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Effects of Incubation Temperature on Growth and Production of Exopolysaccharides by an Antarctic Sea Ice Bacterium Grown in Batch Culture

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Abstract: The sea ice microbial community plays a key role in the productivity of the Southern Ocean. Exopolysaccharide (EPS) is a major component of the exopolymer secreted by many marine bacteria to enhance survival and is abundant in sea ice brine channels, but little is known about its function there. This study investigated the effects of temperature on EPS production in batch culture by CAM025, a marine bacterium isolated from sea ice sampled from the Southern Ocean. Previous studies have shown that CAM025 is a member of the genus Pseudoalteromonas and therefore belongs to a group found to be abundant in sea ice by culture-dependent and -independent techniques. Batch cultures were grown at –2°C, 10°C, and 20°C, and cell number, optical density, pH, glucose concentration, and viscosity were monitored. The yield of EPS at –2°C and 10°C was 30 times higher than at 20°C, which is the optimum growth temperature for many psychrotolerant strains. EPS may have a cryoprotective role in brine channels of sea ice, where extremes of high salinity and low temperature impose pressures on microbial growth and survival. The EPS produced at –2°C and 10°C had a higher uronic acid content than that produced at 20°C. The availability of iron as a trace metal is of critical importance in the Southern Ocean, where it is known to limit primary production. EPS from strain CAM025 is polyanionic and may bind dissolved cations such at trace metals, and therefore the presence of bacterial EPS in the Antarctic marine environment may have important ecological implications.

Keywords: Exopolysaccharides, exopolymer, Antarctica, marine bacteria, sea ice, psychrotolerant, EPS yield

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THE EFFECTS OF INCUBATION TEMPERATURE ON THE GROWTH AND PRODUCTION OF EXOPOLYSACCHARIDES BY AN ANTARCTIC SEA ICE BACTERIUM GROWN IN BATCH CULTURE

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RUNNING TITLE

Temperature effect on EPS production by sea ice strain

ABSTRACT

The sea ice microbial community plays a key role in the productivity of the Southern Ocean. Exopolysaccharide (EPS) is a major component of the exopolymer secreted by many marine bacteria to enhance survival and is abundant in sea ice brine channels, but little is known about its function there.

This study investigated the effects of temperature on EPS production in batch culture by CAM025, a marine bacterium isolated from sea ice sampled from the Southern Ocean. Previous studies have shown that CAM025 is a member of the genus *Pseudoalteromonas*, and therefore belongs to a group found to be abundant in sea ice by culture dependent and independent techniques. Batch cultures were grown at -2^{o}C, 10^{o}C and 20^{o}C and cell number, optical density, pH, glucose concentration and viscosity were monitored. The yield of EPS at -2^{o}C and 10^{o}C was 30 times higher than at 20^{o}C, which is the optimum growth temperature for many psychrotolerant strains. EPS may have a cryoprotective role in brine channels of sea ice, where extremes of high salinity and low temperature impose pressures on microbial growth and survival. EPS produced at -2^{o}C and 10^{o}C had a higher uronic acid content than that produced at 20^{o}C. The availability of iron as a trace metal is of critical importance in the Southern Ocean where it is known to limit primary production. EPS from strain CAM025 is polyanionic and may bind dissolved cations such at trace metals therefore the role of bacterial EPS in the Antarctic marine environment may have important ecological implications.

KEY WORDS

Exopolysaccharides, exopolymer, Antarctica, marine bacteria, sea ice, psychrotolerant, EPS yield

INTRODUCTION

Sea ice is a major component of polar regions, covers millions of km^2 even at the end of the polar summer (21), and provides a home to unique communities dominated by microorganisms (35). Sea ice begins to form as the temperature of seawater, with a salinity of 3.5% drops to –1.8^oC. As ice develops, a salting-out process occurs in which the marine salts excluded from the ice are concentrated in brine pockets. Brine channels develop as the ice rises above sea level. The dense brine drains through the layer of columnar congelation ice by gravity and flows to the underlying seawater (7). The sea ice produces highly variable microenvironments in terms of temperature, salinity, nutrient concentration and light intensities within the columns, which may be as thick as 2 m. Salinity in sea ice brine can range from near that of freshwater to >15% at the ice-sea water interface (35). Temperatures can range from 0 ^oC to –35^oC. These factors contribute to sea-ice being one of the coldest habitats on earth for marine life (22).

