Effect of isolation temperature on the characteristics of extracellular proteases produced by Antarctic bacteria

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Protease-producing psychrotolerant bacteria were isolated from Antarctic biotopes on casein agar plates using different incubation temperatures. Most of the isolates were non-spore-forming Gram-negative motile rods with catalase activity, 30 % were pigmented and none of them were glucose fermenters. All the strains were grown in liquid cultures at 20 °C and protease secretion was tested using the azocasein method. Despite their capacity for production of a clear zone of hydrolysis in agar plates, some strains did not produce detectable levels of proteolytic activity in liquid cultures. The lowest apparent optimum temperature for protease activity found in culture supernatants was 40 °C. Almost all the strains showed activation energy values about 10 - 20 kJ·mol^{-1} lower than that observed for a mesophilic Subtilisin. Most of the proteases showed optimal activity at neutral or alkaline pH values and developed a multiple-band profile on gelatine-SDS-PAGE. It was observed that the lower the strain isolation temperature was, the more stongly cold-adapted—in terms of optimal temperature and activation energy—were the proteases produced by them. This dependence of the characteristics of the proteases on the isolation temperature is an important factor to take into account in the design of screening programmes directed towards the isolation of psychrotolerant bacteria able to produce proteases strongly or weakly adapted to work in the cold. The Antarctic area explored proved to be a promising source of proteolytic bacteria with potential use in industrial processes to be carried out at low or moderate temperatures.

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Enzymes constitute an important industrial product and have reached an annual marketing figure of US$ 1.6 billion. Proteolytic enzymes represent nearly 60 % of this market and have a broad spectrum of applications, mainly in the food industry, laundry products and leather processing (Demain 1999). As enzymes showing high catalytic efficiency under extreme environmental conditions could be useful for many purposes, extremophilic microorganisms became the centre of an important research effort conducted to obtain enzymes adapted to work under high or low temperatures, and in extremely acidic or alkaline media and high salinity (Aguilar 1996). Among extremophiles, psychrophilic and psychrotolerant bacteria, which are adapted to grow at 0 °C, constitute a relevant source of enzymes potentially useful in industrial processes in which working at low temperature represents an advantage (Gerday et al. 2000). As the majority of the industrial proteases currently used are produced by mesophilic microorganisms and show optimum activity around 55 - 60 °C (Kamal et al. 1995; Suhartono et al. 1997; Varela et al. 1997; Asakawa et al.

1998; Han & Damodaran 1998), there is a general interest to find new proteases with lower optimum temperatures, which would result in a considerable energy saving (Horikoshi 1995; Aguilar 1996).

In Antarctica, one of the coldest areas in the world, prokaryotes are the dominant organisms and they are mainly responsible for the hydrogeochemical cycles and the mineralization of organic matter (Karl 1993; Rivkin et al. 1996). In addition, the Antarctic continent is one of the less explored places on Earth and accounts for the high proportion of new taxa that are described when biochemical and molecular techniques are applied to the analysis of bacteria present in samples taken from these environments (Irgens et al. 1996; Reddy et al. 2000). Therefore, Antarctica is one of the most promising sources of new bacterial isolates able to produce cold-adapted proteases with lower optimum temperature than those currently used in industry. Environments in the Antarctic Peninsula are exposed to different temperature ranges. Some of them, such as soil and rock surfaces, are exposed to significant solar irradiation and show a broad range of temperatures which extends from below the freezing point to over 25 °C (Vishniac 1993). On the other hand, environments such as seawater, marine sediments and sea ice have constant temperature values lower than 4 °C (Schloss et al. 1998). Antarctic microorganisms' ecophysiological and biochemical adaptations could be reflecting the different temperature regimes they have to withstand. It is known that isolation temperature strongly influences the psychrophilic/psychrotolerant ratio of the bacteria isolated from a sample (Delille & Perret 1989; Delille 1992). Thus, even among the psychrotolerant bacteria, a low isolation temperature could determine a higher probability to obtain strains with low optimum temperature not only for growth but also for the activity of their extracellular enzymes.

