer pH 8.0, then 2.0 M NaCl was added, followed by dialysis against the previously mentioned Tris-Hce buffer for 24 h and subsequent lyophilization.

Protein determination

Protein concentration was determined by the Folin-Phenol method (Lowry *et al*., 1951) with bovine serum albumin as a standard.

Nitrogen determination

The defermination of nitrogen concentration was carried out by the ninhydrin method (Moore and Stein, 1954) with a mixture of casamino acids and yeast extract as a standard.

Carbohydrate determination

Total carbohydrate was determined by the phenol-sulphuric acid method (Dubois *et al*., 1956) with glucose as a standard.

Determination of dry cell weight

Three ml of the sample was filtered with using 0.2-μm filter membrane under vacuum. After drying at 105°C for 24 h, the dry cell weight was calculated as follows:

$$\text{Dry cell weight} = \frac{(W_1 - W_0) \times 1,000}{3}$$

where, W_o = weight (g) of membrane before filtration ; W_1 = weight (g) of membrane after filtration and drying.

Chemicals

Bacto DNase test agar, with methyl green and casamino acids, was purchased from Difco Laboratories, USA. Yeast extract was purchased from E. Merck, Germany. *Torula* yeast ribonucleic acid, dialysis tubing, bovine serum albumin and Tris (hydroxymethyl) aminomethane wree obtained from Sigma Chemical Co., Ltd. USA. The API system was purchased from bioMerieux, France. Filtration membranes (pore size 0.2 μm) were purchased from Sartorius Co., Ltd. Germany.

RESULTS

Effect of NaCl concentrations on DNase test plates

The first screening of nuclease was carried out using DNase test plates, with deoxyribonucleic acid as a substrate with various concentrations of NaCl. Figure 1 shows a strongly positive ratio of deoxyribonuclease activity at about 6.7 at 0 M NaCl for 4 days. However, at 1 and 2 M, NaCl had little effect, while 3 M NaCl gave a negative ratio. Strain No. 3241 could produce deoxyribonuclease over a range of 0 to 2 M of NaCl. This shows that strain No. 3241 has halotolerant DNase activity, as the activity at 0 M NaCl is higher than those supplemented with 1, 2 and 3 M.

Strain identification

Table 1 shows the physiological properties of the strain, determined by standard methods, in the absence of NaCl. At the first stage of identification the organism was characterized as Gram-negative rods, motile, aerobic (facultative),

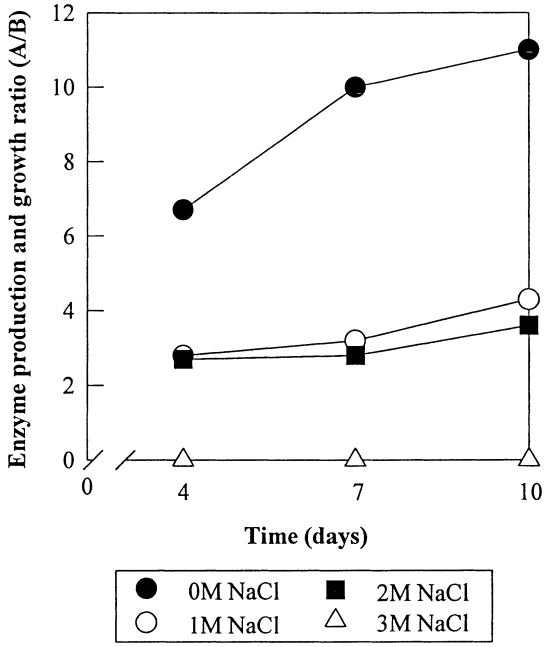

Figure 1 Effect of NaCl concentrations on DNase plate test of strain No. 3241. A : clear zone diameter (cm); B: colony size diameter (cm).

catalase positive and oxidase positive. From these above characteristics, strain No. 3241 was placed into the genus *Pseudomonas*. The second stage of identification using the API system characterized it as the genus *Pseudomonas* with a reliability of 79.5%. In this case, species identification was insufficient, as the API system does not identify all kinds of *Pseudomonas* species. The growth range in 3.0 M NaCl was less than that of at 0 M, so strain No. 3241 was classified as a halotolerant *Pseudomonas* sp. No. 3241. This identification, however, is still preliminary.

