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Production and Characterization of Protease from an Extremely Halophilic *Halobacterium sp.* PB407

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ABSTRACT

In order to accelerate the fish sauce fermentation by using enzyme, the production of protease from *Halobacterium sp.* PB407 isolated from Thai fish sauce was investigated. This strain could produce high protease inM73 medium containing 4 M (24%, w/v) NaCl, pH 7.0 at 37°C in shaking condition for 5days. Its production of protease in 2.5 L mini jar fermentor was investigated at 37°C, 50 rpm agitation and aeration at 0.5 vvm. The protease production time was shorten to only 96 hrs. The optimum pH and temperature for maximal protease activity was 8.0 and 40°C, respectively. This protease had marked halophilic properties, showing maximum activities in the presence of 2.13 M NaCl. In addition, this *Halobacterium sp.* PB407 was further identified. Based on 16S rRNA sequence data, the bacteria was concluded as a *Halobacterium salinarum.*

Key words: protease, production, extremely halophilic bacteria, extremophiles, *Halobacterium salinarum*

INTRODUCTION

Proteolytic enzymes are produced by many varieties of microorganisms. To obtain valuable novel protease, new strains have been screened from nature. So far several well-known proteases such as bromelain, papain, and pepsin have been used as biocatalyst of protein hydrolysis in fish sauce fermentation (Beddow and Ardeshir, 1979; Gildberg, 1989). However, most of these proteases were not sufficiently stable in the presence of high salt concentration. Extremely halophilic bacteria may be considered as models for biological salt tolerance. These organisms have evolved in saline environments and are able to overcome the deterious effects of salt, up to saturating concentration. Therefore, we isolated and screened for extremely halophilic bacteria that can produce high protease activity at high salt concentration both on solid medium and in liquid medium. The *Halobacterium sp.* strain PB407 showed the ability to grow at 3 – 5 M NaCl and produced a high protease activity at 4-5 M NaCl (Kanlayakrit and Bovornreungroj, 2002; 2003).

It is expected that the halophilic protease produced by this strain could catalyse reactions with the presence of high salt concentration in fish sauce production. Therefore, this study focused on the production and characterization of the halophilic protease produced by an extremely halophilic bacterium isolated from Thai fish sauce. In addition, the bacterium was further identified with 16S rRNA sequencing method.

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MATERIALS AND METHODS

Bacterial strain and culture condition

The bacteria used in this study was isolated from fermented fish sauce in Thailand by aerobic enrichment culture technique in SGC medium containing 4 M NaCl, as described in our previously report (Kanlayakrit and Bovornreungroj, 2002). This strain could produce protease in M73 liquid medium (Norberg and Hofsten, 1969) containing 4 M NaCl, pH 7.0, in shaking condition at 200 rpm 37°C for 7 days. The strain was preserved on agar slant of SGC medium supplemented with 4 M NaCl at 4°C. Growth and protease production was carried out by inoculating the bacterial strain in 320 ml M73 broth (Norberg and Hofsten, 1969) in a one liter Erlenmeyer flask and incubating on a rotary shaker at 200 rpm at 37°C for 7-10 days. Growth and halophilic protease were determined as described previously (Kanlayakrit and Bovornreungroj, 2003). The study parameters were NaCl concentration of 3- 5 M in 0.5 M increments; incubation time of 1-7 days; temperature of 30-45°C in 5°C increments and different level of pH 5–8. The optimum parameter from each experiment was utilized in the following experiment until all parameters were optimized.

Production of protease in mini jar fermentor

The bacterium strain PB407 was cultured in 2.5 L mini jar fermentor (New Brunswick Scienctific, USA) containing 2.0 L of M73 broth medium with 4 M NaCl pH, 7.0. Inoculation was performed with 10% (v/v) seed culture of 3 days grown in SGC broth medium. The fermentation conditions were maintained at 37°C, 50 rpm agitation and air supply of 0.5 vvm for 3-5 days. The culture broth was harvested and centrifuged at 8,000 rpm for 20 min at room temperature. Cell free supernatants were used for measuring protease activity. The harvested cells were centrifuged by washing two times with 4 M NaCl for measuring growth. Growth were measured turbidically at 600 nm using a spectrophotometer (UV-1201 Shimadzu, Japan).

