

Figure 5. EDXA spectrum of Mo-Si multilayer.

precipitates was calculated by taking a dark field image (Figure 4a) and was found to be 50–60 nm. The high-resolution micrograph (Figure 4b) shows the lattice fringes with separation of 6.8 Å. The lattice spacing is found to be matching with (110) of Mo_5Si_3 phase. Some regions marked with 'm' show the moiré fringes originating due to the presence of two different lattice spacings, one on the top of the other. These types of moiré fringes are expected in these samples due to the presence of a number of interfaces, one on top of the other. The Mo_5Si_3 phase is stable up to 2180°C, with Si and Mo composition of 37% and 63% respectively. Calculations of Mo and Si composition from the thickness of the Mo and Si layers deposited, gives the same ratio as required to form the Mo_5Si_3 phase. This result was confirmed by energy dispersive X-ray analysis (EDXA; Figure 5). The cross-sectional studies on these multilayers are under investigation, and will be reported separately.

In the present investigation the Mo-Si multilayers were heated from room temperature to 750°C. The as-deposited film was found to be quite smooth. Above 400°C, the interfaces start diffusing into one another. We have observed the formation of Mo_5Si_3 crystalline phases at 750°C in these samples. The formation of Mo_5Si_3 phase was confirmed by high-resolution electron microscopy, selected area diffraction pattern and EDXA.

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Proteolytic heterotrophic bacteria of cyanobacterial assemblage from Schirmacher oasis, Antarctica, capable of growing under extreme conditions

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Physiologically diverse groups of bacteria were isolated from cyanobacterial assemblages of Schirmacher oasis Antarctica. Psychrotrophs have been the most dominant, with little population of thermo-tolerant and halo-tolerant bacteria. One-third of the isolates showed pigmentation, which has been temperature-dependent in thermo-tolerant strains. Almost half of the isolates produced extracellular proteases depending on temperature, pH or salt concentration. Isolates also produced other hydrolytic enzymes, e.g. urease, phosphatase, lipase, etc. Two isolates showed multiple antibiotic resistance; however one halo-tolerant has been sensitive to all the antibiotics tested. Isolates preferred different protein and carbohydrate sources for growth, and in most of the cases protease was maximally induced in the presence of glucose/lactose. The effect of temperature and pH on growth, and enzyme production and activity have also been studied.

PROKARYOTES dominate the Antarctic ecosystems and play a major role in food chains, biogeochemical cycles and mineralization of pollutants. Recent investigations using modern molecular biology techniques as well as classical procedures, have demonstrated that many of the Antarctic microorganisms reported to date, represent new species and broad phylogenetic diversity with representatives from the Archaea and Bacteria domain^{1–6}. Despite the extreme climatic conditions that persist in the icy

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continent of Antarctica, microorganisms have been detected in all the distinctive habitats such as lakes, ponds, rivers, streams, rocks and soil. These habitats differ from one another with respect to nutrients, range of temperature, water activity and other physico-chemical parameters. Since these factors influence the survival and growth of microorganisms, the microbial flora is bound to vary from one habitat to another. Free water is available only in oases during the summer, which experience seasonal variation during late fall and spring. The biology of these freshwater oases is therefore likely to be different from the rest of the continent. Microbiological studies in specific regions of continental Antarctica have been largely directed towards isolation and characterization of psychrotrophic microorganisms and reports of bacteria capable of growing under other extremes, are scanty though psychrophilic and salt-tolerant bacteria have been reported from Antarctic sea ice and hypersaline lakes⁷⁻⁹.

Schirmacher oasis of Antarctica harbours many cyanobacterial assemblages containing concentrated population of bacteria. The present investigation has been undertaken to study the diverse physiological groups of bacteria thriving on cyanobacterial mats of freshwater lakes. The isolates not only exhibited growth in a broad spectrum of cardinal temperatures, but some of them were also capable of growing under extremes of acidic and alkaline pH and high salt concentration. The extracellular hydrolytic enzymes of these diverse groups of bacteria are important for the survival of these organisms, and are of ecological and economic significance. They may provide novel biotechnological applications by combining more than one desirable enzyme characteristics, such as cold-active and alkali-tolerant proteases suitable for detergent formulations for cold wash.