Effects of temperature and NaCl concentration on specific growth rate and enzyme productivity

Figure 2A demonstrates that there was no difference in specific growth rates at various NaCl concentrations at 30°C and 37°C. At higher temperature (45°C) without NaCl, *Pseudomonas* sp. No. 3241 could not grow. Generally, only

Table 1 Physiological characteristics of strain No. 3241.

Determination	Reaction
Gram stain	–
Morphology	Rod
Pigment	CR
Catalase	+
Cytochrome oxidase	+
Growth on MacConkey agar	+
Motility	+
Spore stain	–
Starch hydrolysis	–
Casein digestion	+
Oxidative-Fermentative (OF) medium	N
Anaerobic growth	+
Growth in 0 M NaCl	+
1 M NaCl	+
2 M NaCl	+
3 M NaCl	+
4 M NaCl	–
Growth at 42°C	–
API test	
ONPG (β-galactosidase)	WK
ADH (Arginine dihydrolase)	–
LDC (Lysine decarboxylase)	–
ODC (Ornithine decarboxylase)	–
CIT (Citrate utilization)	–
H_2S (H_2S production)	–
URE (Urease)	–
TDA (Tryptophan deaminase)	–
IND (Indole production)	–
VP (Acetoin production)	+
GEL (Gelatinase)	–
NO_2 production	
Reduction to N_2 gas	–
Sugar fermentation	
GLU (Glucose)	–
MAN (Mannitol)	–
INO (Inositol)	–
SOR (Sorbitol)	–
RHA (Rhamnose)	–
SAC (Sucrose)	–
MEL (Melibiose)	–
AMY (Amygdalin)	–
ARA (Arabinose)	–

Key : rod = rod shape, CR = cream, N = no acid production WK = weak positive.

thermophilic bacteria can grow in such high temperature. However, in this case NaCl enhanced the growth at 45°C. We hypothesize that the sodium cation may stabilize and protect the protein from denaturation in the cell membrane even at such high temperature. Figure 2B demonstrates that increasing of salt concentration and temperature caused a decrease in enzyme productivity. The maximum value was observed using 0 M NaCl at 30°C (0.16 U/ml/h). Ribonuclease from *Pseudomonas* sp. No. 3241 was denatured at 45°C. One conclusion from these results is that *Pseudomonas* sp. No. 3241 can produce a ribonuclease enzyme both in the presence or absence of salt. The best production, however, was observed in SGC medium without NaCl.

Effect of nitrogen sources on specific growth rate and enzyme productivity during shaking

Specific growth rates and enzyme productivity did not increase with increasing nitrogen source (Figure 3), and optimal conditions were observed at a CA:YE ratio of 15:20 g/l in SGC medium without NaCl.

Effect of glucose on specific growth rate and enzyme productivity

Figure 4 shows the effect of glucose as carbon source on the specific growth rate and enzyme productivity in SGC medium containing a ratio concentration of CA:YE at 15:20 g/l. Increasing the glucose concentration resulted in the reduction of specific growth rate and enzyme productivity. These results might stem from the repression of respiratory enzymes by glucose acting as a catabolite repersor (the "Crabtree" effect). (Blanch and Clark, 1997).

Growth and enzyme production in mini jar fermenter

Using nitrogen sources of CA:YE at 15:20 g/l in SGC medium without NaCl, the fermentation process was classified as follows: phase 1 (0 ~ 22 h) ; phase 2 (22 ~ 32 h) ; phase 3 (32 ~ 48 h) (Figure 5). In phase 1, both carbon and nitrogen sources were slightly utilized and decreased in amount ; maximum specific growth rate (μ_{max}) and growth-associated ribonuclease production were observed in this phase (logarithmic growth phase). In the

