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Studies on culture condition and extracellular hydrolase of psychrophilic bacteria from Arctic sea ice

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Abstract Arctic sea ice in the polar region provides a cold habitat for microbial community. Arctic sea ice microorganisms are revealed to be of considerable importance in basic research and potential in biotechnological application. This paper investigated the culture condition and extracellular hydrolase of 14 strains of different Arctic sea ice bacteria. The results showed that optimal growth temperature of strains is 15 ℃ or 20 ℃. The optimal pH is about 8.0. They hardly grow at acid condition. 3% NaCl is necessary for better growth. These strains have different abilities in producing amylase, protease, cellulase and lipase. *Pseudoalteronomas* sp. Bsi429 and *Pseudoalteronomas* sp. Bsi539 produced both cellulose, protease and lipase. These results provide a basis for further developing and exploiting the cold adapted marine enzyme resources.

Key words Arctic sea ice microorganisms, culture condition, *Pseudoalteronomas*, hydrolase.

1 Introduction

Extremely cold environments widely exist in the great ecosystem of the earth. For example, the polar regions which cover 14% of earth's land surface and the deep sea where 90% of seawater has an average temperature of 5℃ or lower[1]. There are cold-adapted microorganisms living in these special environments[2]. The research on cold-adapted bacteria has substantial development with a better understanding of their important use in food industry, medicine, environmental protection, extraterritorial life exploration and basic research[3].

Morita[4] divided the cold-adapted bacteria into two groups according to their growth temperature: One group lived at low temperature and the highest growth temperature was no higher than 20 ℃, the optimum temperature was lower than 15 ℃ and they could grow under 0 ℃. This group is called psychrophiles. The other group could grow at a temperature higher than 20 ℃, the optimum temperature is higher than 15 ℃ and they could grow at 0-5 ℃. This group was called psychrotrophic or psychrotolerants.

Up to now, many psychrophiles and psychrotolerants microorganisms, including viruses, bacteria, yeast and algae have been found in sea ice[5-6]. Many sea ice bacteria display different physiological and biochemical characterization to cope with cold. One of the strategy of sea ice bacteria is to produce psychmphilic enzymes which have high catalytic efficiency at lower temperature and relatively high sensitivity to heat. , such as protease, α-amylase, β-galactosidase, alkaline phosphatase cellulose[7-8].

Present studied were conducted on the characteristics of growth and extracellular hydrolase activities of 14 strains bacteria isolated from the Artic sea ice. The finding in this paper is helpful for the development and utilization of cold adapted bacteria.

2 Materials and methods

2.1 Bacteria strains

14 strains of cold adapted bacteria from Artic sea ice coded Bsi510, Bsi429, Bsi473, Bsi476, Bsi480, Bsi539, Bsi540, Bsi541, Bsi560, Bsi567, Bsi569, Bsi570, Bsi580 and Bsi590 were kindly provided by Polar Research Institute of China. Strain Bsi510 belonged to the genus *Flavobacterium*, Bsi560 belonged to genus *Shewanella* and Bsi567 belonged to the genus *Marinomonas*, others were different strains belonging to the genus *Pseudoalteronomas*.

2.2 Initial screening medium[10]

2.2.1 Artificial sea water(ASW)

KBr 0.096 g, NaCl 24.477 g, $MgCl_2$ 4.981 g, Na_2SO_4 3.917 g, $CaCl_2$ 1.102 g, KCl 0.664 g, $NaHCO_3$ 0.192 g, $SrCl_2$ 0.024 g, H_3BO_3 0.026 g, NaF 0.0039 g, H_2O 1000 mL.

2.2.2 Zobell 2216E agar plate

Polypeptone 0.5 g, yeast extract 0.1 g, $Fe_2(PO_4)_3$ 0.01 g, agar 2 g, ASW 100 mL, pH7.6-7.8

2.2.3 Protease screening medium

Solution A: Polypeptone 0.03 g, yeast extract 0.05 g, agar 3 g, $Fe_2(PO_4)_3$ 0.01 g, ASW 100 mL; Solution B: 10%-20% of skim milk solution. Solution B was sterilized at 110 ℃ for 15 min and solution A was sterilized at 121 ℃ for 20 min. Both solutions were cooled to about 50 ℃ and mixed with each other in equal volume.