Although cloning of the genes involved in protease production could be a necessary step in the optimization of the production process at industrial scale, the screening of new strains and the characterization of the proteases produced by them represent an important initial activity. Since, from a biotechnological point of view, an industrial process based on psychrotolerant bacteria is more simple and cost-efficient than one based on psychrophiles, the influence of the isolation temperature on the optimum temperature for growth and protease activity is an important point to consider to improve the search for this kind of strains.

In the present work, our aim was to isolate neutral or alkaline protease-producing psychrotolerant bacteria from Antarctic environments and compare the effect that isolation temperature has on the basic properties of these extracellular proteases.

Materials and methods

Sampling area

The samples were taken during Argentine summer Antarctic Research Expedition (ARE) 1995–96 near Jubany scientific station (62° 14' S, 58° 40' W) on King George Island, South Shetland Islands. Samples were collected from soil, fresh and marine waters, marine sediments and remains of organic matter of animal and plant origin. In addition, strains isolated during the ARE 1991–92 (Vazquez et al. 1995) were included for comparison and further study.

Screening and isolation of psychrotolerant bacterial strains

Samples from soil and organic matter were placed in a screw-capped bottle containing 5 g of sterile sand and 15 ml of sterile diluent (1 $g \cdot l^{-1}$ of peptone solution) and shaken for 15 min. After shaking, serial 10-fold dilutions were prepared in the same diluent and 0.1 ml of each dilution was spread on the surface of nutrient agar plates (Merck) for counting total heterotrophic bacteria. Proteolytic bacteria were screened by spreading 0.1 ml of dilutions on casein agar plates at pH 7.0, in accordance with Hoshino et al (1997). Proteolytic activity was detected as clear zones of hydrolysis around the colony. Samples from marine origin were processed in the same way but diluent and media were prepared using 75 % v/v seawater. Samples were incubated at 20 °C and at 4 - 6 °C for 20 days, in order to further analyse the effect of incubation temperature on the characteristics and growth of the isolated strains and on the activity of the proteases produced by them. Different proteolytic colonies were isolated and purified by re-streaking twice. General characteristics of previously obtained proteolytic strains isolated at 10 - 13 °C during ARE 1991–92 (Vazquez et

al. 1995) are included for comparison in the Results section.

Characterization of bacterial strains

All proteolytic bacteria were characterized from 48 h old pure cultures grown at 20 °C. The strains were Gram stained and shape and size of the cells were examined under the light microscope. Their mobility was investigated in hanging drops. The colour of the colonies was observed on nutrient agar plates. Catalase, cytochrome oxidase activity and aerobic and anaerobic utilization of glucose (Hugh-Leifson medium) were also tested.

Liquid culture conditions

Growth experiments were performed in nutrient broth (Oxoid CM1) supplemented with 0.3 $g \cdot l^{-1}$ $CaCl_2.2H_2O$, pH 8.0. For marine strains the medium was re-hydrated with 100 % v/v artificial seawater (Lyman & Fleming 1940). Experiments were carried out in 300 ml Erlenmeyer flasks with 60 ml of medium and incubated in a rotatory shaker at 240 rpm at 20 °C. Inocula were grown in the same medium, adjusted to an optical density at 580 nm (OD_{580}) of 0.100 and added to broth at 1 % v/v proportion (final OD under the detection limit). Samples were taken after 24, 48 and 72 h of incubation and used to measure growth, pH and proteolytic activity. All the experiments were performed in duplicate. Growth was monitored by measuring the OD_{580} of the samples in an UV-Visible spectrophotometer and expressed as dry weight using previously tested calibration curves. Dry weight was determined (in triplicate) by weighting the dried biomass (48 h at 105 °C) after centrifugation of aliquots of the culture.

Protease assay

Proteolytic activity was measured by digestion of azocasein, in accordance with Charney & Tomarelli (1947). An appropriate dilution of culture supernatant (400 µl) was incubated with 400 µl of 1 % w/v azocasein in 0.1 M Tris/HCl buffer (pH 8.0) and 0.5 mM $CaCl_2.2H_2O$ at 20 °C for 30 min. The reaction was stopped by adding 800 µl of 5 % w/v trichloroacetic acid. After centrifugation of the reaction mixture, absorbance of the supernatant was measured at 340 nm. Samples were assayed in duplicate and the activity was expressed in relative enzymatic units (EU). One EU was defined as the amount of enzyme that produces an increase of 0.100 in A_{340} under the assay conditions.