Determination of protease activity

Protease activities were routinely determined by measuring the release of TCA-soluble material from azocasein (Sigma chemical Co.) as substrate according to a method of Sarath *et al*. (1990) modified by Kanlayakrit and Bovornreungroj (2003). One arbitrary unit (AU) of protease activity was defined as the amount of enzyme required to produce an increase in absorbance at 440 nm of 0.1 in a 1 cm cuvette, under the assay conditions.

Effect of temperature, pH and salt concentration on halophilic protease activity

The optimal pH for halophilic protease activity was carried out by using various buffers (0.05 M acetate buffer, pH 4.0, 5.0; 0.05 M Tris-acetate buffer, pH 6.0, 7.0; 0.05 M Tris-HCl buffer, pH 8.0, 9.0; 0.05 M Glycine-NaOH buffer, pH 10.0, 11.0, 12.0). The optimal temperature during 30-45°C was investigated whereas salt concentrations of NaCl were varied from 0- 4 M.

Strain identification by using 16S rRNA sequence analysis

Determination of 16S rRNA sequence was performed by direct sequencing of enzymatically amplified DNA with specific oligonucleotide primers (Pfeifer and Betlach, 1985). The archaebacteria primer for PCR was constructed using DNA synthesizer achieved by cloning PCR product of 16S r DNA into *E. coli* (Ochsenreiter, *et al*., 2002). Sequence analysis was performed using an automatic DNA sequence. In order to construct the phylogenetic tree, complete 16S rRNA sequences were determined and compared to 16S rRNA sequences with other archaebacteria. Analyses were performed using BLAST2 search.

RESULTS AND DISCUSSION

Growth and protease production by *Halobacterium sp.* PB 407

The study of optimal conditions for growth and protease production in shaking flask was carried out at the fifth days of incubation. As described in previous report, growth and protease production of the strain PB407 did not occur at less than 3 M NaCl (Kanlayakrit and Bovornreungroj, 2003). Therefore, effect of salt concentration was studied from 3 to 5 M NaCl. In Figure 1, it was observed that the strain PB407 was capable growth in medium with 4 M (24 % w/v) NaCl although the growth was not so high. On the other hand, protease production was highest in medium with 4 M (24 % w/v) NaCl. In these respects, the strain PB407 resembled *Halobacterium halobium* ATCC 43214 (Kim and Dordick,1997), but the former might be regarded as having less resistance to NaCl. *Halobacterium sp.* PB407 produced protease even in the presence of 5 M (30 % w/v) NaCl. The optimum temperature and pH for both growth and protease production were 37°C and pH 7.0, respectively. These optimum conditions were similar to that of the *H. halobium* ATCC 43214 (Ryu, *et al.*, 1994).

Production of protease in fermentor

The protease production of the *Halobacterium sp.* PB407 carried out in mini jar fermentor was shown in Figure 2. Protease activity was observed after 30 hrs incubation whereas pH was slightly increased from 7.0- 8.2 as the culture became slightly orange in color. Growth and protease production increased and maximized similarly after 96 hrs incubation, followed by a slightly decrease in both growth and enzyme production as same as the proteolytic enzyme from *H. salinarium* (Norberg and Hofsten,1969) and *H. halobium* ATCC 43214 (Kim and Dordick,1997 and Ryu *et al.*, 1994). The protease