In spite of these extremes, up to 20-30% of primary production in sea ice cycles through heterotrophic bacteria (24, 30). Complex microbial communities, dominated by diatoms in close association with bacteria (30), are concentrated in the lower 10-20 cm of sea ice where nutrients are available from the sea water and light is available from the surface (35). Abundant bacterial populations have been found in thick annual pack ice with psychrophilic bacteria being particularly common in samples of pore waters and ice colored brown by the presence of algal pigments (15). During ice formation microalgal cells are scavenged by sea-

ice crystals floating up to the sea surface (17), and bacteria attached to algal cells may be incorporated into new ice in conjunction with some algal species (18). Bacterially-produced EPS may provide a means by which bacteria can adhere to the microalgal cells (36). Studies of both the Arctic (26) and Antarctic (36) sea ice communities suggest that exopolymer production by both phytoplankton and bacteria make a significant contribution to organic carbon in the sea-ice and ice-water interfaces.

Little is known about EPS produced by Antarctic marine bacteria or about its role in this extreme environment. Sea ice bacteria maintained in laboratory culture can secrete copious amounts of mucus (20, 28). The sea ice isolate CAM025, examined in previous studies belongs to the genus *Pseudoalteromonas* and class "*Gammaproteobacteria*" (28). Studies of polar sea ice communities using cultivation dependent and independent techniques have shown that the "*Gammaproteobacteria*" are among the dominant taxonomic groupings (6, 8, 10, 35). CAM025 exhibits growth in the temperature range -2^{o}C to 30^{o}C, on media containing 1% to 12% (w/v) sea salts and exhibits an enhanced mucoid morphology on marine media with added glucose (29).

When grown in batch culture at temperatures near the predicted optimum for this strain, CAM025 produced exopolymer and chemical analyses showed the purified EPS was composed primarily of neutral sugars (glucose, arabinose, fucose and galactose), uronic acids (glucuronic acid) and sulfates (29). Sulfates carry a net negative charge at seawater pH (27); uronic acids also contain an acidic carboxyl group that is ionisable in these conditions. The presence of these

two groups (uronic acids and sulfates) is known to result in an EPS with a polyanionic quality (12). Due to these chemical characteristics, microbial exopolymer and EPS like those produced by CAM025 have been shown to accumulate cations such as metals (4, 9). The ecological role of EPS is, therefore directly related to its chemistry. The frequency and type of functional groups present in the EPS impact on the tertiary structure and over-all physicochemical characteristic of the polymer in the surrounding aqueous environment (12).

EPS of strain CAM025 had a molecular weight of 5.7×10^6 Daltons, which is high relative to EPS produced by many other marine bacteria ($1 – 3 \times 10^5$ Daltons, 12) but similar to molecular weights of EPS produced by bacteria from deep-sea hydrothermal vents (19). The physical, rheological and chemical properties of the structure and properties of EPS are influenced by the length of the polymer chain, that is, the molecular weight (11). As the length of the polymer increases, there is a greater opportunity for complex entanglement of polymer chains and intramolecular associations, and these contribute to the tertiary structure and physical behavior of the polymer (37). A fungal strain, *Phoma herbarum,* isolated from Antarctic soil produced a homosaccharide of glucose with a molecular weight of 7.4×10^6 Daltons (33).The authors suggested the fungal EPS could provide a cryoprotective role in the harsh Antarctic environment where the availability of liquid water and temperatures are extremely low.

Exopolymer produced by CAM025 in laboratory cultures incubated at 20°C, has provided some insight into the possible ecological role for these biopolymers (29). Although 20°C is near the predicted optimum for growth of this psychrotolerant strain, it is much higher than that of the natural environment from which this strain was isolated. This study was undertaken to investigate the effects of a range of temperatures on the production of exopolymer and purified EPS by sea ice isolate *Pseudoalteromonas* strain CAM025.

