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Effect of temperature on extracellular enzymes occurring in permanently cold marine environments*

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Introduction

More than 80 % of the earth's biosphere is permanently cold with temperatures below 5 °C (BAROSS and MORITA 1978), nevertheless, our knowledge of degradation processes and extracellular enzymatic activities in the cryosphere is limited. Cold marine ecosystems are locally and temporarily confronted with high amounts of particulate matter, introduced for example by algal blooms, krill swarms or sea ice melting. In spite of the generally activity restricting low temperatures, the decomposition of this input can obviously be accomplished adequately to its production. Bacterial floras inhabiting bottom sediment as well as sea ice of the polar oceans are found for the most part to be well adapted to cold conditions with respect to growth (not yet published). In the present work extracellular enzymes of psychrophilic bacteria (the term psychrophilic is used after the definition of MORITA 1975) and of field samples of the cryosphere are studied for their adaptation to cold temperatures.

Material and methods

Organisms and cultural conditions

The bacteria used in this study were isolated from the upper layer of marine sediments, from gut or surface material of marine animals, and from sea ice sampled during the "Polarstern" cruises ANT II/3, ANT III/2, ANT V/2 and ARK III/3, during cruise no. 56 of R.V. "Meteor", during cruises no. 196 and 200 of R.V. "Anton Dohrn", and during cruise no. 4/85 of R.V. "Victor Hensen".

Viable counts were determined at 1 and 20 °C on chitin agar (WEYLAND et al. 1970) by means of the spread plate technique. Temperature optima and maxima of the bacterial isolates were determined in liquid ZoBell medium 2216. Degradation of starch, casein, and gelatin was tested after WEYLAND et al. 1970.

To obtain extracellular enzymes necessary for activity tests, the bacterial strains were grown at 3 °C (psychrophiles) or 18 °C (mesophiles) in ZoBell medium 2216 with the organic nutrient concentration reduced to a third but supple-

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mented with casein-Hammarsten (Merck, Darmstadt) (final concentration 0.01 %) for protease production or with starch (final concentration 0.5 %) for amylase formation. At the end of the logarithmic growth phase the cells were separated from the culture medium by centrifugation at 11,000 x g for 20 min. at 4 °C. The supernatants were used for the enzyme assays.

Enzyme assays

The decomposition rates caused by crude bacterial proteases and α-amylases respectively were followed using the particulate model substances Azocoll and Amylopectin Azure (Calbiochem, Frankfurt) according to the method of KIM and ZOBELL 1974 as well as by means of the dissolved fluorogenic substrate analogues L-leucine-4-methylcoumarinyl-7-amide HCL, (leu-MCA), (Serva, Heidelberg) and methylumbelliferyl-α-D-glucopyranoside (Sigma, München) according to HOPPE 1983. The protease activity of natural samples was examined with the dissolved substrate analogue leu-MCA only.

The following natural samples were studied: brackish surface water sampled from the estuary of the river "Weser", sea ice of the Southern Ocean collected at 63°28.22'S; 54°16.63'W and at 65°36.20'S; 03°03.28'W, sediment sampled from the Southern Ocean (60°46.6'S; 45°47.9'W), Northern Ocean (79°59.94'N; 07°30.92'E), and the German Bight (53°38.05'N; 08°26.56'E). The enzyme assays were carried out using 4 different substrate concentrations at each temperature. Each test contained 15 ml of the natural sample or in the case of sediment a suspension of 2 ml sediment per 100 ml artificial seawater. Fluorescence measurements were conducted after HOPPE 1983. Sediment suspensions were centrifuged at 8,000 x g for 2 min. prior to buffer addition and fluorescence determination.

Results and discussion

Amylases and proteases of 35 psychrophilic and 13 mesophilic bacteria were examined. From these results only some representative temperature-activity data are demonstrated in Fig. 1 as Arrhenius plots. Although the psychrophilic organisms studied have maximum growth temperatures not exceeding 20 °C their extracellular enzymes functioned with the particulate model substrates (Fig. 1a and b) optimally in the temperature range of 30 to 50 °C and maximum temperatures were in the range of 40 to 60 °C. However, in comparison to the enzymes of the mesophiles, optimum and maximum temperatures were about 10 to 20 degrees lower for the psychrophiles. Furthermore, at 10 °C the amylases from the psychrophiles achieved about 37 % of their maximal activity compared to only 7 % for those of the mesophiles, and the proteases showed 3 % and 0.2 % of maximum activity for the psychrophiles and mesophiles, respectively. A further indication of an adaptation to low temperature of the psychrophiles is the smaller slope of the linear descending section of their Arrhenius curves.