Effect of pH and temperature on activity of proteases

The effect of pH and temperature on protease activity was determined by using the protease assay described above. Determination of the optimum pH was performed at 20 °C with the following buffer systems (0.1 M each): KH_2PO_4/Na_2HPO_4 (pH 5 - 7); Tris/HCl (pH 8 - 9) and Na_2HPO_4/NaOH (pH 10 - 12). For determination of the optimum temperature, the reaction was carried out at different incubation temperatures (between 0 °C and 60 °C) and pH 8.0. Activation energies were calculated from the linear part of Arrhenius plots as described by Pirt (1985).

Polyacrilamide gels

Extracellular protease profiles were analysed by SDS-PAGE minigels containing gelatine as copolymerized substrate (Heussen & Dowdle 1980). Proteolytic activity was evident as bands depleted of gelatine.

Results

Even though we sampled in biological systems where natural changes in the bacterial population make comparison among samples difficult, a common pattern was observed (Table 1). Samples from ecosystems exposed to wide thermal oscillation (like soil surface and organic matter exposed to solar radiation) showed higher total bacterial counts at 20 °C when compared with those obtained at 4 °C. Conversely, samples taken from areas subjected to low and almost constant temperature (like seawater or marine sediments) had lower counts at 20 °C than at 4 °C. The number of Gram-positive strains varied with the different isolation temperatures, corresponding to 6 - 14 % when the isolation was made at 13 °C or lower, but rising to 40 % when the strains were isolated at 20 °C. The presence of spore-forming bacteria was observed when the screening was carried out at 20 °C (Table 2) but they were not isolated at 4 °C, probably because this temperature was not suitable for the germination of the spores which were likely to be present.

On the basis of the formation of a clear zone in casein agar, 89 proteolytic strains (40 isolated at 20 °C and 49 at 4 - 6 °C) were selected. In Table 2, we summarize the general characteristics of the selected protease-producing psychrotolerant strains isolated during ARE 1995–96 and of other strains previously isolated during ARE 1991–92 (Vazquez et al. 1992). All the strains were capable of growth at 0 °C but had an optimum growth temperature higher than 15 °C, corresponding to psychrotolerant microorganisms (Morita 1975). The majority of them were non-spore forming Gram-negative rods, though some strains were Gram variable. This was probably due to the presence of certain components on their cell walls which may interfere with the cell permeability to the stain. The pigmented strains (30 % of total isolates) were mostly yellow and orange coloured. Motile strains with cytochrome oxidase activity predominated. Almost all the strains had catalase activity and none of them were glucose fermenters.

When all the newly-isolated 89 proteolytic strains plus the 34 additional strains selected in a previous screening were cultured in nutrient broth, some showed a protease secretion pattern associated with growth (Fig. 1b), while other strains presented their enzyme secretion partially associated with growth (starting at the early stationary phase) or not associated with growth at all (Fig. 1a).

Some strains were discarded for further stud-

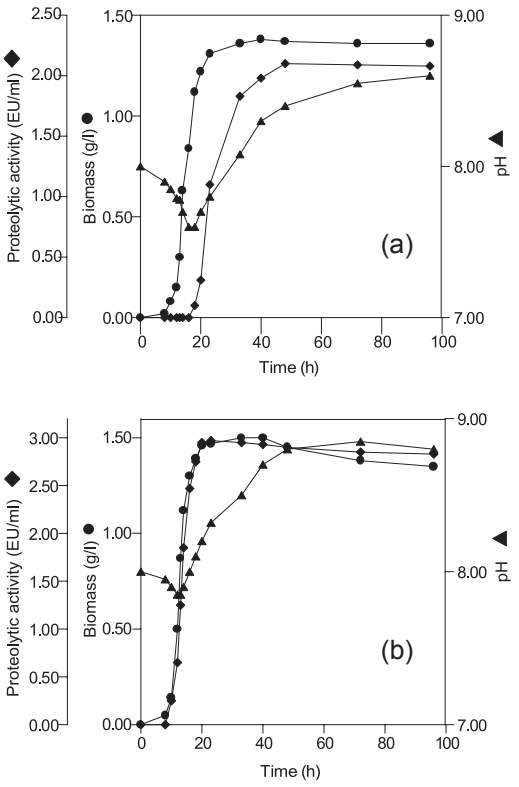

Fig. 1. Examples of two different patterns of growth and protease production kinetics observed in the selected Antarctic strains. When strains were cultivated in liquid culture, protease production showed two different behaviours. (a) Protease secretion delayed with respect to growth, starting at the early stationary phase (P-96-34). (b) Enzyme secretion associated with growth (P95-16).