MATERIALS AND METHODS

Batch cultures at different temperatures

The CAM025 was grown in duplicate broth cultures at -2°C, 10°C and 20°C. Culture broth was composed of 1 g yeast extract, Oxoid L21; 5 g bacteriological peptone, Oxoid L37; 32 g artificial sea salts, Sigma S9883; 4.76 g HEPES, Sigma H7637; and 1000 ml distilled water. The pH of the broth was adjusted to 8 prior to autoclaving. A glucose solution was prepared and autoclaved separately before being combined with the above media for a final concentration of 3% glucose (w/v). One litre baffled flasks containing 220 ml of the above media were inoculated with 20 ml of the exponentially growing batch cultures of the bacterial isolate. Approximately 5 ml of the broth culture was removed aseptically from each of the flasks to sterile McCartney bottles for pH, optical density (600 nm), viable cell counts, viscosity and glucose measurements (described below) at inoculation and then once daily through the experiment. Flasks were incubated in oscillating water baths (Ratek Pty Ltd, Australia) fitted

with refrigeration units that cooled and circulated antifreeze liquid at the desired temperature. All incubations took place in a room maintained at 20^{o}C. Batch cultures at -2^{o}C, 10^{o}C were harvested after 2 weeks incubation. The cultures at 20^{o}C were harvested after 1 week.

Enumeration

Optical density of culture material was measure at 600 nm at 24 hr intervals using a Smart Spec 3000 spectrophotometer (Bio-Rad, Regent Park, NSW, Australia). Aliquots (1 ml) of culture media were removed from flasks at 48 hr intervals, serially diluted in sterile artificial sea salts solution (3.2% w/v in distilled water) and aliquots (150 µl) were spread onto replicate agar plates (Marine agar with 3% (w/v) added glucose; MA+Glu) with a Spiral Biotech automated plater (Bethesda, USA). Cultures were incubated at 12^{o}C for 4 days before colonies were enumerated for viable cell count determination. The pH of culture material at 20^{o}C was measured at 24 hr intervals with an Orion pH electrode (Boston, USA)

Chemical and physicochemical measurements

The viscosity of a one ml aliquot of the culture broth, warmed to room temperature (20^{o}C), was measured daily with a Brookfield LVT microviscometer fitted with a cone and plate assembly. (Middleboro, MA, USA). One ml aliquots of culture broth, removed every 24 hr, were centrifuged at 15,000 g for 10 min (Eppendorf, Hamburg, Germany). The supernatant was removed and frozen. Glucose was quantified using a Boehringer Mannheim kit (Darmstadt, Germany).

Isolation, purification and characterization of CAM025 EPS

Culture broth was centrifuged at 30,000 g for 2 hr at 4^{o}C (Beckman Coulter, Pasadena, USA). The cell pellets were freeze-dried and weighed. Culture broth was filtered and exopolymer was purified by filtration and dialysis and then freeze-dried and weighed. Uronic acid, protein and total neutral carbohydrate content was determined and analysis of EPS monosaccharides was performed according to procedures described in detail (29).

Statistical treatment of data.

Univariate analysis of variance was performed on data gathered from measurements of cell yield, EPS yield, glucose consumption (initial glucose concentration minus final glucose concentration; $[Glu]_{init} - [Glu]_{final}$), crude chemical analysis and monosaccharide analyses of EPS. The statistical analysis was carried out using General Linear Model package of SPSS (34).

RESULTS

Growth of CAM025 in batch cultures

Viable cell counts showed that the highest rate of exponential growth occurred in cultures incubated at 10 and 20^{o}C in the first 24 hr (Figure 1). Cultures at -2^{o}C reached a maximum viable cell count by day 5 of incubation. The final optical density reading (600 nm) for each culture was used as an indication of the cell yield. The cell yield was significantly different between cultures incubated at -2^{o}C and 10^{o}C (Table 1).

