Chinese Journal of Polar Science, Vol. 12, No. 1, 69 - 74, June 2001

Isolation and characterization of a marine bacterium producing protease from Chukchi Sea, Arctic

Zeng Yinxin (曾胤新), Cai Minghong (蔡明红), Chen Bo (陈 波) and He Jianfeng (何剑锋)

Polar Research Institute of China, Shanghai 200129, China

Received March 1, 2001

Abstract A Gram negative bacterium Ar/W/b/75°25′N/1 producing extracellular alkaline protease was isolated from surface water of latitude 75°25′N, and longitude 162°25′W in Chukchi sea, Arctic. The strain can grow at the temperature range from 7 ℃ to 30 ℃, and grow better at 30 ℃. It can not grow at 40 ℃. Keeping certain salinity concentration in medium is necessary for cell growth. It grows well in medium containing salinity concentration from 0.5% to 10% sodium chloride. Glucose, sucrose and soluble starch can be utilized by the strain, among which glucose is the optimal carbon source. Peptone is the optimal organic nitrogen source for cell growth and protease producing, and ammonium nitrate is the optimal inorganic nitrogen source. About 75.7% of total protease of the strain are extracellular enzyme. Optimal temperature for proteolytic activity is at 40 ℃. Protease of the strain keeps stable below 40 ℃, and shows high proteolytic activity within the pH range from 7 to 11.

Key words protease, marine bacterium, Arctic.

1 Introduction

Microorganisms have been discovered that live in constantly low temperature environments, such as Antarctic and Arctic. In polar region there exist abundant microorganisms adapted to growth in cold environments, including psychrophiles and psychrotrophes. These microorganisms are possible sources of enzymes active at lower temperatures. Along with the producing microorganisms, these enzymes may offer a great potential in biotechnology (Gounot 1991; Feller *et al*. 1996; Master and Mohn 1998).

Proteases are one of the important industrial enzymes. Low temperature active enzymes have some advantages for food, detergent, environment protection and other industries. Studies on proteases active at low temperature from yeast (Ray *et al*. 1992) and bacteria (Qiu *et al*. 1991; Vazquez *et al*. 1995; Hoshino *et al*. 1997) have been reported.

In China, the scientific research on microorganisms in Antarctic began in the early 1980's, and much attention was paid to the respects of microbial ecology and taxonomy. Seldom reports about the characteristics of physiology and biochemistry of microorganisms from Antarctic and the exploitation of their metabolites have been published. As to microorganisms in Arctic, less researching work has been carried out because of the limita-

tion of large scale of explorations.

On the basis of the first Chinese Arctic Expedition from July 1 to September 9, 1999, a large number of Arctic marine microorganisms have been obtained. We have isolated some marine bacteria with protease activity. And some bacteria grow on the plates with distinct colors such as yellow, red or orange, which may possess potential in pigment production or other applications through further research.

In this paper we report the isolation and some characteristics of a psychrotolerant bacterium capable of producing alkaline protease with optimum temperature of 40 ℃.

2 Materials and methods

2.1 Medium and culture condition

Samples were obtained from the surface water of latitude 75°25′ N, and longitude 162°25′ W in Chukchi Sea, Arctic on Aug. 25, 1999. In the sampling site, temperature was − 1.1 ℃, and salinity of the water was 28.1 ‰.

A screening medium containing 1.0% casein and 2.0% agar was used to isolate protease-producing bacteria by incubating samples at 15 ℃ for 48 h. Isolates showed a zone of hydrolysis were selected for further examination. The selected isolates were grown in differently responsible media at 15 ℃ with vigorous shaking. After 48 h, the cultured broth was directly used for total enzyme activity assaying, and the supernatant fluid without cells after centrifugation was used as a crude enzyme preparation for extracellular enzyme activity.

Zobell 2216E medium (Bianchi 1973) was used for growth of the isolates, and Luria-Bertani medium (Sambrook *et al.* 1989) with different sodium chloride concentrations was used for determining bacteria' salinity tolerance. Basic carbon source utilization medium containing 1% NaCl, 0.5% different carbohydrate, 0.32% Na_2HPO_4, 0.13% KH_2PO_4, 0.1% $NaNO_3$, 0.08% Na_2SO_4 and 0.05% $(NH_4)_2HPO_4$, and basic nitrogen source utilization medium containing 1% NaCl, 0.5% glucose, 0.7% K_2HPO_4, 0.3% KH_2PO_4, 0.1% different nitrogen source material, 0.05% sodium citrate and 0.01% $MgSO_4 \cdot 7H_2O$ were used for examining nutrition utilization of the isolates.

