Chinese Journal of Polar Science, Vol. 15, No. 2, 118-128, December 2004

Extracellular enzymatic activities of cold-adapted bacteria from polar oceans and effect of temperature and salinity on cell growth

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Received November 10, 2004

Abstract The potential of 324 bacteria isolated from different habitats in polar oceans to produce a variety of extracellular enzymatic activities at low temperature was investigated. By plate assay, lipase, protease, amylase, gelatinase, agarase, chitinase or cellulase were detected. Lipases were generally present by bacteria living in polar oceans. Protease-producing bacteria held the second highest proportion in culturable isolates. Strains producing amylase kept a relative stable proportion of around 30% in different polar marine habitats. All 50 Arctic sea-ice bacteria producing proteases were cold-adapted strains, however, only 20% were psychrophilic. 98% of them could grow at 3% NaCl, and 56% could grow without NaCl. On the other hand, 98% of these sea-ice bacteria produced extracellular proteases with optimum temperature at or higher than 35℃, well above the upper temperature limit of cell growth. Extracellular enzymes including amylase, agarase, cellulase and lipase released by bacteria from seawater or sediment in polar oceans, most expressed maximum activities between 25 and 35℃. Among extracellular enzymes released by bacterial strain BSw20308, protease expressed maximum activity at 40℃, higher than 35℃ of polysaccharide hydrolases and 25℃ of lipase.

Key words Enzyme, cold-adapted, bacteria, polar ocean

1 Introduction

Temperature is one important environmental factor for life, as it influences the basic components of a cell and most biochemical reactions of an organism. Organisms usually grow between 0 and 100℃, with the greatest diversity being observed between 15 and 45℃ (Cummings and Black 1999). However, more than 70% of the Earth's surface is occupied by cold ecosystems, including the ocean depths, polar and alpine regions, with a temperature usually below 5℃ (Feller and Gerday 2003). In Arctic and Antarctic marine habitats, temperature of seawater and sediments is near -1℃, sea ice even low to -35℃ in winter time (Margesin and Schinner 1994; Deming 2002). Despite the severe environmental conditions, investigations on microbial communities in polar oceans have shown that numerous microorganisms, prokaryotic and eukaryotic, with their own strategies for cold adaptation,

have successfully colonized in these permanently cold habitats, from surface sea ice and water to deep-sea sediment (Helmke and Weyland 1995; Priddle *et al.* 1996; Thomas and Dieckmann 2002; Purdy *et al.* 2003; Ruger *et al.* 2000).

Cold-adapted microorganisms, including heterotrophic bacteria, yeast and cyanobacteria, play a significant role in the cycling of carbon, nitrogen and other elements and materials in polar marine ecosystems, and the in-situ biodegradation and bioremediation (Rivkin and Legendre 2001; Huston and Deming 2002; Cummings and Black 1999; Deming 2002; Margesin and Schinner 2001; Nichols 2003). They are essential components of the polar pelagic marine food webs. In marine environments, a portion of non-living organic matter is consumed directly by grazers. The rest is subject to degradation by heterotrophic bacteria. Most of the organic matter in aquatic environments is too large for direct uptake by bacteria (Thurman 1985; Chrost 1991), hence degradation by extracellular enzymes is necessary first (Rogers 1961; Billen 1984). Extracellular enzyme activity has been regarded as the rate-limiting step in microbial degradation of high molecular weight organic matter in the marine environment (Hoppe 1991; Meyer-Reil 1991).

In addition to their important roles in polar marine environments, microbial extracellular enzymes with optimal activity at low temperature provide opportunities to study the adaptation of life to cold and the potential for biotechnological exploitation (Cavicchioli *et al.* 2002; Gerday *et al.* 2000; Nichols *et al.* 1999). Bacteria that produce cold-active, heat-labile enzymes are recognized as attractive candidates for the applications such as waste digestion and cleaning agents in cold environments, food processing and preservation, molecular biology and industrial processing that benefits from rapid inactivation of enzymatic reactions.