Viscosity of culture media from 20°C incubations reached a maximum of 1.7 milliPascal-seconds (mPa·s) after 72 hr (Figure 2). A decrease on day four was followed by no change on the subsequent three days. Viscosity of cultures at 10°C increased gradually to a maximum of 2.7 mPa·s on day 14 and then decreased slightly. Cultures incubated at -2°C showed the greatest increase in viscosity, reaching a maximum of 3.4 mPa·s after 11 days.

The pH of cultures at 10°C decreased 0.7 pH units within the first 24 hr of incubation then decreased gradually until day 15 (Figure 3). The pH of cultures incubated at -2°C decreased gradually over the first six days and then gradually increased until day 13. The pH of 20°C cultures decreased 1.5 pH units by day three and after a slight increase on day four and five, decreased over the subsequent two days. This decrease in pH was occurring without increase in viscosity. The -2°C and 10°C cultures, in comparison, showed increases in viscosity and smaller decreases in pH (Figure 3).

The concentration of glucose in culture media was measured throughout the experiment (Figure 4). The difference in glucose concentration in the batch cultures between the start and end of the incubation was calculated to represent the amount of glucose consumed at each temperature. Maximum glucose consumption occurred in cultures incubated at 10°C, where the final glucose concentration was 80% lower than at the start of the experiment. In contrast, a glucose consumption of 15% occurred in cultures incubated at -2°C. The difference in values representing glucose consumption at these two incubation temperatures was significant (Table 1).

Yield and characterization of EPS

Yield of purified EPS at each temperature is expressed as mg EPS per g dry weight of cell material. The yield of EPS at -2°C and 10°C was approximately 30 fold higher than at 20°C (Table 1). The crude chemical composition of EPS produced by replicate cultures incubated at three temperatures is shown as a percentage of the total (Figure 5). Differences in total neutral sugar and protein content in the EPS produced by cultures at the three incubation temperatures were not significant. In contrast, uronic acid content in EPS expressed as percentage of total EPS produced at -2°C and 10°C was significantly different ($p < 0.05$) from and higher than at 20°C. Analysis of individual monosaccharides in EPS harvested from duplicate cultures incubated at -2°C, 10°C and 20°C did not reveal any significant differences in the percentages of arabinose, ribose and fucose. Percentages of mannose and glucose were higher in cultures incubated at -2°C than at 10°C or at 20°C, whereas percentages of galactose and rhamnose were lower at -2°C than at 10°C or at 20°C. Percentages of galacturonic acid were lowest in cultures incubated at 20°C and highest in those incubated at –2°C (Table 2).

DISCUSSION

Previous studies reported that the sea ice bacterium *Pseudoaltermonas* strain CAM025 exhibited growth in the temperature range -2 to 30°C but no growth occurred on solid media at 37°C (29). Other closely related *Pseudoalteromonas* species were shown to be psychrotolerant, that is, able to grow at 4°C and with a growth temperature optimum of approximately 22-25°C

(5). Psychrotolerant bacteria appear to be common in both sea ice and in underlying water (10, 14, 20) with *Pseudoalteromonas* strains being the most frequently isolated within this group (6).

In 10^{o}C cultures, exponential growth phase occurred in the first 24 hours and was followed by a stationary phase in which viscosity increased. Cultures grown at 10^{o}C also showed maximum optical density readings, highest cell yield, yield of EPS and glucose utilization compared to cultures at -2^{o}C and 20^{o}C (Table 1). A high yield of EPS accompanied by high cell yield appears to indicate balanced growth was occurring at this temperature compared to -2^{o}C and 20^{o}C. Maximum viscosity was reached during stationary phase in all cultures, with cultures growing at -2^{o}C and 10^{o}C showing the highest viscosity.