Samples of blue-green algal mat from freshwater lakes of Schirmacher oasis, Antarctica were collected in sterilized polypropylene bags during the fourteenth Indian scientific expedition and stored at -20°C . Ten-fold serial dilutions of samples in sterilized, normal saline were surface-spread on ABM agar plates containing peptone, 5 g; yeast extract, 3 g; agar, 15 g and distilled water, 1000 ml. For isolation of halo-tolerants and psychrotrophs, pH of the medium was adjusted to 7.0, while for acid-tolerant and alkali-tolerant bacteria it was adjusted to 5.0 and 10.0 respectively. The ABM agar was supplemented with 10% NaCl for halo-tolerant bacteria. Plates were incubated at 20°C for two days, except those for psychrotrophs and thermo-tolerants, which were incubated at 10°C for two days and 55°C for one day respectively. Morphologically unique colonies were picked up and subjected to SDS-PAGE for total cell-protein profile by the method of Blackshear¹⁰. Isolates were monitored for colour and size of colony, and the cell morphology was studied under light microscope after Gram-staining. Motility of culture was determined by hanging-drop technique. The biochemical tests were performed in the

appropriate media by following the procedures of Holding and Collee¹¹, Stainer *et al.*¹² and Stolp and Gadhari¹³. The sensitivity of the cultures to different antibiotics was carried out using HiMedia Antibiotic Discs and by observing the zone of inhibition. The API microbial identification kit was also used for the tentative identification of the microbes.

Qualitative screening of protease-producing bacteria was carried out in ABM agar containing 0.4% casein. Purified colonies were streaked on the respective casein agar medium and incubated under described conditions. The zone of hydrolysis was observed by staining the plates with 0.04% Coomassie brilliant blue (in 40% methanol) for 15 min, followed by destaining with 40% methanol. Quantitative protease estimation was performed by the method of Arima and Tawasaki¹⁴.

Isolates were grown on casein agar medium under varying conditions of salt (0, 5 and 10% NaCl), temperature (5, 10, 20, 30, 37 and 55°C) and pH (5, 7 and 10). After incubation, they were monitored qualitatively for growth and zone of proteolysis. The ability of the microbes to grow in broth media with different carbon and nitrogen contents was evaluated by inoculating freshly-grown cultures in 100 ml of ABM, nutrient broth (NB), diluted nutrient broth (1:3) and casein broth (K_2HPO_4 , 2 g; KH_2PO_4 , 1.5 g; yeast extract, 2 g; ammonium sulphate 2 g; magnesium sulphate 0.15 g; calcium chloride, 0.015 g, and distilled water, 1000 ml). Growth ($\text{OD}_{600\text{ nm}}$) and protease activity were recorded at various phases of growth at specified temperature of incubation. Similarly, the effect of different inducers, viz. casein, gelatin, BSA, lactose and glucose for production of protease was observed by adding 0.4% of the inducers to selected broth. The optimum temperature, salt and pH for growth and enzyme production were monitored by growing the cultures in selected broth. Enzyme was partially purified from culture supernatant by ammonium sulphate fractionation (30–60%) and dialysis at 4°C . Enzyme activity was assayed at temperatures ranging from 5 to 55°C and pH ranging from 5.0 to 12.0 (in 0.1 M appropriate buffer). Respective blanks were used under each assay condition. To assess the acid and alkali stability of protease, 100 μl of enzyme was mixed with 0.2 N NaOH or 0.2 N HCl and kept overnight at 37°C prior to enzyme estimation. For thermal stability, the enzyme was kept at 60°C in a water bath and the activity was monitored at 30 min interval.

Studies from the two different samples from Schirmacher oasis showed that the cyanobacterial mats contain varying bacterial population, which are capable of growing under extreme conditions of temperature, salt and pH. The psychrotrophs along with the acid/alkali-tolerant groups were the predominant populations in the two samples (Figure 1). Interestingly, thermo-tolerant bacteria were also present in substantial numbers ($1-2 \times 10^2$ cfu/ml) in both the samples. The halo-tolerant bacteria were present only in one sample (BG1) and were

almost negligible in the other, when isolated under the selection pressure of 10% NaCl.