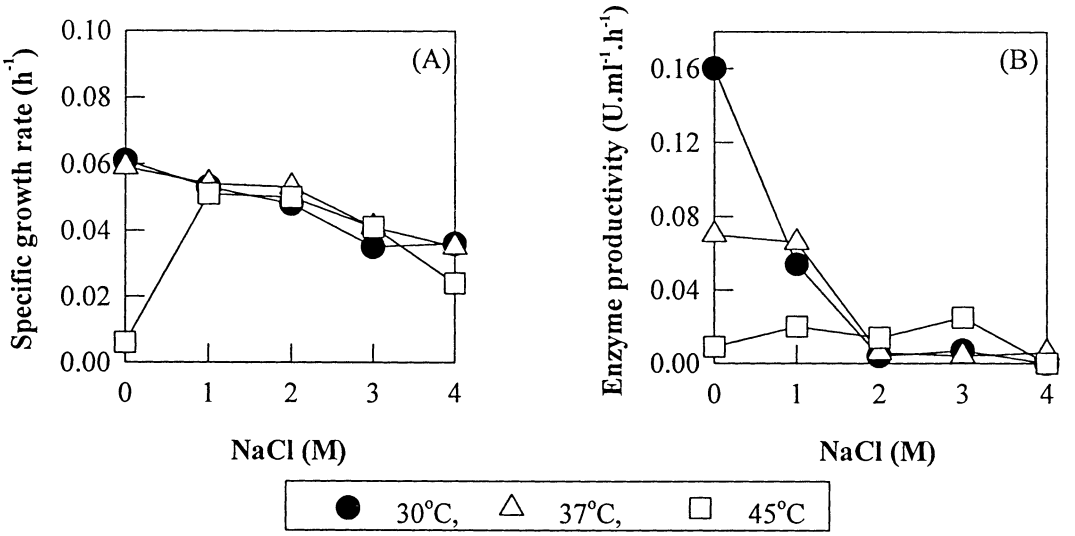

Figure 2 Effects of temperature and NaCl concentration on specific growth rate and enzyme productivity of *Pseudomonas* sp. No. 3241. Key (A) : Specific growth rate; (B): Enzyme productivity.

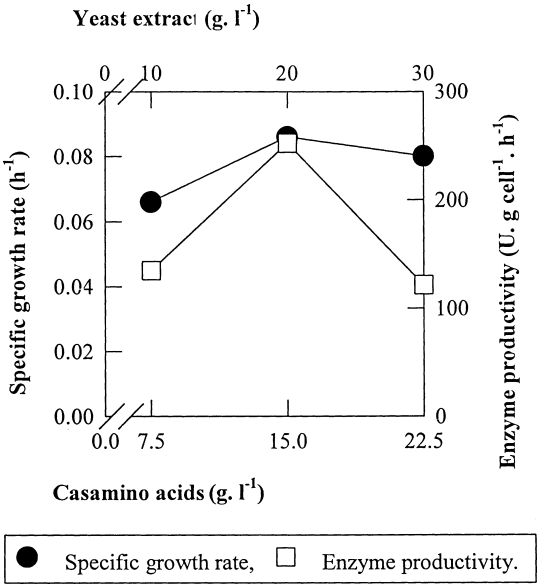

Figure 3 Effect of nitrogen sources on specific growth rate and enzyme productivity of *Pseudomonas* sp. No. 3241.

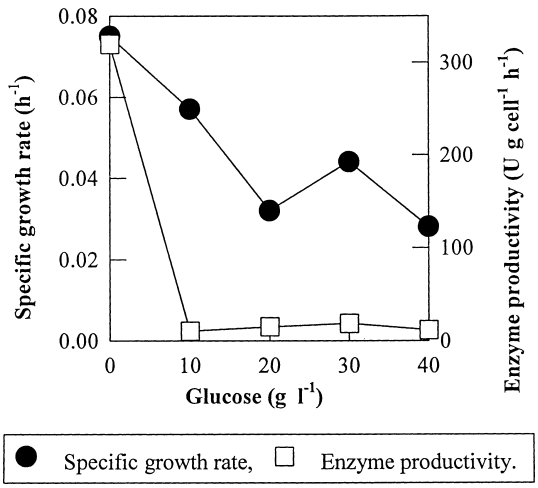

Figure 4 Effect of glucose on specific growth rate and enzyme productivity of *Pseudomonas* sp. No. 3241.

second (stationary) phase, the carbon source was not utilized completely, but on the other hand nitrogen sources were still consumed. The maximum enzyme production (18.18 U/ml) of halophilic ribonuclease was observed at 32 h. In the last phase, growth was still stationary, but enzyme activity decreased. It is possible that cell lysis occurred, or that lost of activity due to a high concentration of ribonuclease (product inhibition).

Partial purification

A summary of the partial purification of the halophilic ribonuclease enzyme from *Pseudomonas* sp. No. 3241 is shown in Table 2. The best fractionation of the enzyme was obtained at a final concentration of 50% of ethanol. The final specific activity of ribonuclease was 77.5 U/mg protein with 95% total yield.