In batch culture studies of deep-sea hydrothermal vent isolate, HYD 1545, EPS production began in late exponential phase and continued during stationary phase (38). Deep-sea hydrothermal vent strain *Alteromonas* sp. strain 1644 produced an EPS at the beginning of stationary phase and this suggested that synthesis was induced by restricted growth conditions (31). In the current study, cultures of CAM025 incubated at 20^{o}C, pH decreased by 1.7 units while viscosity remained low during early stationary phase. EPS production at 20^{o}C seems to be restricted compared to production of EPS in cultures incubated at -2^{o}C and 10^{o}C.

In growth experiments with *Lactobacillus sakei* strain 0-1, low temperature combined with glucose as a carbohydrate source enhanced EPS production (13). In the observed growth temperature range of 15 - 42^{o}C, 15 and 25^{o}C were

the best for bacterial growth and EPS production for this mesophile. Specific production of EPS decreased with increasing temperature. Depletion of glucose indicated the C/N ratio of the media was optimal (13). In the current study, glucose was depleted by 80% in cultures incubated at 10^{o}C, and EPS and cell yield were the highest at this temperature. Cultures at -2^{o}C showed the lowest glucose consumption and cell yield. At this temperature, CAM025 produced a high yield of EPS (Table 1). At -2^{o}C, CAM025 may have had more energy to produce EPS as less was being used for production of cellular components than at higher incubation temperatures.

EPS production occurred during stationary phase as demonstrated by increasing viscosity after exponential growth had ceased. In addition, increased EPS yield at -2^{o}C and 10^{o}C indicated EPS production was occurring in these cultures at suboptimal temperatures. Optical density measurements for strain CAM025 indicated a higher cell yield for cultures incubated at 10^{o}C compared to those incubated at 20^{o}C. Previous studies of sea ice isolates in the genus *Pseudoalteromonas* and closely related to CAM025 suggested that the optimum temperature for growth would be in the 20^{o}C – 22^{o}C range (5). Further work is necessary before the precise optimal temperatures for cell growth and EPS production are known for strain CAM025.

In continuous fermentation studies with the mesophile *Pseudomonas* NCIBI 11264, EPS production was influenced by media composition, temperature, pH and the growth rate of the organism. However, the polysaccharide varied little in overall composition irrespective of pH,

temperature, nitrogen, carbon and phosphate content of the growth medium (39). Culture conditions generally did not affect the types of monosaccharides in an EPS produced by halophilic bacterium *Halomonas maura* (2). Crude chemical analysis of EPS produced by CAM025 showed that there was a higher percentage of uronic acids in the EPS produced at -2^{o}C and 10^{o}C than at 20^{o}C, while neutral sugar content did not vary significantly (Figure 5). Further analyses of individual monosaccharide also showed the highest percentage of galacturonic acid in EPS produced by cultures incubated at –2^{o}C relative to cultures incubated at 10^{o}C or at 20^{o}C. This coincided with a higher total neutral monosaccharide content at higher growth temperatures. Further study is required to more thoroughly assess the effect of growth temperature on individual monosaccharide content.

During each Austral summer, approximately four fifths of the sea ice surrounding Antarctica melts (41), releasing dissolved and particulate material, living cells and aggregations of cells into the surrounding waters (7). Arctic sea ice studies (25, 26) demonstrated that microbially produced neutrally buoyant EPS was carried large distances by prevailing under-ice currents and ice drifts. Studies in more temperate waters showed marine bacterial EPS production played a major role in the aggregate formation (12, 3). When released into the water column, a combination of biological, chemical and physical forces caused this colloidal material to form aggregates (1), which became centers of high heterotrophic microbiological activity (23). The availability of iron as a trace metal is of critical importance in the Southern Ocean where it is know to limit primary

production (32). Since 99% of dissolved iron in the ocean is bound to organic ligands (40), polyanionic EPS such as those produced by CAM025 may have an important role in the Antarctic marine environment. Microbial EPS potentially act to keep iron in solution and accessible for primary productivity (16) or as the framework of sinking aggregates, transporting bound iron out of the euphotic zone (40).