2.2.4 Amylase screening medium

1% soluble starch was added in the Zobell 2216E agar plate.

2.2.5 Lipase selection medium

1% Tween 80 was added in the Zobell 2216E agar plate.

2.2.6 Cellulase screening medium

0.5% carboxymethylcellulose sodium (CMCNa) was added in the Zobell 2216E agar plate.

2.3 Cultivation condition

2.3.1 Temperature of the strain growth

14 strains were inoculated by toothpick onto Zobell 2216E agar plates. The agar plates were incubated at 4 ℃, 15 ℃, 20 ℃, 25 ℃, 30 ℃ and 37 ℃ respectively up to 8 days. The diameters of the colonies were measured and the growth condition was observed everyday. The optimum growth temperature was decided by the growth rate according to the diameter of the colony.

2.3.2 pH of the strains growth

The bacteria were inoculated onto Zobell 2216E agar plates at pH 4.5, 6, 7, 8 and 10 respectively incubated up to 7 days. The diameters of the colonies were measured and the growth condition was observed everyday. The optimum pH was decided by the growth rate according to the diameter of the colony.

2.3.3 Salt resistance test

The strains were inoculated onto Zobell 2216E agar plates containing 0%, 3%, 5% of NaCl. The diameters of the colonies were measured and the growth condition was observed everyday.

2.4 Characterization of enzyme activities

2.4.1 Plate-screening of protease activity

Bacteria were inoculated onto protease initial screening medium and incubated for 4-7 d at 20 ℃. A positive reaction was noticed when clear zone around the colony was directly visible.

2.4.2 Plate-screening of amylase activity

Bacteria were inoculated onto amylse initial screening medium and incubated for 3 d at 20 ℃. Drop iodine solution to the plate and observe the clear zone. A positive reaction was noticed when clear zone around the colony was observed. Selecting the strains according to the ratio of clear zone and colony diameter.

2.4.3 Plate-screening of lipase activity

Bacteria were inoculated onto lipase initial screening medium and incubated for 2-3 d at 20 ℃. The strains with lipase decomposed Tween80 and generate white precipitation zone. Selecting the strain according to the ratio of precipitation zone and colony diameter.

2.4.4 Plate-screening of cellulase activity

Bacteria were inoculated onto cellulose initial screening medium and incubated for 2-4

d at 20 ℃. Add suitable quantity of 1 mg/mL Congo red dye onto the plate and wait for 1 h; the dye was then discarded and 1 mol/L NaCl was added to fix it. Clear zone around colony was observed for the strain that had cellulase activity.

2.4.5 Influence of temperature to enzyme production

Strains producing cellulase and amylase were inoculated onto the selection medium at 20 ℃ and 4 ℃. The diameter of clear zone (D) and colony (d) were measured. The enzyme activity was determined by the D/d ratio.

3 Results and discussion

3.1 Basic characteristics of the strains

3.1.1 The optimum growth temperature of strains

Measuring the colony diameter of the strains under different temperature from 4 to 37 ℃. The optimum temperature was determined by the growth rate (table 1). The specific temperature was different among strains: the optimum growth temperature of the strains Bsi429、Bsi476、Bsi539、Bsi541、Bsi569、Bsi580 and Bsi590 was 20 ℃, whereas for strains Bsi473、Bsi480、Bsi510、Bsi540、Bsi560、Bsi567 and Bsi570 that was 15 ℃. The optimum growth temperature of the strains from *Pseudoalteromonas* was between 15 and 20 ℃. Most of the strains (80%) could grow at 37 ℃. For strain Bsi560 (*Shewanella*) and strain Bsi567 (*Marinomonas*) the optimum temperature was 15℃ and highest growth temperature lower than 37℃. They were both psychrophilic bacteria according to Morita's definition of psychrophilic bacteria.