Cold-adapted microorganisms are one important exploitable source for cold- active enzymes. In recent years a large numbers of cold-adapted microorganisms have been isolated and purified from different habitats in Arctic and Antarctic oceans, and a series of studies on screening for cold-active enzyme products have been conducted (Deming 2002; Cavicchioli *et al.* 2002).

It is the objective of this study to investigate the potential of hundreds culturable cold-adapted bacteria isolated from polar oceans to produce various enzymes at low temperatures. A large number of isolates from Arctic and Antarctic oceans were found to have capacity of producing a variety of enzymes, including protease, cellulase, lipase, agarase, gelatinase, chitinase or amylase. Parts of enzymes were characterized by determining the effect of temperature on enzymatic activities.

2 Materials and methods

Sample collection, bacterium isolation and cultivating conditions

Sea ice samples were collected from Canada Basin and Greenland Sea in 2002, sediment samples obtained from Chukchi and Bering Sea in 1999, and seawater sampled from Antarctic Prydz Bay between 1999 and 2002. Seawater samples were spread directly on pre- cooled ZoBell 2216E agar plates and incubated at 4– 6℃ for 40 days. Sea ice samples firstly were melt in sterile seawater at 4℃ in the dark, then were processed by spread plate

method for bacterium isolation. After suspension and dilution with sterile seawater, marine sediment samples were processed using the same isolation method as seawater sample. Zo-Bell agar plate was prepared as described by *Truong et al.* (2001) with the following modified composition: 1 liter in-situ seawater containing 5 g peptone, 1 g yeast extract, 0.1 g $FePO_4$ and 15 g agar. A total of 324 marine bacterial isolates were collected.

Screening for enzymatic activities

Using similar plate-screening techniques on marine bacteria, amylase, cellulase, chitinase, protease, gelatinase and lipase activities were tested on Zobell 2216 E agar plates supplemented with starch, carboxymethylcellulose, colloidal chitin, casein, gelatin (each compound 0.5%, wt/vol) and tributyrin (0.5%, vol/vol) as substrate, respectively. After 5-20 days at 5-10℃, a positive reaction was noticed when clear zone around the colony was directly visible or detected after precipitation or coloration of the undegraded substrate (Castro et al. 1993; Teather and Wood 1982). In the case of agarase activity, a positive reaction was noticed when colonies sunken occurred on Zobell 2216E agar plate.

Growth temperature range

Suspensions of microbial cells (pre-grown in ZoBell 2216E media at 6℃) were inoculated onto ZoBell 2216E agar plates. Agar plates then were incubated at 6, 20, 35 and 42℃, respectively. Growth was monitored up to an incubation time of 7 days.

Growth salt concentration range

According to a ratio of 1∶100 (vol∶vol), microbial cells growing in ZoBell 2216E media were inoculated into Luria-Bertani media (Sambrook et al. 1989) containing 0, 3, 5 and 10% (wt/vol) NaCl, respectively. Bacteria grew at 10℃ for 2 days. Cell growth was monitored by the increase in optical density at 600nm (OD_{600}).

Characterization of enzyme activities

Cells were grown in Zobell 2216E medium, and harvested by centrifugation at 4000 rpm, 4℃ for 10min. Supernatant was used as crude enzyme preparation. To test the effect of temperature on enzymatic activity, enzyme-substrate mixture was incubated at 5-55℃ for 30 min, respectively.

Protease

1 ml of crude enzyme preparation was added to 1.5 ml of 50 mmol l^{-1} phosphate buffer (pH 7.0) containing 1% (wt/vol) casein. After incubating at 35℃ for 30min, the reaction was stopped by adding 2.5 ml of 0.4 mol l^{-1} trichloroacetic acid (TCA). 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). Absorbance at 680 nm was measured with a spectrophotometer. One unit of protease activity was defined as the amount of the enzyme releasing 1 μg of tyrosine per 1 min at 35℃.