2.2 Protease activity measurement

1 ml of 0.2 mol/L phosphate buffer (pH7.0) containing 1% casein were added to 1 ml of crude enzyme preparation. After incubating at 40 ℃ for 1 h, the reaction was stopped by the addition of 2 ml of 0.4 mol/L trichloroacetic acid (TCA). Then mixture was filtered, and the filtrate was used to estimate the liberated proteolytic peptides by the method of Folin phenol regent (Lowry *et al.* 1951). One unit of protease activity was defined as the amount of the enzyme releasing 1 μg of tyrosine per 1 min at 40 ℃.

3 Results and discussion

3.1 Isolation and morphological characteristics of protease-producing bacteria

One bacterium isolated from the surface water sample showed strong proteolytic activity and was chosen for further research. The strain showed negative by Gram staining was designated as Ar/W/b/75°25′N/1. Cells of the isolate were rod shape with size (0.1 – 0.3) × (1.8 – 2.1) μm.

3.2 Physiological and biochemical characteristics of strain Ar/W/b/75°25′N/1

3.2.1 Effect of temperature on cell growth

After appropriate dilution, the 2-d cultured broth of the bacterium was inoculated onto Zobell 2216E agar plates by spread plate method. Cell growth at different cultivation temperature was observed.

Results show that on the plates incubated at 20 ℃ and 30 ℃, visible colonies can be found only after 24 h, and the size of colonies formed at 30 ℃ is generally twice that at 20 ℃. At 7 ℃. visible colonies can be formed late after 6 d. No growth occurred at 40 ℃. It suggests that the optimum temperature for cell growth is about 30 ℃, and the strain is psychrotolerant. Considering that the environment temperature of sampling site was just – 1.1 ℃, the marine bacterium can be deduced to originate from other warmer environments.

3.2.2 Effect of salinity on cell growth

Cell growth was examined after inoculating the strain into Luria-Bertani media containing different sodium chloride concentrations and incubating at 7 ℃ for 5 d.

Fig. 1 shows that the bacterium can grow well in media containing salinity range from 0.5% to 10% sodium chloride concentrations. And the strain grows better when salinity ranges from 1% to 7.5% sodium chloride concentrations, showing some halophilous characteristics. It suggested that the strain really originated from oceanic conditions.

3.2.3 Carbon source utilization of the strain

The strain was inoculated into carbon source utilization media with various carbohy-

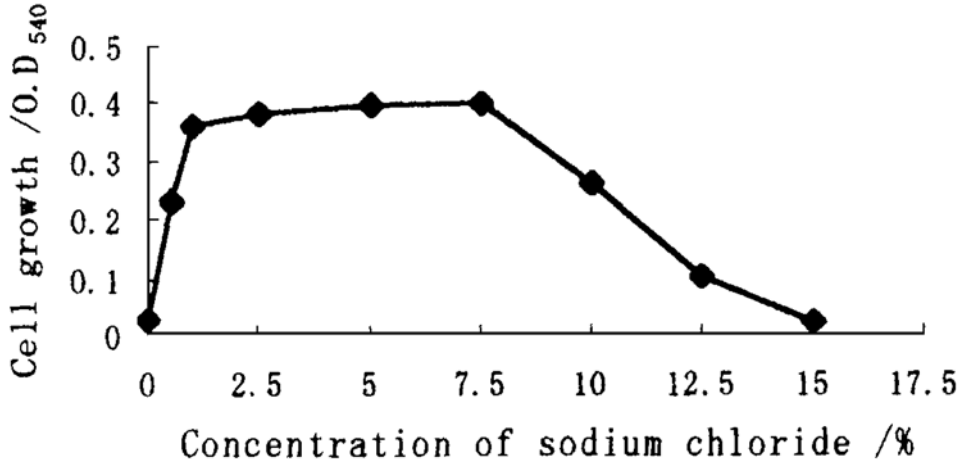

Fig. 1. Effect of salinity on cell growth.

drates. After incubation at 15 ℃ with vigorous shaking for 48 h, cell growth and protease activity were examined.