Table 1. The growth rate of different strains at different temperatures

Strain	4 ℃	15 ℃	20 ℃	25 ℃	30 ℃	37 ℃
Bsi429	++++	+++++	++++	+++	++	+
Bsi473	+++	+++++	++++	+++	++	+
Bsi476	+	+++	+++++	+++	+++	+
Bsi480	+++	+++++	++++	+++	++	+
Bsi539	++	++++	+++++	+++	++	-
Bsi540	++	+++++	++++	++	+	-
Bsi541	++	++++	+++++	+++	++	-
Bsi569	+++	++++	+++++	+++	++	+
Bsi570	++	+++++	++++	++	++	+
Bsi580	++	++++	+++++	+++	++	+
Bsi590	++	++++	+++++	+++	++	+
Bsi510	++	+++++	++++	+++	++	+
Bsi560	++++	+++++	+++	++	+	-
Bsi567	++	+++++	+++	+++	+	-

Note: '-' referred to no growth, '+++++, ++++, +++, ++, +' referred to a growth rate from high to low.

3.1.2 Optimum growth pH of the strains

The colony diameters were measured at 20 ℃ when the strains were cultured in plates of different pH(table 2). The optimum growth pH of the strains was 7-8, which was in accordance with the weak alkaline environment in the seawater. It suggested the adaptability of Arctic bacteria to ocean environment. The range of growth pH of *Pseudoalteromonas* differed among strains, which suggested the broad origin of the strains from *Pseudoalteromonas*.

Table 2. The growth rate of different strains at different pH

Strain	pH4.5	pH6	pH7	pH8
Bsi429	+	++	+++	++++
Bsi473	-	-	++	+++
Bsi476	-	-	+++	++
Bsi480	-	-	++	+++
Bsi539	-	-	++	+++
Bsi540	-	-	+++	++
Bsi541	-	-	++	+++
Bsi569	-	-	++	+++
Bsi570	-	-	++	+++
Bsi580	-	-	++	++++
Bsi590	-	-	+++	++
Bsi510	-	-	+	++
Bsi560	-	-	++	+++
Bsi567	-	-	+	++

Note: '-' referred to no growth, '+++++, ++++, +++, ++, +' referred to a growth rate from high to low.

3.1.3 NaCl tolerance of the strains

According to the results (table 3), the strains required NaCl to grow and 3% NaCl was the optimum concentration to yield highest growth rate. Strains Bsi429 andBsi539 could grow well with 5% NaCl in the medium.

Table 3. Growth condition of different strains at different concentration of NaCl

NaCl(%)	i429	i473	i476	i480	i539	i540	i541	i569	i570	i580	i590	i560	i567	i510
0	0	0	0	0	0	0	0	0	0	0	0	0	0	4.0
3	8.5	8.5	8.1	7.5	12.0	4.5	8.0	9.2	7.5	7.0	8.0	5.5	7.1	3.2
5	4.0	1.0	0	0	6.0	1.9	2.2	0	0	0	0	0	0	0

Note: value referred to diameter(mm).

3.2 Characterization of enzyme activities

3.2.1 The enzyme activities of the strains

Protease, amylase, lipase and cellulase activities were tested among the selected 14 strains of bacteria. Strains Bsi560, Bs i590, Bsi580, Bs i541, Bsi480, Bs i473 and Bsi540 had amylase activity (Plate 1-1,2); Strains Bs i569, Bs i570, Bs i580, Bs i473, Bs i540, Bs i480, Bsi539, Bsi590, Bs i429 had lipase activity (Plate 1-3,4); Strains Bs i429 and Bs i539 had cellulase activity (Plate 1-5,6); Strains Bsi429 and Bsi539 had pro-

tease activity (Plate 1-7). Strains Bsi429 and Bsi539 were of particular interest because they had protease, cellulase and lipase activities.