CONCLUSIONS

CAM025 is a halotolerant, psychrotolerant sea ice isolate, belonging to the genus *Pseudoalteromonas*, which is known to abound in the sea ice microbial community. The enhanced production of a high molecular weight polyanionic EPS at sub-optimal incubation temperatures lends support to theories that EPS may have a cryoprotective role in the high salinity, low temperature habitat of sea ice brine channels. The importance of the role of EPS in sea ice as well as in the Southern Ocean, where the availability of trace nutrients such as iron limits primary production and CO_2 sequestration, requires further attention.

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FIGURE LEGENDS

Figure 1 Viable cell count for batch cultures of sea ice isolate CAM025, incubated at -2°C (♦) 10°C (■) and 20°C (▲).

Figure 2 Viscosity for batch cultures of sea ice isolate CAM025, incubated at -2°C (♦), 10°C (■) and 20°C (▲).

Figure 3 pH for batch cultures of sea ice isolate CAM025, incubated at -2°C (♦), 10°C (■) and 20°C (▲).

Figure 4 Glucose concentration for batch cultures of sea ice isolate CAM025, incubated at -2°C (♦), 10°C (■) and 20°C (▲).

Figure 5 Crude chemical analysis of EPS produced by sea ice isolate CAM025 produced in batch cultures incubated at -2°C, 10°C and 20°C, reported as percentage of total EPS. Pairs of symbols * and # denote significant ($p < 0.05$) differences in percentages.

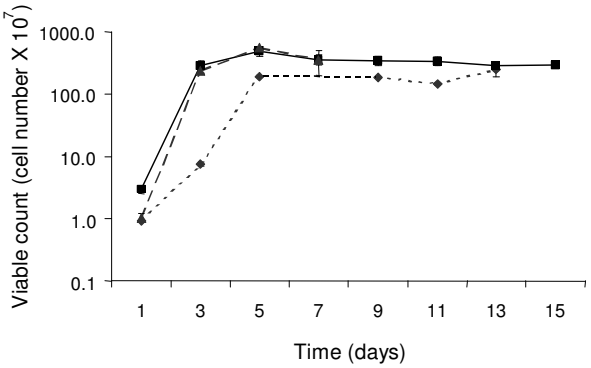
Viable count (cell number X 10^7)
1000.0
100.0
10.0
1.0
0.1
1
3
5
7
9
11
13
15
Time (days)

Figure 1

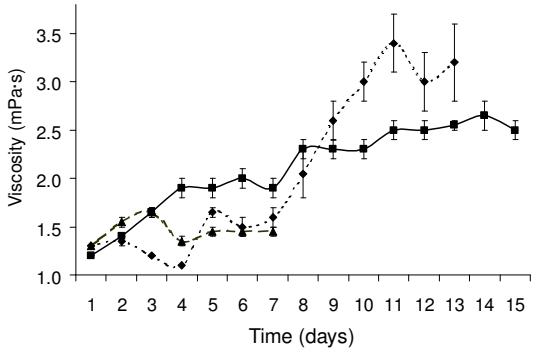
Viscosity (mPa·s)
3.5
3.0
2.5
2.0
1.5
1.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Time (days)

Figure 2

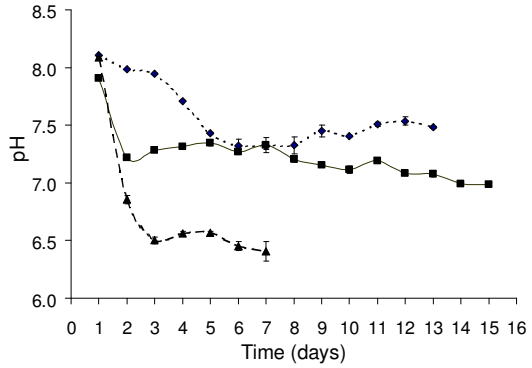

Figure 3

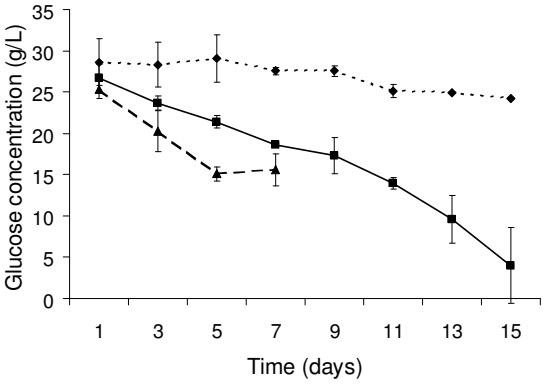
Glucose concentration (g/L)
35
30
25
20
15
10
5
0
1
3
5
7
9
11
13
15
Time (days)