In comparison to the particulate model substrates (Azocoll and Amylopectin Azure), the reactions with the natural products casein and starch were quite similar (results not shown). Deviating results were obtained with dissolved fluorogenic model substrates (Fig. 1c and d). Most of the proteolytic supernatants of the psychrophilic bacteria hydrolyzing Azocoll and casein, did react with leu-MCA at slow rates only and with this substrate the temperature optima shifted clearly to higher temperatures (Fig. 1c). However, similar to the particulate substrates, the activity in the low temperature range related to the maximal

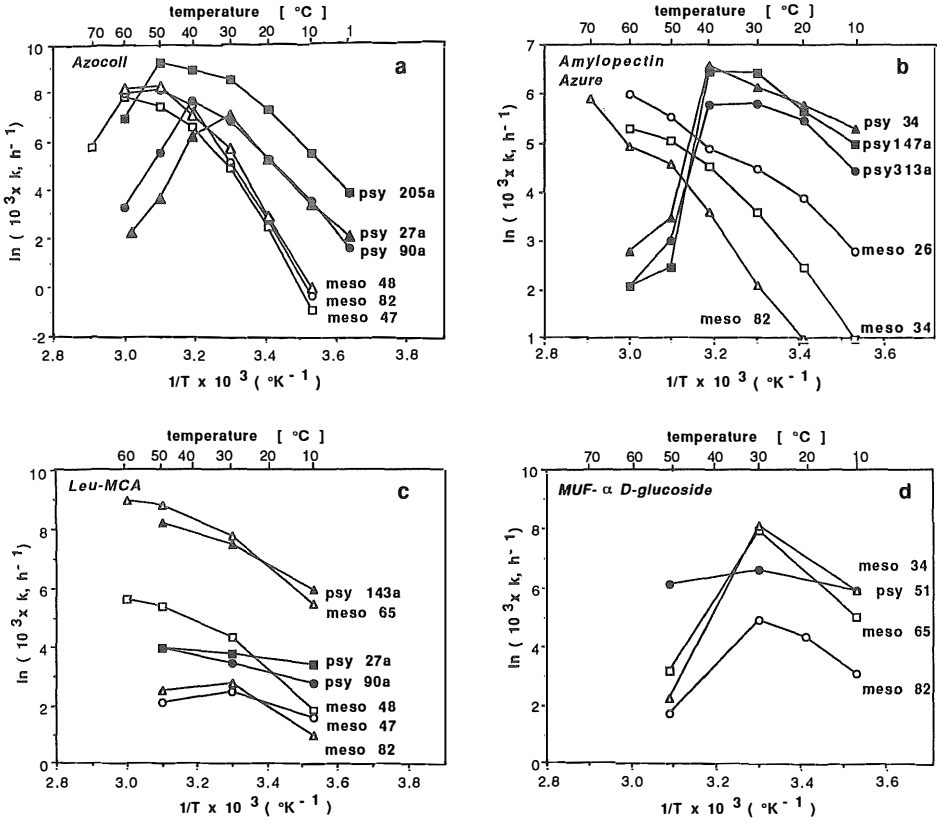

Fig. 1. Effect of temperature on the activity of crude proteases (a, c) and amylases (b, d) of different psychrophilic and mesophilic bacteria assayed by means of the particulate model substrate Azocoll (a) and Amylopectin Azure (b) or the dissolved methylumbelliferyl-derivatives leu-MCA (c) and MUF-α-D-glucopyranoside (d) (final concentration of the MUF-substrates 8 µmol l^{-1}).

activity was rather high and the Arrhenius-slopes were less than those of the proteases of the mesophiles.

Similarly, the results obtained with MUF-α-gluc. were not analogous to those obtained with Amylopectin Azure and starch. Most of the amylolytic supernatants of the psychrophiles were unable to hydrolyze MUF-α-gluc. and the enzymes of the mesophiles exhibited surprisingly low optimal temperatures (Fig. 1d).