Table 1. Heterotrophic bacterial counts incubated at 20 °C and 4 - 6 °C from different samples taken during summer Antarctic Research Expedition 1994–95.

Sample origin	pH	CFU[a]	
		20 °C	4 - 6 °C
Lichens (*Usnea antarctica)*	5.5	$1.8 \cdot 10^6 \pm 5.1 \cdot 10^4$	$1.0 \cdot 10^5 \pm 4.5 \cdot 10^4$
Moss mat (near pond coast)	6.0	$4.3 \cdot 10^6 \pm 4.6 \cdot 10^5$	$3.0 \cdot 10^4 \pm 4.5 \cdot 10^3$
Dead elephant seal skin (Potter Cove coast)	nd[b]	$4.0 \cdot 10^6 \pm 4.9 \cdot 10^5$	$3.5 \cdot 10^4 \pm 4.1 \cdot 10^3$
Seawater (Potter Cove)			
Surface	7.8	$1.2 \cdot 10^2 \pm 6.9 \cdot 10$	$3.8 \cdot 10^3 \pm 6.9 \cdot 10^2$
5 m deep	7.9	$7.6 \cdot 10^1 \pm 7.0 \cdot 10$	$2.9 \cdot 10^3 \pm 6.5 \cdot 10^2$
10 m deep	7.9	$8.3 \cdot 10^1 \pm 6.2 \cdot 10$	$3.1 \cdot 10^3 \pm 6.0 \cdot 10^2$
20 m deep	7.8	$3.5 \cdot 10^2 \pm 1.5 \cdot 10^1$	$2.7 \cdot 10^3 \pm 3.4 \cdot 10^2$
30 m deep	7.8	$1.2 \cdot 10^2 \pm 9.0 \cdot 10$	$2.9 \cdot 10^3 \pm 5.4 \cdot 10^2$
Soil (near Fourcade glacier)	nd[b]	$2.6 \cdot 10^5 \pm 4.1 \cdot 10^4$	$1.0 \cdot 10^4 \pm 3.7 \cdot 10^3$
Algae (*Desmarestia antarctica*)	7.0	$5.0 \cdot 10^5 \pm 5.3 \cdot 10^4$	$4.6 \cdot 10^5 \pm 5.7 \cdot 10^4$
Sediment			
Pond (1 m deep)	7.2	$7.5 \cdot 10^3 \pm 7.4 \cdot 10^2$	$1.7 \cdot 10^3 \pm 5.7 \cdot 10^2$
Potter Cove (30 m deep)	7.7	$5.9 \cdot 10^3 \pm 7.0 \cdot 10^2$	$2.4 \cdot 10^4 \pm 4.1 \cdot 10^3$

[a] Colony forming units of the cultivable heterotrophic bacteria expressed as gram of dry weight except for the seawater samples, which are expressed as ml of sample.
[b] Not determined.

ies, as they showed no proteolytic activity at any time in liquid culture, measured by the azocasein method. After centrifugation of cells, the proteases present in culture broth supernatants were partially characterized. When the strains isolated at 20 °C were tested, only 14 % of cell-free supernatants showed optimal activity at 40 °C (the lowest apparent optimum temperature found). Nevertheless, when the strains were isolated at 10 - 13 °C or at 4 - 6 °C, the percentages raised to 28 % and 46 %, respectively (Table 2). On the basis of their apparent optimum temperature for substrate utilization (Table 3), 25 protease-containing culture supernatants were selected. The isolation temperature of the 25 protease-producing strains is indicated in Table 4. These supernatants showed their maximal activity at 40 °C (Table 3) and contained neutral and alkaline proteases with regards to their optimum pH for activity (Table 4). The activation energy values for proteolytic activity were, in general, about 10 - 20 $kJ \cdot mol^{-1}$ lower than those observed for the mesophilic protease Carlsberg Subtilisin (Table 3).