Lipase

Lipase activity was determined by titrimetry according to Deerl and Akpinar (2002) with little modification. Lipase assay was performed with an olive oil substrate consisting of 4 ml of 25% (vol/vol) olive oil emulsion and 5 ml of 25mmol l^{-1} phosphate buffer (pH 7.5). Olive oil containing 2% (wt/vol) polyvinyl alcohol was first emulsified. 1 ml of crude enzyme solution was added to olive oil substrate solution. The enzyme-substrate mixture was incubated at 35℃ for 15 min, and the reaction was terminated by adding 15 ml of 95% (vol/vol) alcohol. After adding 3 drops of phenolphthalein indicator, the liberated fatty acids were titrated with 50mmol l^{-1} NaOH till the mixture turned into pink color. One unit of activity was defined as the quantity of enzyme necessary to liberate the equivalent of 1 μmole of acid (H^+) from olive oil per min for per ml under the conditions of the assay.

Cellulase

Cellulase activity was assayed by analyzing reducing sugars released as a result of enzyme reaction with substrate of CMC. 1 ml of crude enzyme preparation were added to 1.5 ml of 50 mmol l^{-1} phosphate buffer (pH 7.0) containing 0.5% (wt/vol) CMC. After incubation at 35℃ for 30min, the reaction was stopped by adding 2.5 ml of 3, 5-dinitrosalicylic acid (DNS) solution and was incubated in boiled water for 6 min. The reductive sugars released from the reaction were determined using the DNS method (Miller 1959), with glucose as standard. The absorbance at 530 nm was measured. One unit of cellulase activity was defined as the amount of enzyme releasing 1 μg of glucose per 1 min at 35℃.

Amylase

Amylase activity was measured by incubating 1 ml of crude enzyme solution with 1.5 ml of 0.5% (wt/vol) soluble starch in 50 mmol l^{-1} phosphate buffer (pH 7.0) at 35℃ for 30min. The release of reducing sugars was examined using the DNS method. One unit of amylase activity was defined as the amount of enzyme releasing 1 μg of glucose per min under the assay conditions.

3 Results and discussion

3.1 *Screening for enzymatic activities*

Plate assay showed that a large number of bacteria isolated from various habitats in polar oceans, including Arctic sea ice and marine sediment, Antarctic seawater, produced a variety of extracellular enzymes at low temperature (Table 1, 2 and 3). Lipases were generally present by bacteria living in polar oceans, enzyme-producing strains holding the highest proportion in isolates, from 72% in Arctic sea ice increasing to 94% in Arctic marine sediment. It indicates that marine bacteria in polar oceans are important potential source for novel cold-active lipases.

Though less than lipase-producing strains, protease-producing bacteria was also common in polar oceans, with a proportion between 37 and 65%. It was mentioned that in Arctic sea ice the proportion was much higher than that in Arctic marine sediment or Antarctic

surface water.

Comparing with bacteria producing lipase or protease, strains producing amylase kept a relative stable proportion of around 30% in different polar marine habitats.

Table 1. Potential of 162 bacterial strains (= 100%) isolated from Arctic sea ice to produce enzymes at 5℃

Enzymatic activity	Lipase	Protease	Gelatinase	Amylase	Chitinase	Agarase
Bacteria No.	117	106	95	50	13	0
(100% = 162) %	72.2	65.4	58.6	30.9	8	0

Table 2. Potential of 88 bacterial strains (= 100%) isolated from Antarctic surface seawater to produce enzymes at 10℃

Enzymatic activity	Lipase	Protease	Amylase	Cellulase	Agarase
Bacteria No.	71	33	28	10	6
(100% = 88) %	80.7	37.5	31.8	11.4	6.9

Table 3. Potential of 74 bacterial strains (= 100%) isolated from Arctic marine sediment to produce enzymes at 5℃

Enzymatic activity	Lipase	Protease	Amylase	Cellulase	Agarase
Bacteria No.	70	31	18	9	0
(100% = 74) %	94.6	41.9	24.3	12.2	0