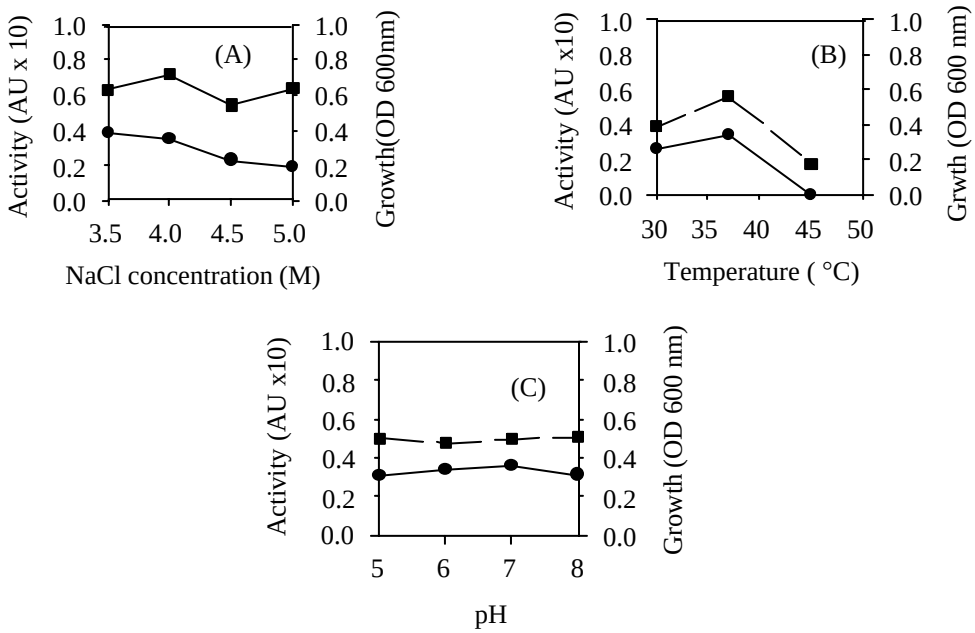

Figure 1 Effect of NaCl (A), temperature (B) and pH (C) on growth and protease production of *Halobacterium sp.* PB407 in M73 liquid medium, pH 7.0 in shaking flask at 200 rpm and 37°C for 5 days. Symbols: growth (●) and protease production (■).

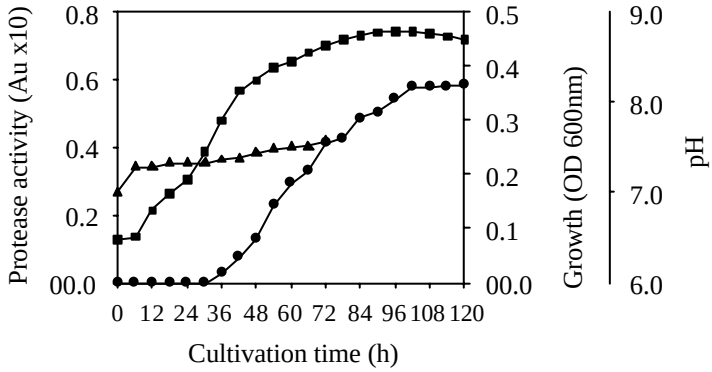

Figure 2 Time course of growth (■), protease production (●) and pH (▲) of the *Halobacterium sp*. PB407 in mini jar fermentor.

production in shaked flask was similar to the production of protease in fermentor, but the cultivation time in fermentor was shorten (Kanlayakrit and Bovornreungroj, 2003). As a result, the maximum protease activity was obtained at 96 hrs.

Characteristics of *Halobacterium sp.* PB407 protease

Effects of NaCl, temperature and pH on protease activity were shown in Figure 3. As previous report, the protease was the salt-dependent (Kanlayakrit and Bovornreungroj, 2003). It showed a maximum protease activity at 2.13 M NaCl, and remained its activity more than 60 % at 4 M NaCl (Figure 3A). For optimum temperature, the activity increased with an increase of temperature up to 40°C. The activity of protease decreased slightly from 40°C to 50°C and dropped rapidly after 50°C (Figure 3B). This bacterial protease showed a high activity in pH range of 7-8, shown in Figure 3C.

From these characteristics, it is interesting that these optimal values are roughly the same as the actual fermentation conditions of fish sauce production in Thailand except pH value. This was higher than those of fish sauces where they regarded from 5-6 (Saisithi, 1987). However, this protease could have activity more than 90% at pH 6.

There was a report which classified a pattern behavior of enzyme from extreme halophiles toward salts into three types: (i) The halophilic enzymes that need at least 1 M monovalent salts for activity and show maximum activity in 2- 4 M salt concentration, (ii) the enzymes shown maximum activity at 0.5-1.5 M are inhibited at higher salt concentration, (iii) the enzymes shown the highest activity in the absence of salt are strongly inhibited by high salt concentration. (Kushner, 1978). From these characteristics, the halophilic protease from *Halobacterium sp.* PB407 was similar to the type (i).

Strain identification using 16 S rRNA nucleotide sequence data

The whole sequence of 16S rRNA of *Halobacterium sp.* PB407 was aligned with the sequence of other *Halobacterium sp*. and some other archaebacteria using BLAST2 search (data not shown). The complete 16S rDNA sequence of *Halobacterium sp.* PB407 was compared with type strain of *Halobacterium* and other archaebacteria from the similarity matrix, calculated by number of base differences, the highest level of similarity observed was *Halobacterium salinarum.* Distance matrix trees were reconstructed as shown in Figure 4.