In all, twenty-seven unique bacteria belonging to different physiological groups were isolated, based on colony characteristics and SDS-PAGE protein profile of cell lysates. The morphological features and qualitative growth characteristics of the isolates are shown in Table 1. One-third of the isolates were pigmented, though no pigmentation was seen in any of the bacteria capable of thriving at low and high pH. Two thermo-tolerant strains (B2a and B2e) exhibited temperature-dependent variation in pigmentation, forming orange colonies on nutrient agar plates at 55°C but not at 37°C or below (data not shown). With the exception of one halo-tolerant strain (S1), all the isolates were rod-shaped and long chain formation was observed in both the thermo-tolerant bacteria. Isolates belonging to the halo-tolerant and thermo-tolerant groups were all Gram-positive and of the acid/alkali-tolerant group were Gram-negative, whereas the psychrotrophic population was a mixture of the two types. When the strains were qualitatively tested for their ability to grow at different temperatures in broth medium, a broad spectrum of cardinal temperatures was observed, representing stenopsychrotrophic (8), eupsychrotrophic (10), mesophilic (6) and slightly thermophilic (2) groups (data not shown).

In all, nine proteolytic isolates were selected based on their ability to grow under various extremes. Two of the halo-tolerant strains (S1 and S2) produced zones of proteolysis up to 5% NaCl and grew well at 10% salt concentration (Table 1). Among acid/alkali-tolerant isolates, four produced extracellular proteases as seen on casein

agar plate, among which P7 exhibited growth in a broad pH range (5–10). The remaining two isolates selected in this group, P5 and P8, had preference for low (5.0–7.0) and high (7.0–10.0) pH respectively, and were also capable of producing protease on their respective extremes. In the psychrotrophic group, four isolates produced extracellular protease among which two (T7 and T9) exhibited luxuriant growth $\leq 10^\circ\text{C}$. Only T7 produced the enzyme at 5°C , whereas both the isolates did not grow above 30°C . The two thermo-tolerant bacteria (B2a and B2e) were proteolytic and grew in the temperature range of 20 to 60°C .

The selected isolates were subjected to biochemical characterization and screened for production of other hydrolytic enzymes. All the nine cultures were oxidase and catalase positive among which S1 did not produce any other hydrolytic enzymes tested. The two alkali-tolerant isolates (P7 and P8) produced urease, which was also observed in the thermo-tolerant B2e. The two psychrotrophs produced lipase, apart from phosphatase by T7 and urease by T9. Amylase and β -galactosidase activity was seen in both the thermo-tolerant bacteria, and the latter was also produced by S2. The selected isolates were tested for resistance to 19 different antibiotics. All the isolates showed sensitivity to chloramphenicol, neomycin, gentamycin, kanamycin, polymyxin B, tetracyclin, streptomycin and carbencillin. Two isolates, T7 and P5, notably had resistance to eight antibiotics, including penicillin, ampicillin, vancomycin, bacitracin, amoxycillin, cephalexin, and methoprim. The *Planococcus* strain S1 was sensitive to all the antibiotics tested, whereas S2 was resistant only to ceftazidime. Resistance to nalidixic acid, bacitracin and methoprim was more common among these isolates.

The optimum conditions for growth and protease production at different temperatures, pH, and salt concentrations were evaluated using different carbon sources and media composition. Maximal enzyme production was observed in the presence of glucose or lactose, except in isolates T9 and P8, where casein was found to be more suitable. Isolates exhibited optimal enzyme production in the range of $20\text{--}45^\circ\text{C}$ and correlated with their temperature optima for growth, except in two of the thermo-tolerant strains and T7, where it was slightly lower. The optimum pH for growth and enzyme production remained more or less neutral (6.5–7.5) in most of the isolates. Two halo-tolerant strains (S1 and S2) preferred a higher pH (10.0–10.5), whereas it was 8.0–8.5 in case of P8. The requirement for NaCl varied from 0 to 2.5%, with the only exception of S1 which produced maximum protease at 7.5% salt concentration. The enzyme titer in the supernatant varied from 112 to 432 units/ml, with P8 and S1 being the best protease producers, followed by B2a and P5.