Figure 4

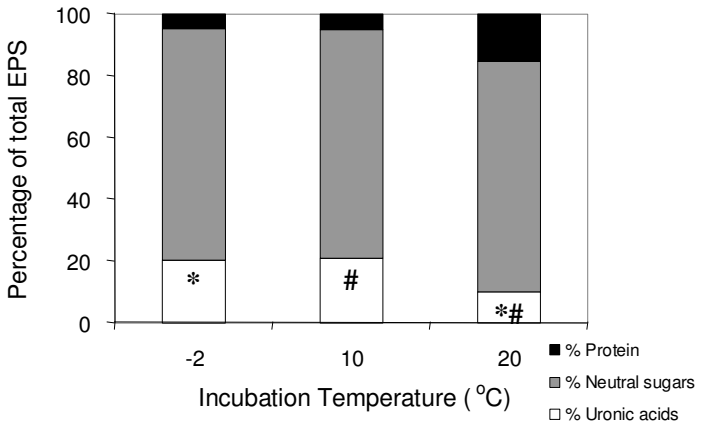
100
80
60
40
20
0
Percentage of total EPS
*
#
*#
-2
10
20
Incubation Temperature (°C)
% Protein
% Neutral sugars
% Uronic acids

Figure 5

TABLE 1. Growth parameters of sea ice bacterium *Pseudoalteromonas* strain CAM025 grown in batch cultures

Parameter	Units	Incubation temperature					
		-2°C		10°C		20°C	
		Ave	SE (n=2)	Ave	SE (n=2)	Ave	SE (n=2)
Cell yield	Final Absorbance (600 nm)	4.68[a]	0.280	10.13[a]	0.390	7.705	0.965
EPS yield	mg/g dry weight cells	97.2[b]	9.3	99.9[c]	8.0	3.6[b,c]	0.2
Glucose consumption $[Glu]_{initial}$ - $[Glu]_{final}$	g/L	4.10[d]	2.5	22.8[d]	2.9	9.7	0.8

[a] Significant difference ($p \leq 0.071$), [b] ($p \leq 0.024$), [c] ($p \leq 0.024$), [d] ($p \leq 0.076$)

TABLE 2. Monosaccharide composition of EPS from duplicate cultures incubated at three temperatures. Data expressed as percentage of total[a].

	Incubation temperature		
Monosaccharides	-2°C	10°C	20°C
Arabinose	3.1 (1.0)	3.7 (1.9)	11.3 (2.5)
Ribose	1.2 (0.9)	1.6 (0.2)	0.0 (0.0)
Rhamnose	4.8 (0.5)[b,c]	18.8 (3.1)[b]	25.9 (5.8)[c]
Fucose	1.6 (0.4)	7.7 (0.4)	6.9 (2.9)
Galacturonic Acid	15.8 (0.4)[e,f]	8.1 (1.0)[e,g]	0.2 (0.2)[f,g]
Mannose	16.5 (0.7)[h,j]	9.6 (1.5)[h]	8.9 (0.8)[j]
Galactose	5.7 (0.3)[k,l]	19.7 (3.2)[k]	23.6 (2.0)l
Glucose	51.3 (1.8)[m,n]	30.9 (5.4)[m]	23.3 (9.2)[n]

[a] Standard errors of the means are given in parentheses.

[b-n] Pairs of letters denote significant differences ($p < 0.05$) betweens percentages of EPS monosaccharides from duplicate cultures.