A concept has been accepted that hydrolytic enzymes in aquatic environments are induced by the presence of polymeric substrates (Chrost 1991; Vetter and Deming 1999). Most microorganism cells have the ability to alter their membrane fatty acid composition as temperature changes in order to keep their membrane at nearly the same fluidity and permeability (Russell 1990). Besides the increasing of lipid content in cell (Margesin and Schinner 1994), the unsaturated to saturated fatty acid ratio in cell membrane increased when temperature dropped from 20 to 5 degrees ℃ (Erdal *et al.* 2003; Zsiros *et al.* 2000). People have begun to consider the bacterial roles for the input of PUFA (polyunsaturated fatty acid) to marine food webs, and regard that the PUFA-producing bacteria may play an important role in the food web of global polar marine ecosystems (Nichols 2003). The majority of PUFA-producers are characterized as being psychrophilic and halophilic (Russell and Nichols 1999; Kato and Nogi 2001; Kato 1999). These physiological traits have influenced the ecological distribution of PUFA-producing bacteria in the marine environment. A high proportion of lipase-producing strains in culturable isolates from polar oceans may reflect a fact of lipid material including PUFA widely existing in polar cold marine ecosystems.

Extracellular proteases play an important role in influencing the nitrogen content of or-

ganic matter regardless of being in cold or temperate marine environments, and the dominance of protease activity over other extracellular enzymatic activity has been documented (Christian and Karl 1995, 1998; Hoppe *et al.* 1998; Huston and Deming 2002; Smith *et al.* 1992). The observation that a high proportion of protease-producing bacteria occurred in isolates from polar oceans indicates the importance of proteases and enzyme-producing bacteria in polar marine ecosystems.

3.2 *Effect of environmental factors on cell growth of sea-ice bacteria*

Sea ice provides a unique habitat for bacteria and microscopic plants and animals that are encased in an ice matrix (Thomas and Dieckmann 2002; Staley and Gosink 1999). Temperature and salinity are two important environment factors influencing bacterial survival and activities (Ackley and Sullivan 1994). 50 bacteria isolated from Arctic sea ice showing strong extracellular protease activity were chosen for investigating the effect of environment factors on bacterial growth.

No sea-ice bacteria grew when temperatures higher than 42℃ (Table 4), suggesting all bacteria were cold-adapted strains. Only 20% of them with an upper growth limit at 20℃ were psychrophilic organism. Research showed that even in permanently cold environments, at least 50% of the bacteria were not psychrophilic (Delille and Perret 1989). However, the proportion of psychrophiles in Arctic sea-ice bacteria was much lower than that in Antarctic sea ice for 45% of the isolated strains are psychrophilic (Bowman *et al.* 1997).

Table 4 Effect of temperature on cell growth of 50 Arctic sea-ice bacteria

Temperature(℃)	6	20	35	42
Percentage of growing strains(%)	100	80	24	0

Sea-ice bacteria usually originate from bacterioplankton in seawater (Thomas and Dieckmann 2002; Staley and Gosink 1999). In comparison with the Antarctic with an isolated geography characteristic, the Arctic not only has higher annual mean temperatures, but also is more influenced by surrounding continents. At the same time, the Arctic ocean experiences frequently exchanging of material and energy between Arctic cold waters and warm waters inflowing from the Pacific and the Atlantic. These factors may contribute to a low proportion of psychrophilic bacteria in Arctic seawater, then result the proportion of psychrophilic bacteria in Arctic sea ice lower than that in Antarctic sea ice.

98% of these sea-ice isolates grew at 3% NaCl (Table 5), which was equal to the mean seawater salinity. On the other hand, 56% of them could grow in media without any sodium chloride, suggesting a possibility that these bacteria originated from Arctic terrestrial habitats. The Arctic is actually an ocean surrounded by continents, and every year inflowing freshwater can bring rich terrestrial organic matter, nutrition and microorganisms into the ocean. A terrestrial influence on Arctic pack ice community was suggested by the presence of limnic phylotypes (Brinkmeyer *et al.* 2003).