Crude enzyme characteristics

A quantity of 270 ml of crude ribonuclease enzyme was obtained through batch fermentation and stored at –20°C. The crude enzyme activity had an optimal pH and temperature of 10.0 and 50°C, respectively (Figure 6A and B). The enzyme was stable between pH 6.0 and 9.0 at 40°C for 24 h and was stable for 30 min between 30 and 40°C at pH 8.0 (Fig. 7 A and B). The effect of NaCl on the activity of crude ribonuclease enzyme is presented in Figure 8 This enzyme had marked halophilic properties, which required an optimal NaCl level of 3.0 M. In addition, activity at 3.5 M NaCl was higher than that at 2.5 M. From these properties, it is concluded that ribonuclease from halotolerant *Pseudomonas* sp. No. 3241 enzyme is a form of halophilic ribonuclease.

DISCUSSION

There are only a few reports of halophilic nuclease production by halophiles, including *Micrococcus varians* var. *halophilus* (Kamekura and Onishi, 1974) and *Bacillus halophilus* (Onishi *et al*., 1983, Ventosa, 1991). In these experiments, halophilic nuclease from slightly or moderately

Table 2 Partial purification of ribonuclease from halotolerant *Pseudomonas* sp. No. 3241.

Purification steps	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Recovery (%)
Culture filtrate	1,320	4,224	10,982.4	2.6	100
50% ethanol precipitation	220	176	10,467.6	59.48	95.31
Dialysis with 0.01 M Tris-HCl buffer+2M NaCl	270	135	10,459.8	77.48	95.24

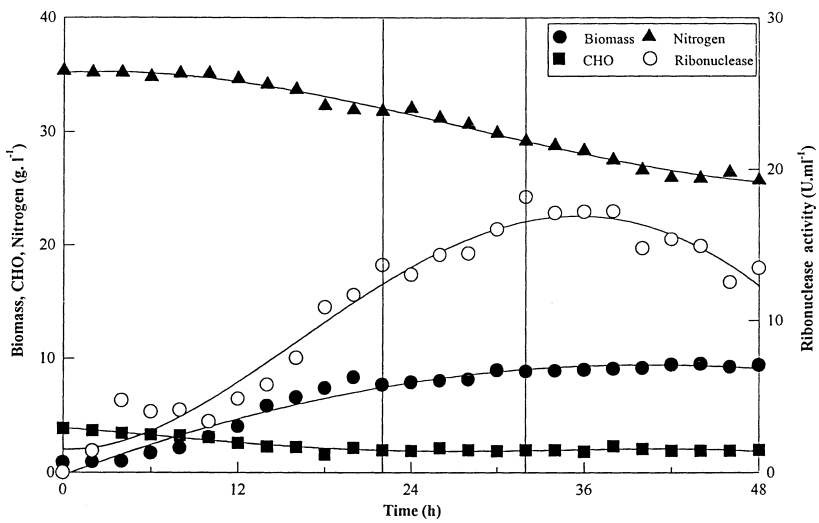

Figure 5 Batch cultivation of halotolerant *Pseudomonas* sp. No. 3241 in 2-L mini jar fermenter.

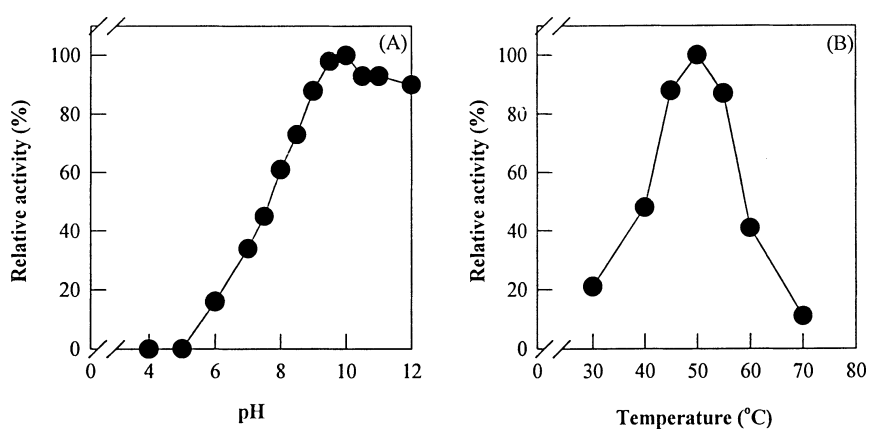

Figure 6 Effects of pH and temperature on activity of the crude ribonuclease from halotolerant *Pseudomonas* sp. No. 3241. Key (A) : Optimal pH; (B) : Optimal temperature.