Fig. 2 shows that the strain can utilize glucose, sucrose and soluble starch as sole carbon source for cell growth, among which glucose is the best both for cell growth and protease producing. Sucrose and soluble starch are good for cell growth, but have little to do with protease producing of the bacterium.

3.2.4 Nitrogen source utilization of the strain

The strain was inoculated into various nitrogen source utilization media. After incubation at 15 ℃ with vigorous shaking for 48 h, cell growth and protease activity were examined.

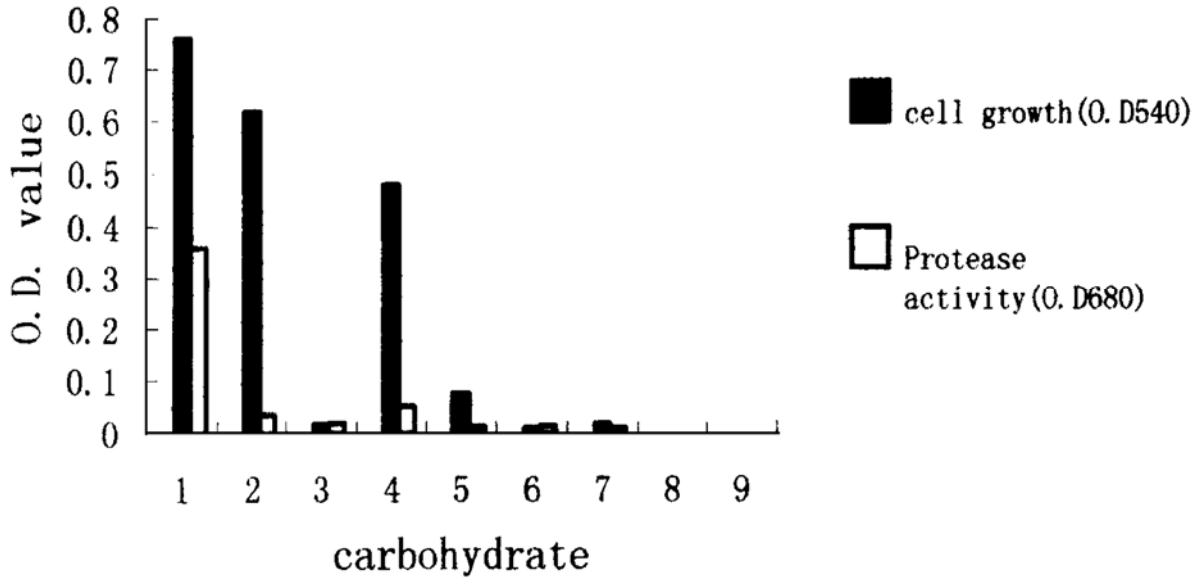

Fig. 2. Carbon source utilization of the strain.

1. Glucose; 2. Sucrose; 3. Lactose; 4. Soluble starch; 5. Potato starch; 6. Maltose; 7. Sodium carboxymethylcellulose(CMC).

Fig. 3 shows that peptone is the optimal nitrogen source both for cell growth and protease producing of the strain for its natural and complicated nutrition components. Ammonium nitrate is the optimal inorganic nitrogen source.

3.3 Characteristics of protease

3.3.1 Ratio of extracellular and total protease activity

It was observed that about 75.7% of total protease activity of the strain were extracellular enzyme. It will be of benefit for simplifying the procedure of enzyme extracting and purifying, and has potential role in biotechnology.

3.3.2 Temperature activity profile

The enzyme reaction was carried out at a various temperature range from 10 ℃ to 60 ℃ for 1 h with 1% casein as the substrate. Fig. 4 shows that maximum activity occurring at 40 ℃. And the activity was reduced by 50% at 50 ℃ when enzyme preparation was preincubated for 30 min (Fig. 5).