The deviating results between the enzyme assays performed with methylumbelliferyl-derivatives and particulate substrates suggested an examination of an extensive collection of bacterial strains for their ability to decompose the substrates in question (Tab. 1). Leu-MCA hydrolysis was almost a universal feature of the strains tested, even if rates were sometimes low. In contrast casein degradation was accomplished by only 27-38 % of the strains and gelatin decomposi-

Table 1. Comparison between the decomposition of the particulate substrates casein, gelatin, and starch and the dissolved model substrates leu-MCA and MUF-α-gluc. by a collection of bacterial isolates from the polar oceans. The degradation of the particulate substrates was tested at 1 °C after WEYLAND et al. 1970. The hydrolysis of the MUF-substrates were determined with the supernatant of liquid cultures grown at 1 °C and applied to the substrates (final concentration 8 µmol l^{-1}) at room temperature.

substrate hydrolyzed	% bacterial strains isolated from sediment		sea ice	
	Northern Ocean n=280	Southern Ocean n=263	Northern Ocean n=116	Southern Ocean n=108
leu-MCA	90	93	78	71
casein	27	37	31	38
gelatin	38	67	n.a.	n.a.
both, leu-MCA and casein	25	36	27	24
			n=150	n=67
MUF-α-gluc.	41	27	33	4
starch	41	44	37	29
both, MUF-α-gluc. and starch	24	20	16	3

n.a. = not applied

tion by only 38 % of the strains from the Northern Ocean and 67 % from the Southern Ocean. MUF-α-gluc. and starch were hydrolyzed by similar percentages of the isolates from the Northern Ocean but only about half of these strains were able to hydrolyze both. Considering the strains of the Southern Ocean the discrepancies between the portions of strains hydrolyzing starch and MUF-α-gluc. are even more pronounced.

In comparison to the extracellular enzymes of bacterial pure cultures, proteolytic enzymes of sea ice and bottom sediment samples of the Northern and Southern Ocean were studied along with material from temperate habitats. Predominance of psychrophilic bacteria in the culturable parts of the sea ice and sediment floras was proven by means of viable counts at 1 and 20 °C (Tab. 2) and by temperature growth investigations conducted with a representative collection of sea ice and sediment isolates (Fig. 2B and Fig. 3B).

In spite of the different bacterial floras of the sediments tested (Tab. 2 and Fig. 2B) the extracellular enzymes of the different samples responded quite similar to temperature using leu-MCA (Fig. 2A). An adaptation to low temperatures might only be indicated by the higher V_{max} at 1 °C of the sediment from the Southern Ocean (Fig. 2A). The results obtained with sea ice and water samples (Fig. 3A) deviated considerably from those of the sediment samples (Fig. 2A). The proteases of the sea ice and water samples exhibited remarkably low temperature optima and maxima lower than those of psychrophilic pure cultures. On the other hand the responses of the enzymes of the water sample originating from the temperate region (Fig. 3Ac), however, resembled those of at least one

Table 2. Comparison of viable counts determined at 1 and 20 °C of sediments from different oceanic regions.

sediment sample of	viable counts determined at 1 °C (x 10^4 ml^{-1})	20 °C	proportion 1 °C/20 °C
Southern Ocean	78	2	38
Northern Ocean	196	24	8
German Bight (winter season)	55	212	0,2

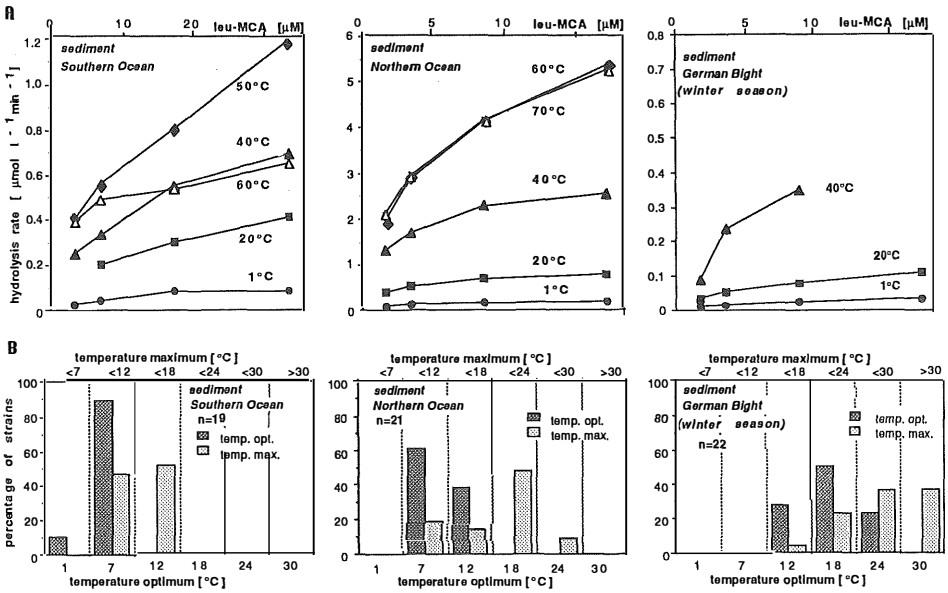

Fig. 2. Effect of temperature on the extracellular proteases of sediment samples from different oceanic regions (A). Growth response to temperature of a representative collection of bacterial strains isolated from the specific sediment samples (B).

sea ice sample (Fig. 3Ab). A relatively high V_{max} at 1 °C was found with the other sea ice sample (Fig. 3Aa).