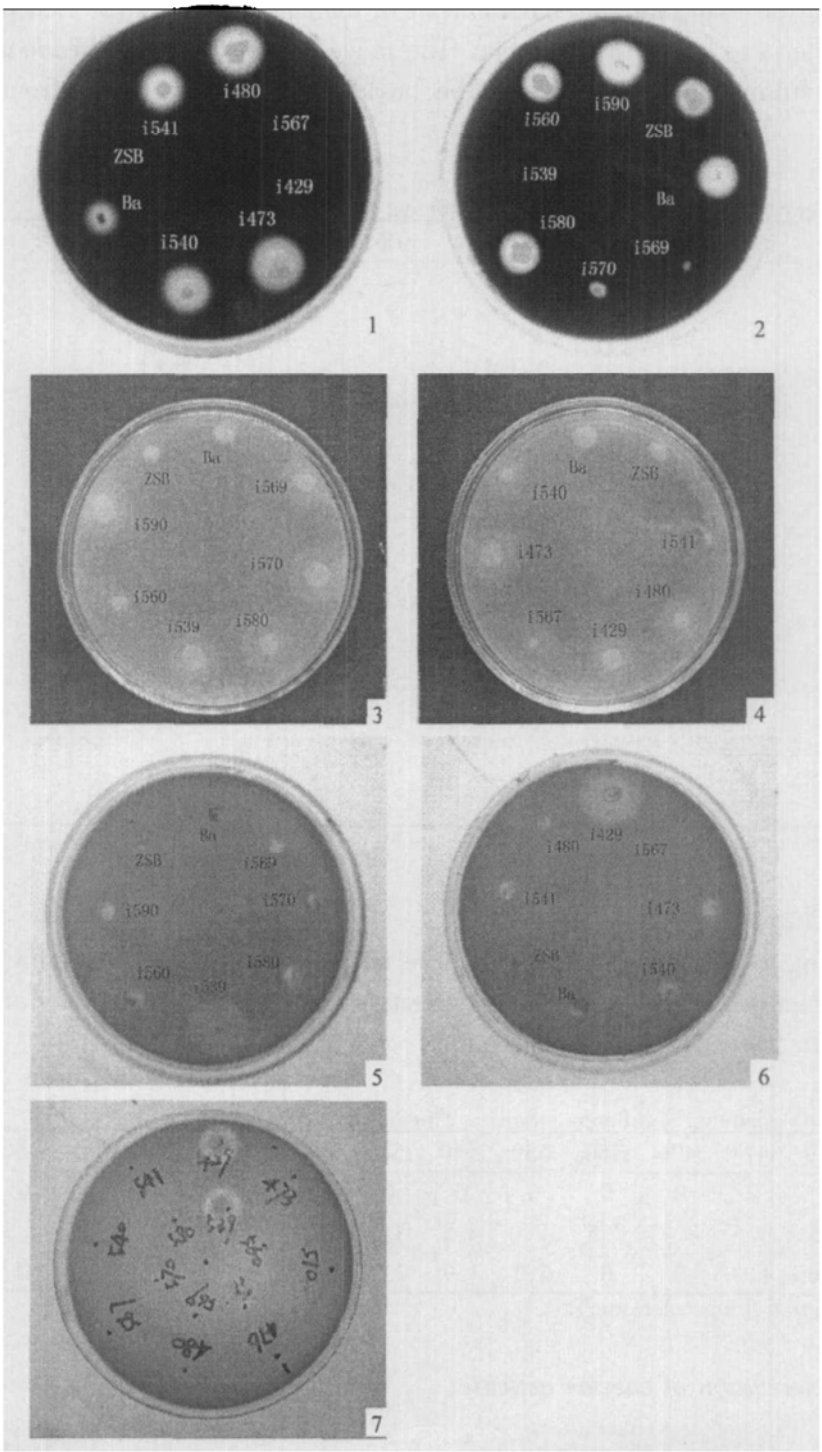

Plate 1. Screening of excellular hydrolase from Arctic sea ice bacteria.