Enzymes usually have an optimum temperature range from 35 ℃ to 60 ℃. Microorganisms living in cold environments are possible sources of enzymes active at lower tem-

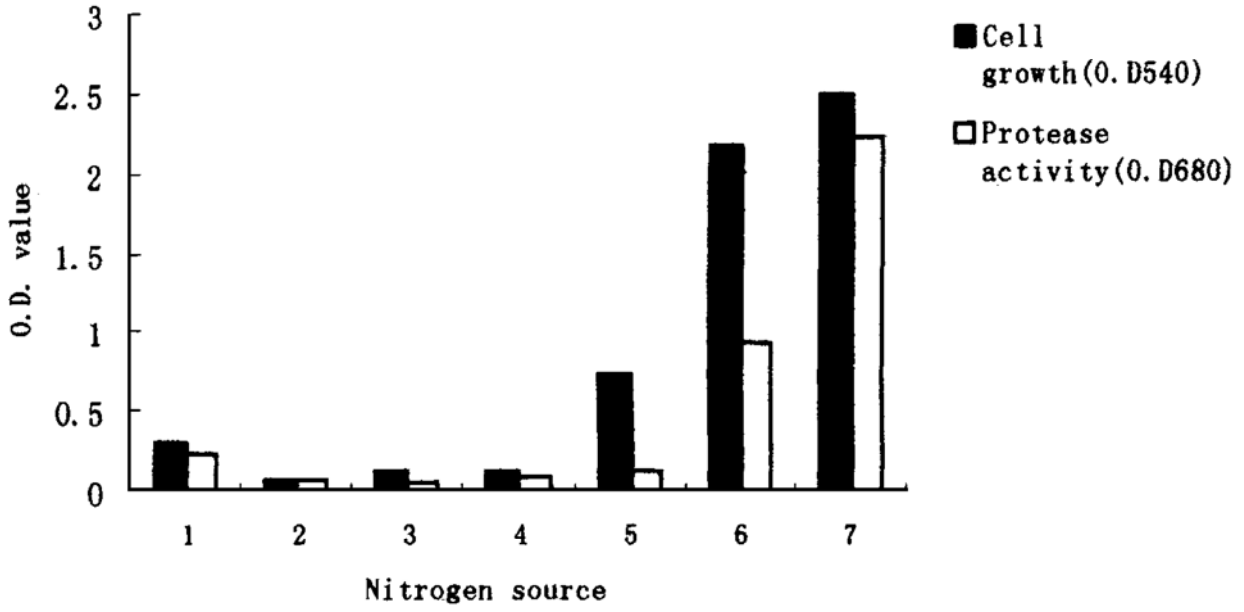

Fig. 3. Nitrogen source utilization of the strain.

1. Ammonium nitrate; 2. Potassium nitrate; 3. Ammonium sulfate; 4. Urea; 5. Casein; 6. Yeast extract; 7. Peptone.

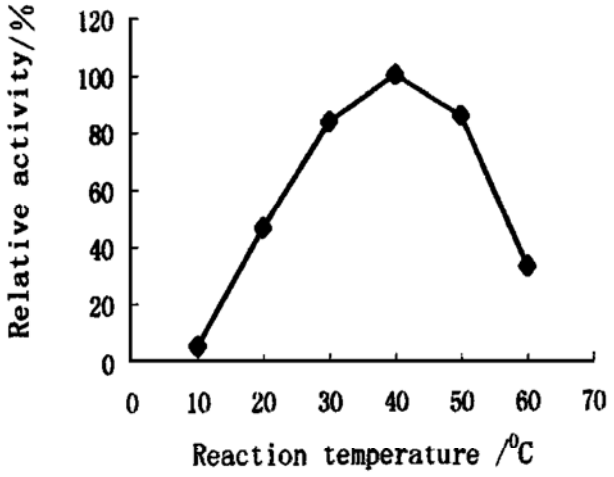

Fig. 4. Effect of temperature on protease activity.

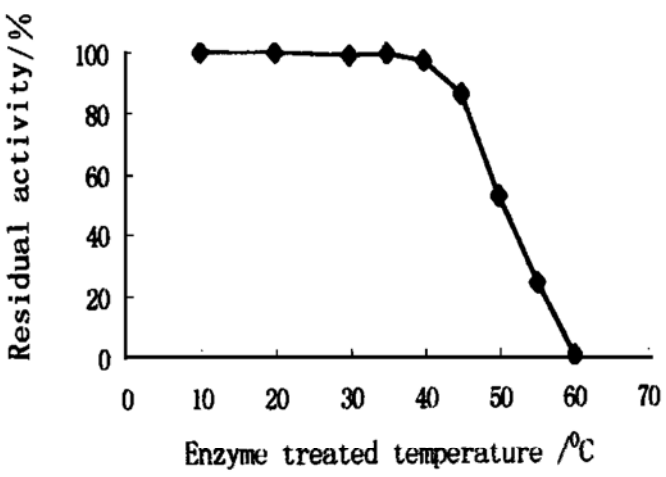

Fig. 5. Thermal stability of protease.