To our knowledge only few studies are related to the effect of temperature on extracellular enzymes from psychrophilic bacteria or natural samples from cold habitats. A purified protease as well as few phosphatases and chitinases of psychrophiles had been analyzed (KATO et al. 1972, MORITA 1975, KOBORI et al. 1984, HELMKE and WEYLAND 1986). In the present study a series of crude amylases and proteases was investigated and it could be shown that adaptation to cold temperatures was realized to a certain extent, indicated by lower temperature optima and maxima and especially by high reaction rates in the low tem-

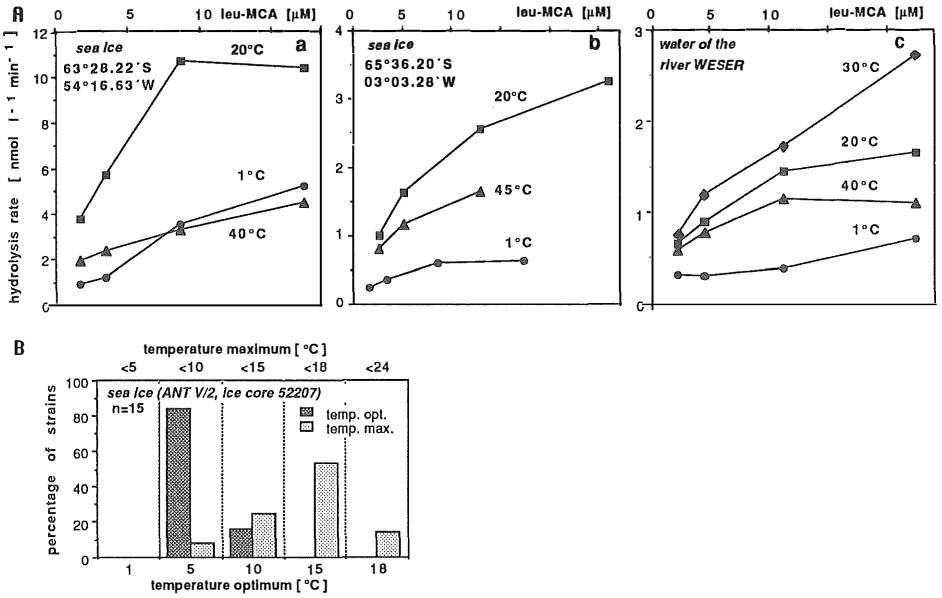

Fig. 3. Effect of temperature on the extracellular proteases of Antarctic sea ice and of brackish water from temperate region (A). Growth response to temperature of a representative collection of bacterial strains isolated from a densely populated ice core (B).

perature range compared to responses of enzymes of mesophilic bacteria. However, the adaptation to cold temperatures is clearly less pronounced with respect to extracellular enzymatic activity than with respect to growth.

The response to temperature of extracellular enzymes was recorded using particulate substrates as well as dissolved MUF-derivatives. Although an adaptation in the low temperature range was obvious with both types of substrate, the responses with respect to temperature optima and maxima deviated clearly, indicating that apparently different enzymes were recorded depending on the type of substrate used. A screening test on the decomposition of substrates conducted with more than 700 marine polar isolates supported the finding that MUF-substrates are inappropriate substrate analogues for the polymers tested in studies with psychrophilic bacterial floras. These results contrast with experiments conducted with bacterial isolates from the Baltic Sea and from the Atlantic (KIM and HOPPE 1984) suggesting further studies on the suitability of MUF-derivatives as model substrates. In this context it is worth mentioning that we found with protease activity measurements using leu-MCA four of ten Antarctic sediment samples not obeying Michaelis-Menten kinetics (unpublished data). No tests were performed if this was due to specific reactions of the psychrophilic flora, to a competitive inhibition effect of natural occurring substrates or to sediment properties (chelating agents, adsorption, chemical denaturation), however, the phenomenon indicates that activity measurements using substrate analogues in natural samples have to be interpreted very cautiously. A specific temperature adaptation to the cold habitat became not obvious when using water or sediment

of different environments as test samples in a comparative study. This may be due on the one hand to leu-MCA and on the other hand to interfering factors in complex systems like sediment which may mask a typical characteristic.

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