A crude enzyme preparation after ammonium sulphate fractionation (30–60%) and dialysis was used for optimization of temperature and pH, and also to evaluate its stability. The temperature optima for enzyme activity markedly varied in different strains and was maximum

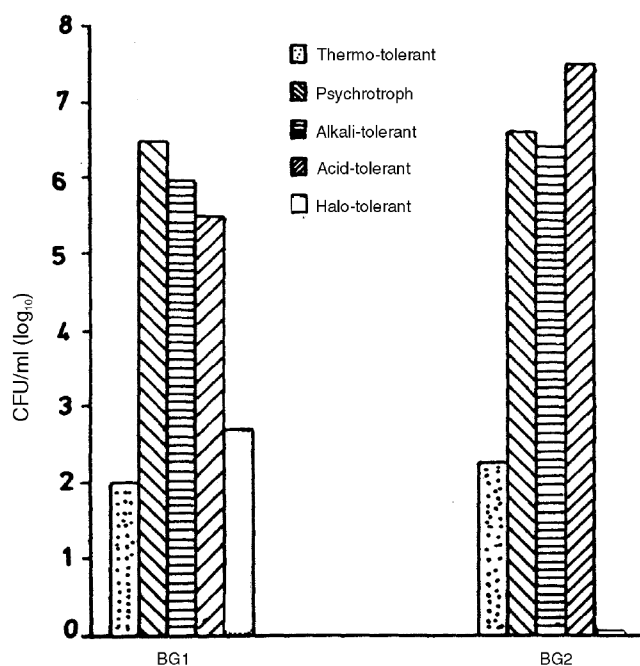


Figure 1. Bacterial count in the two cyanobacterial mats.

Table 1. Morphology and growth characteristics of the isolates

	S1	S2	S3	S4	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
Cell morphology	Cocci	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods
Gram-staining	+	+	+	+	-	-	-	-	-	-	-	-	-	-
Motility	+	-	-	-	+	+	-	+	-	+	+	+	+	+
Pigment	O	Y	-	Y	-	-	-	-	-	-	-	-	-	-
Growth/protease production														
0% NaCl	+	+++	+	+	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
	(-)	(+++)	(-)	(-)										
5% NaCl	+++	+++	+	±	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
	(+)	(+++)	(-)	(-)										
10% NaCl	++	++	±	±	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
	(-)	(-)	(-)	(-)										
pH 5	NT	NT	NT	NT	±	-	±	±	+	-	++	-	±	-
					(-)	(-)	(-)	(-)	(+)	(-)	(+)	(-)	(-)	(-)
pH 7	NT	NT	NT	NT	+++	+++	±	+	+	-	++++	+++	±	+++
					(-)	(-)	(-)	(-)	(++)	(-)	(++)	(+)	(-)	(+)
pH 10	NT	NT	NT	NT	++	++	+	+	-	+++	+++	+++	+	++
					(-)	(-)	(-)	(-)	(-)	(±)	(-)	(+)	(±)	(±)
	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	B2a	B2e	
Cell morphology	Rods	Rods	Cocci	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	
Gram-staining	-	-	+	-	-	-	-	-	+	+	-	+	+	
Motility	+	+	-	-	-	-	+	+	-	-	+	+	-	
Pigment	Y	-	O	-	-	-	-	-	YG	YO	-	O	O	
Growth/protease production														
5°C	-	+	-	±	±	+	++	+	+	+	+	-	-	
	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(-)	(-)	(-)	(-)	(-)	(-)	
10°C	±	++	±	+	+	++	+++	+++	+++	+++	++	+	-	
	(-)	(-)	(-)	(-)	(-)	(-)	(+++)	(-)	(++)	(-)	(-)	(-)	(-)	
20°C	++	+++	+	++	++	++	+++	+++	+++	+	++	+	+	
	(-)	(-)	(-)	(-)	(-)	(-)	(+++)	(-)	(++)	(-)	(-)	(-)	(-)	
30°C	++	+++	++	++	+	+	++	+++	+	-	+	+++	++	
	(-)	(+)	(-)	(-)	(-)	(-)	(-)	(+)	(+)	(-)	(-)	(+)	(+)	
37°C	+++	+++	+	++	-	-	-	++	-	-	-	+++	+++	
	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(++)	(-)	(-)	(-)	(+++)	(+++)	
55°C	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	+++	+++	
												(++)	(++)	

Signs in parentheses indicate zone of proteolysis. Colony pigmentation; O, Orange; Y, Yellow; YG, Yellow-green; YO, Yellow-orange; NT, Not tested.

(45°C) in the two alkali-tolerant strains and S1. The enzymes from the two psychrotrophs and the acid-tolerant strain exhibited maximum activity at 25°C, were active in the neutral range of pH and were thermolabile. Protease from thermo/halo-tolerant bacteria exhibited maximum hydrolysis in the pH range of 10.0–11.0 and was also moderately thermostable, except B2e. Only two proteases (S2 and T9) displayed resistance to both acid and alkali, while the one from P7 was moderately alkali-tolerant.