peratures. Extracellular acidic protease from Antarctic yeast *Candida humicola* has an optimum temperature of 37 ℃ for enzyme activity (Ray *et al*. 1992). The optimum temperature of proteases of *Pseudomonas maltophilia* from Antarctica (Vazquez *et al*. 1995), and *Pseudomonas* species from fish intestine (Hoshino *et al*. 1997) were 20 ℃ and 25 ℃ respectively. Considering the relative higher temperature for protease activity, the marine bacterium we isolated possibly originates from other warmer oceanic environments.

3.3.3 Effect of pH on protease activity

Optimum pH of protease activity was determined. The enzyme reaction was carried out in 0.2 mol/L phosphate buffer in the pH range from 4 to 12 at 40 ℃ for 1 h. Fig. 6 shows the optimum pH for enzyme activity is about pH 8, and the protease can express fairly high enzyme activity in the pH range from 7 to 11.

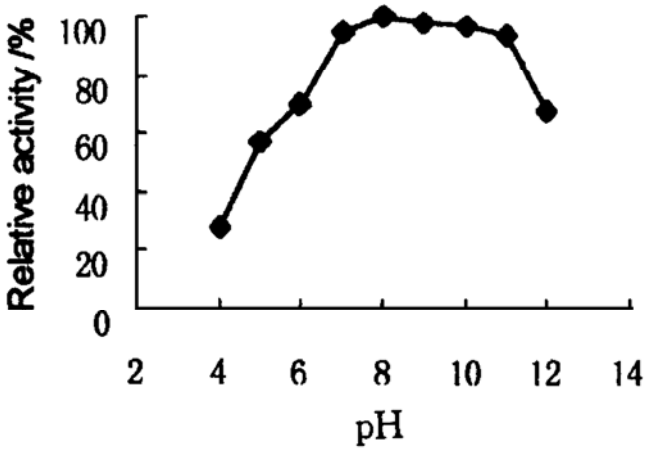

Fig. 6. Effect of pH on protease activity.

Acknowledgements This research was supported by Oceanographic Science Fund of State Oceanic Administration(No. 99614), as well as the First Chinese Arctic Expedition.

References

Bianchi JAM(1973): Variations de la concentration bacterienne dans les eaux et les sediments littoraux. Marine Biology, 22: 23 - 29.

Feller G, Narinx E, Arpigny JL, Aittaleb M, Baise E, Genicot S, Gerday C(1996): Enzymes from psychrophilic organisms. FEMS Microbioloy Reviews, 18: 189 - 202.

Gounot AM (1991): Bacteria life at low temperature: physiological aspects and biotechnological implications. J. Appl. Bacteriol., 71: 386 - 397.

Hoshino T, Ishizaki K, Sakamoto T, Kumeta H, Yumoto I, Matsuyama H, Ohgiya S(1997): Isolation of a *Pseudomonas* species from fish intestine that produces a protease active at low temperature. Letters in Applied Microbiology, 25: 70 - 72.

Master ER, Mohn WW (1998) Psychrotolerant bacteria isolated from Arctic soil that degrade polychlorinated biphenyls at low temperatures. Appl. Environ. Microbiol, 64: 4823 - 4829.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ(1951): Protein measurement with the Folin phenol regent. Journal of Biological Chemistry, 193: 265 - 275.

Qiu XB, Li T, Dai H, Yuan Y(1991): Study on proteases from psychrophilic marine microorganisms. Microbiology, 18: 138 - 141 (in Chinese).

Ray MK, Devi KU, Kumar GS, Shivaji S(1992): Extracellular protease from the Antarctic yeast *Candida humicola*. Appl. Environ. Microbiol., 58: 1918 - 1923.

Sambrook J, Fritsch EF, Maniatis T(1989): Molecular cloning: A laboratory manual. 2nd ed. Cold Spring Harbor Laboratory Press.

Vazquez SC, Rios Merino LN, MacCormack WP, Fraile ER (1995): Protease-producing psychrotrophic bacteria isolated from Antarctica. Polar Biol., 15: 131 - 135.