Effects of temperature and salt concentration on sea-ice bacterial growth reflected the result of environmental selective pressures on microorganisms existing in polar oceans.

Table 5. Effect of salt concentration on cell growth of 50 Arctic sea-ice bacteria

Salt concentration(NaCl%)	0	3	5	10
Percentage of growing strains(%)	56	98	71	2

3.3 *Temperature profile of extracellular proteases of sea-ice bacteria*

In general cold-active enzymes have maximal catalytic activity at temperature below 40℃ (Nichols et al. 1999). However, among extracellular proteases produced by these 50 Arctic sea-ice bacteria, 98% had highest activity at 35℃ or higher (Table 6), 62% even at 45℃ or higher in which conditions no sea-ice bacteria could grow (Table 4).

Table 6. Temperature profile of extracellular proteases produced by 50 Arctic sea-ice bacteria

Optimum temperature(℃)	25	35	45	55
Percentage of total bacteria (%)	2	36	46	16

The less cold-optimized proteases measured in Arctic sea-ice bacteria were consistent with previous studies indicating that psychrophilic and psychrotrophic bacteria release enzymes with activity optima between 25 and 45℃, well above the upper growth limit of enzyme-producing organisms (McDonald *et al.* 1963; Helmke and Weyland 1991; Schinner *et al.* 1992; Huston *et al.* 2000). It indicates that in contrast to bacterial growth, extracellular proteases of bacteria from polar oceans are less well adapted to cold temperatures (Reichardt 1987). It suggests that the evolution progress of enzyme molecule for cold adaptation is not consistent with that of bacterial physiology or metabolism.

However, extracellular protease may not express maximal activity at a lower temperature optimum for bacterial growth, it may be sufficient to support cell growth in cold environment (Brenchley 1996).

3.4 *Temperature profile of extracellular enzymes of bacteria from seawater or sediment*

As for extracellular enzymes including amylase, agarase, cellulase and lipase released by bacteria from seawater or sediment in polar oceans, most expressed maximum activities between 25 and 35℃, lower than that of proteases by Arctic sea-ice bacteria (Table 7 and 8).

Table 7. Temperature profiles of extracellular amylases produced by 18 Arctic sediment bacteria

Optimum temperature(℃)	25	35	45
Percentage of total bacteria(%)	33.3	44.5	22.2

In the extracellular enzymes released by bacterial strain BSw20308, protease expressed maximum activity at 40℃ higher than 35℃ of polysaccharide hydrolases and 25℃ of lipase (Table 8). Analysis by 16S rRNA sequencing (Genbank access number AY646431) indicated that strain BSw20308 belonged to the genus *Pseudoalteromonas* (data not shown).

Table 8 Temperature characteristics of enzymes produced by bacteria from seawater or sediment in polar oceans

Strain	Environment	Enzyme	Optimum Temperature (℃)
BSw20308	Arctic seawater	Protease	40
		Lipase	25
		Amylase	35
		Cellulase	35
		Agarase	35
BSw10009	Antarctic seawater	Cellulase	35
BSw10011	Antarctic seawater	Cellulase	35
BSs20022	Arctic sediment	Lipase	25
BSs20039	Arctic sediment	Lipase	25

Does there exist any relationship between optimum temperature and the category of extracellular enzymes in polar marine bacteria? And is it a common phenomenon that the temperature at which proteases of polar marine bacteria express maximal activities is higher than that for polysaccharide hydrolases and lipases? The difference of temperature characteristic of extracellular enzymes maybe has connection with the origin of bacterium and environmental selective pressures, including temperature and nutrition conditions.

Acknowledgments This work was supported by grants from the National Science Foundation of China (30200001, 40376001), the National Science and Technology Ministry of China (2001DIA50040, 2003DEB5J057), Oceanographic Science Fund of State Oceanic Administration (2004201) and Polar Research Institute of China (JDQ200401). We are grateful to M. Cai and J. He for collecting field samples, and same grateful to the captain and crew of Chinese polar exploration icebreaker *Xuelong* for their technical and moral support under challenging conditions.

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