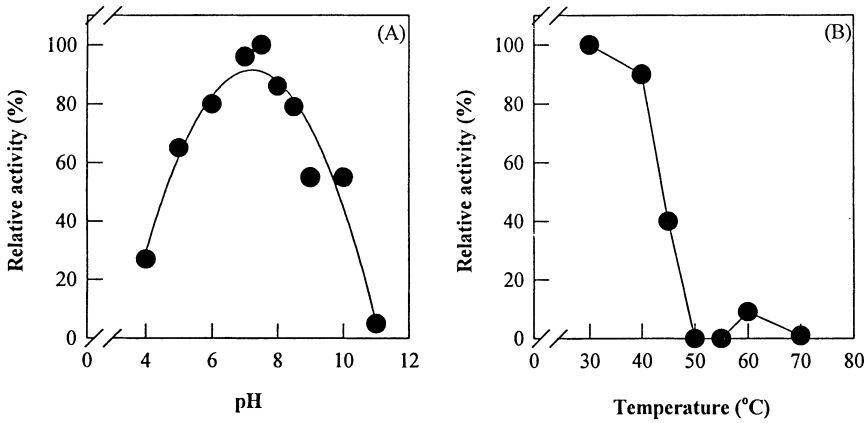

Figure 7 pH and temperature stability of crude ribonuclease from halotolerant *Pseudomonas* sp. No. 3241. Key (A) : pH stability; (B) : Temperature stability.

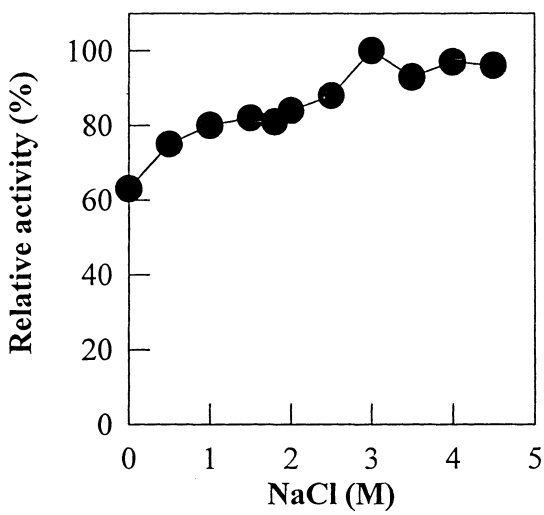

Figure 8 Effect of NaCl on activity of crude ribonuclease from halotolerant *Pseudomonas* sp. No. 3241.

halophilic bacteria, but not from halotolerant bacteria, were used. In the present study a halophilic ribonuclease from a halotolerant bacterium was isolated and investigated for the first time. Halotolerant DNase from halotolernat *Pseudomonas* No. 3241 was first found by the plate test, then halophilic RNase from halotolerant *Pseudomonas* No. 3241 was investigated following partial purification of enzyme.

One unusual outcome from these experimental results is that growth medium containing a high concentration of NaCl enabled the bacterium to grow even at high temperature (Figure 2A). Interactions between the effects of temperature and NaCl concentration on growth of halophilic bacterium have been reported by Ohno *et al.* (1979). In the case of moderately halophilic species, *Pseudomonas halosaccharolytica*, one possible explanation of the regulatory mechanism of membrane structure and function in high NaCl concentration is as follows. The total amount of negatively charged phospholipids in cells increases with higher concentrations of NaCl in the medium. High content of negatively charged phospholipids may contribute to the regulation of cation permeability in a salty environment. Negatively charged phospholipids may be increased selectively in the permeability of cations in a cell. One reason for this is the presence of a regulatory mechanism to maintain suitable proportions of individual phospholipids for cation shielding, and to adjust adequately the internal concentration of cations in the presence of NaCl at high temperature. It was observed that, while the growth of *Pseudomonas* No. 3241 was rescued or stabilized in the presence of NaCl at high temperature, enzyme production

was inhibited. Formerly, there were no other reports of halophilic nucleases from halotolerant *Pseudomonas* species, and this is the first research reported on a halophilic ribonuclease from halotolerant *Pseudomonas* sp. isolated from fish sauce. In future research it is proposed to purify this enzyme and apply the crude enzyme to Thai fish sauce production for taste improvement.

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