1,2. Amylase initial screening plate, strains Bsi473, Bsi480, Bsi540, Bsi541, Bs i560, Bsi580 and Bsi590 had positive result; 3,4. Lipase initial screening plate, strains Bsi429, Bsi473, Bsi480, Bsi539, Bsi540, Bs i569、Bsi570、Bsi580 and Bsi590 had positive result; 5,6. Cellulase initial screening plate, strain Bsi429 and Bsi539 had obvious clear zone; 7. Protease initial screening plate, strainsBsi429 and Bsi539 had positive result

3.2.2 Influence of incubation temperature on amylase activity of the strains

Amylase screening agar plates were incubated at both 20 ℃ and 4 ℃. The results were shown in Fig. 1. The amylase activity was higher at 4 ℃.

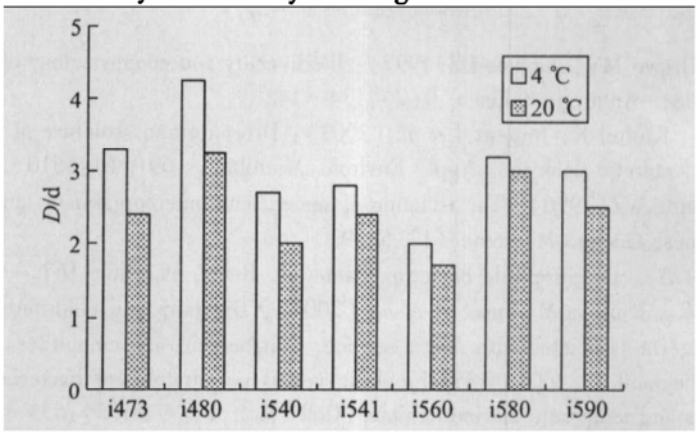

Fig. 1 The influence of temperature on amylase activity of different strains.

4 Discussion

Temperature and salinity are two major environment factors that control the survival and activity of sea ice bacteria. Only those tolerate cold and high salinity can survive in this environment and take advantage in quantity and distribution[11].

In this study, the incubation condition of 14 Artic sea ice strains belonging to 4 different genus was studied. The optimum growth temperature of the bacteria was between 15 and 20 ℃. More than half of the bacteria (64%) could grow at 37 ℃, which suggested that they were cold adapted bacteria. Strain *Marinomonas* sp. Bsi567 could not grow at 37 ℃ and its growth was inhibited by 5% NaCl and it could not decompose some carbohydrates used in this experiment, which was obviously different from other strains. *Flavobacterium* sp. Bsi510 could grow without NaCl. *Pseudoalteromonas* widely existed in the marine environment, they required NaCl in their growth environment and have a wide hydrolases and could grow at pH7-10, 4-37℃ and endure up to 5% NaCl. Among them, 2 strains produced protease, 9 strains produced lipase and 2 strains produced cellulase, both Bsi429 and Bsi539 have three kind of enzyme which revealed *Pseudoalteromonas* have the wide adaptability to different environments.

Feller *et al.* (1994) found that cold-adapted bacteria usually secreted bioactive products only under low temperature[12]. Hellio *et al.* (1993) found that the optimum temperature for the cold-adapted bacteria to produce extracellular enzymes was usually lower than their optimum growth temperature[13]. Similar phenomenon was observed in this study on the amylase and cellelular. The enzyme activity at 4 ℃ was higher than that at 20 ℃. The present research found that amylase activity of the amylase producing strains kept increasing at 4 ℃, which revealed the stability of the enzyme at low temperature.

Cold adapted enzymes are of great importance in applied and theoretical research. *Pseudoalteromonas* strains Bsi429 and Bsi539 have three kinds of hydrolase, so further investigation the characterization of the enzymes would bring about potential application in detergent and environment protection.

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