The development of proteolytic activity of culture supernatants on SDS-PAGE with gelatine as a copolymerized substrate showed mainly multiple-band profiles. Among the 25 protease-containing supernatants with the lowest optimum temperature, only 8 developed a single proteolytic band.

Discussion

Microbiological studies related to Antarctic environments are frequently focused on the role of the bacterial community in heterotrophic production, in the cycling of matter, or in the relationship between bacterial activity and the dynamics of food webs. However, the majority of these studies do not discriminate between the different groups of bacteria that constitute the total flora responsible for one particular activity. When analyses of the bacterial flora of particular Antarctic environments were carried out, Gram-negative bacteria proved to be highly predominant in marine habitats (Delille 1993), coastal zones (Tearle & Richard 1987; Line 1988) and terrestrial habitats covered with vegetation (Heal et al. 1967). This is consistent with the results presented here and could explain the fact that, in spite of the known capacity of Gram-positive bacteria to produce and secrete proteases, few of the selected strains were Gram-positive. Among marine isolates, Gram-positive bacteria were found mostly in sediments while among non-marine isolates they were predominant in soil, mosses and lichens. Also, the number of spore-forming and pigmented strains was higher when the incubation temperature was 20 °C. The predominance of these strains during the summer could be reflecting their capability to withstand the relatively high temperatures and levels of UV radiation that prevail in this period, especially taking into account that the explored area is affected by the thinning of the ozone layer during spring and summer (Tong & Lighthart 1997). It is worth mentioning that most microorganisms, especially in aquatic environments, are not culturable on conventional agar media and using conventional techniques. The considerations expressed above relate to the part of the bacterial community that can be isolated with the culture conditions used, corresponding to the aerobic chemoorganotrophic psychrophilic and psychrotolerant culturable bacteria.

The differences observed in the total counts obtained at 4 - 6 °C and at 20 °C with samples from environments having broad and narrow ranges of temperature reflect the different degrees of adaptation showed by bacteria living in Antarctic areas with or without thermal oscillations.

Table 2. Characteristics of the psychrotolerant Antarctic proteolytic bacteria, collected during two Antarctic Research Expeditions (ARE), isolated on casein agar using three different incubation temperatures.

	ARE 1995–96 Isolation 20 °C	ARE 1991–92 Isolation 10 - 13 °C	ARE 1995–96 Isolation 4 - 6 °C
Pigmented strains	40 %	15 %	22 %
Spore-forming	13 %	1 %	0 %
Form			
rod-shaped	87 %	97 %	88 %
cocci-shaped	13 %	3 %	2 %
Gram stain			
positive	40 %	6 %	14 %
negative	60 %	94 %	86 %
Detection of proteases in liquid culture at 20 ºC	53 %	73 %	67 %
Optimum temperature for proteolytic activity			
40 °C	14 %	28 %	46 %
45 °C	29 %	7 %	46 %
>50 °C	57 %	65 %	8 %
Number of isolated strains	40	34	49

Since psychrotolerants grow faster at 20 °C than at 4 °C and we previously tested that the incubation time was long enough to allow the development of psychrophiles and psychrotolerants colonies, the higher counts obtained with non-marine samples when the incubation was made at 20 °C might be related to the predominance of this type of microorganism in areas with a wide daily and seasonal variations in temperature (Delille 1990). On the contrary, with seawater and marine sediment the counts obtained at 4 °C were higher than those obtained at 20 °C, reflecting the appropriateness of these constantly cold environments for the predominance of psychrophiles.

The selected strains showed variable proteolytic activity levels in liquid cultures. Even when some of the proteases from psychrotolerants had their maximal activity at similar temperatures than their corresponding mesophile, the majority of them showed curves of activity as a function of temperature shifted towards low temperatures, with apparent optimum values between 10 and 15 °C lower than Carlsberg Subtilisin. Other authors found similar results for proteases (Helmke & Weyland 1991; Turkiewicz et al. 1999) and other enzymes (Feller et al. 1997; Hoyoux et al. 2001) produced by marine bacteria from permanently cold environments. This observation reveals a clear adaptation of the enzyme-producing strains to their habitats. The low activation energies shown by many of the crude proteases also represents a way of adaptating to work in the cold and constitute a requirement for substrate hydrolysis in cold environments and, thus, for survival (Margesin et al. 1991; Feller & Gerday 1997). These enzymes compensate the low kinetic energy in their environments by reducing the activation energy barrier.