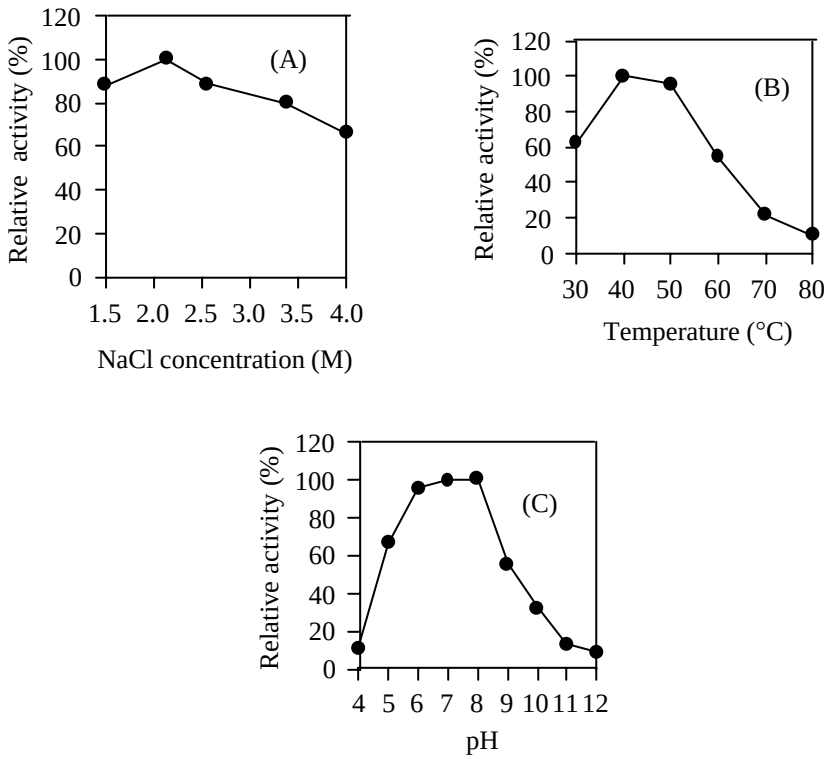

Figure 3 Effects of NaCl concentration (A), temperature (B) and pH (C) on protease activity from *Halobacterium sp.* PB407.

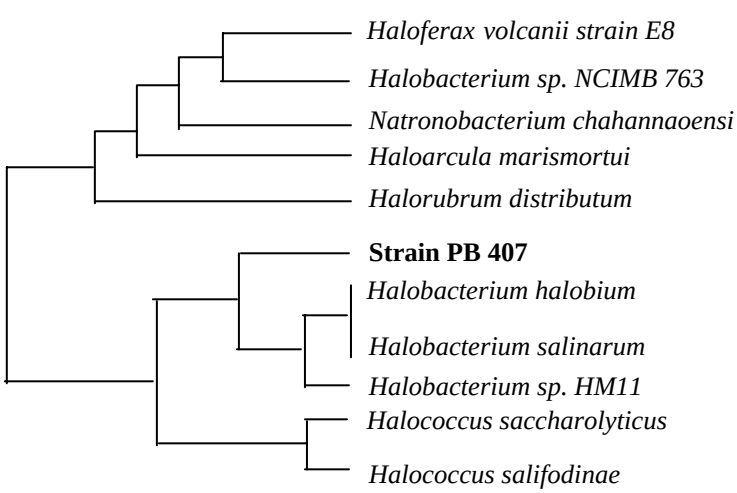

Figure 4 Phylogenetic tree showing the relationship of *Halobacterium sp.* PB407 with 16S rRNA of other archaebacteria.

CONCLUSION

Based on 16S rRNA sequence data, the *Halobacterium* sp.strain PB407, isolated from Thai fish sauce was identified as *Halobacterium salinarum*. This strain had ability to produce halophilic protease in M73 medium with 4 M NaCl, pH 7.0 at 37°C under shaking condition for 5 days. In addition, the protease production by this strain in 2.5 L mini jar fermentor was the same as in shaking flask, but the production time was shorten. The protease of the strain also showed high activity at high salt concentration of 1 to 4 M NaCl, pH 8.0 at 40°C. These results lead to the consideration for utilization of this bacterial enzyme in fish sauce production.

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