The present investigation shows that cyanobacterial assemblages of the freshwater bodies of Antarctica harbour a variety of aerobic, heterotrophic bacteria which not only differ in their nutritional requirement and growth conditions, but their enzymes also show diverse characteristics with respect to temperature, pH requirement and stability. The bacterial count in the two cyanobacterial samples revealed the presence of not only psychrotrophs but also fairly large number of other popu-

lations like halo-tolerant, acid/alkali-tolerant and thermo-tolerant bacteria. In another study, Astwood and Wais¹⁵ have reported psychrotrophic bacteria from a constantly warm, tropical environment. Diversity and association of bacteria in Antarctic sea ice has been studied earlier by Bowman *et al.*⁷ with sole emphasis on psychrotrophic bacterial population, ignoring other physiological groups. It was proposed that the cyanobacterial assemblages provide niches conducive for the proliferation of a diverse array of psychrophilic bacterial species. Our findings indicate that bacterial association in such niches is more intricate than ever thought and probably provides spatially separated microenvironments which greatly differ in the prevailing conditions of temperature, salt and pH. The occurrence of microorganisms with cardinal temperatures uncharacteristic of Antarctic bacteria, is worth investigating in detail.

Prevalence of pigmented bacteria is in accordance with earlier observation by Shivaji and co-workers^{16–18}, but the

absence of pigmentation in the acid/alkali-tolerant group is interesting. The production of extracellular enzymes by the isolates like amylase, lipase, β -glucosidase, etc. may play a key role in organic-matter cycling of the Antarctic freshwater lakes. Enzymatic activities of amino peptidase and β -glucosidase in Antarctic Ross Sea sediments have been investigated by Fabiano and Danovaro¹⁹, and have shown to play an important role in organic-matter diagenesis.

Based on their morphological features, biochemical characteristics and profile on API identification kit, the selected isolates were tentatively designated to various genera. The halo-tolerant strain S1 is a *Planococcus* sp., which was also confirmed by its 16S rRNA gene sequencing (data not shown). Two isolates, T7 and P5, appear to be *Pseudomonas* sp., among which T7 shows 99.9% homology to *Pseudomonas fluorescense* by API profile and software analysis. The two thermo-tolerant strains are designated as *Bacillus* species. The preliminary morphological studies and biochemical characterization are not sufficient to even make a tentative identification of the remaining isolates. The isolates, by and large, are sensitive to antibiotics, except T7 and P5, showing resistance to multiple antibiotics, though their association with any plasmid could not be ascertained. In another study by Ray *et al.*²⁰, a total of 31 bacterial isolates from Antarctica were screened for occurrence of plasmid and antibiotic resistance conferred by them. The frequency and size of plasmids were found to be like those from unpolluted sites²¹.

The isolates preferred different carbohydrates and proteins as carbon sources for growth and inducers for protease. Both the thermo-tolerant strains preferred carbohydrates for growth and enzyme production. The maximum induction of proteases on substrates other than protein in most of the cases is unusual. We made similar observations earlier with *Aeromonas hydrophila*, isolated from low-temperature, high-altitude regions of India²². In a similar finding, O'Reilly and Day²³ have reported better protease induction by *A. hydrophila* in the presence of sucrose. In another study, protease production by *Vibrio* sp. has been suggested to be under an inducer catabolite repression that is actually reflected by the growth rate and energy status of the cell²⁴. The proteases from the two psychrotrophic strains are cold-active, thermolabile and function in a narrow range of pH, though a more careful examination from purified enzyme is necessary to ascertain their characteristics. The thermolabile nature of most of the cold-active enzymes has been attributed to their flexible nature, which enables them to catalyse at low temperatures²⁵. The protease of the thermo-tolerant B2e being thermolabile and sensitive to alkali treatment, though active in the alkaline range, is surprising. The strain S1 produced thermo-tolerant protease active in the alkaline range, in the crude preparation.

The proteases from the limited number of isolates revealed great variation in their requirement for pH and temperature. The proteases and other enzymes produced by these organisms could be of immense biotechnological applications, since they are likely to possess more than one desired characteristics, e.g. cold-active and halo-tolerant/alkali-tolerant proteases. These enzymes have been shown to have a potential role in detergent, food processing, tanning of hides, development of biosensors, etc.

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