In addition, all proteases with the lower optimum temperature (40 °C) had a neutral or alkaline optimum pH, probably influenced for the neutral pH of the isolation culture media or the pH of the habitats explored. Although the broad pH range in which some of our proteases showed maximum activity agree with the ones reported for other cold-adapted proteases (Hamamoto

Table 3. Optimum temperature (OT), percent of relative activity measured at 20 °C with respect to the activity at optimum temperature (RA) and activation energy for azocasein hydrolysis (E_{act}) of crude proteases from 79 Antarctic psychrotolerant strains.

Strain	OT (°C)	RA (%)	E_{act} (kJ·mol^{-1})	Strain	OT (°C)	RA (%)	E_{act} (kJ·mol^{-1})	Strain	OT (°C)	RA (%)	E_{act} (kJ·mol^{-1})
Ele-2	40	34	40	P95-8	45	17	45	P96-20	45	21	51
Ele-3	40	19	54	P95-9	45	22	46	P96-23	45	19	47
TCN-2	45	13	51	P95-16	45	20	49	P96-27	45	14	55
GUD-5	50	12	50	P95-17	45	26	49	P96-28	45	9	52
GUD-8	50	17	52	P95-18	45	29	49	P96-29	40	29	53
ANT-1-1	55	6	55	P95-19	45	24	44	P96-33	40	21	52
ANT-3-1	50	6	50	P95-20	>50	12	52	P96-35	40	35	40
ANT-7-1	55	6	55	P95-21	>50	25	47	P96-37	50	22	46
YOA-3	60	5	60	P95-24	40	30	36	P96-38	45	37	39
814	40	32	40	P95-27	40	10	52	P96-39	45	34	42
PIEL-1	40	29	40	P95-28	>50	10	52	P96-41	40	14	54
91	50	35	50	P95-29	>50	21	38	P96-43	40	31	47
435	50	30	50	P95-31	>50	27	33	P96-44	50	26	38
337	50	9	50	P95-32	>50	9	55	P96-45	45	14	50
273	50	13	50	P95-33	>50	13	41	P96-46	45	7	55
A1	50	6	50	P95-37	>50	25	51	P96-47	45	27	47
Prot-2	50	17	50	P95-38	>50	15	49	P96-48	45	21	50
Prot-4	50	20	50	P95-39	>50	19	55	P96-49	40	23	50
Prot-5	45	18	45	P95-40	>50	9	48	P96-50	40	35	48
Prot-8	40	34	40	P96-1	45	30	37	P96-51	40	16	87
Prot-9	40	13	40	P96-3	45	28	38	P96-52	40	22	74
Prot-10	50	11	45	P96-4	45	29	39	P96-53	40	24	55
Prot-11	40	14	40	P96-5	40	23	48	P96-54	40	22	76
Prot-12	55	7	55	P96-6	50	11	52	P96-55	45	40	29
Prot-14	45	29	45	P96-7	40	33	51	P96-56	45	21	49
P95-1	40	21	42	P96-12	40	13	51				
P95-6	40	15	47	P96-18	40	24	50	Subtilisin	55	12	60

et al. 1994), this characteristic differs from that observed by other authors for low-temperature active proteases (Davail et al. 1994; Kärst et al. 1994) where a discrete value of optimum pH was found. In other cases, even though a discrete optimum pH value was reported, there was a broad range where the proteases showed more than 80% of their maximum activity (Margesin & Schinner 1992). The pH values for maximum activity of the studied proteases make them suitable for use in detergents and other industrial products requiring alkaline proteases, though the stability of the proteases at the pH of such products and processes must be confirmed.

By developing proteolytic activity of culture supernatants on gelatine-SDS-PAGE, it was possible to observe that some strains produced only one protease. These strains were selected for further studies (when their optimum temperature for activity was of interest) because they are easier to purify and characterize than a mixture of proteins. However, the majority of the strains produced a multiple-band profile, which might have been the result of the activity of more than one protease produced by the strain, isoenzymes or the active fragments of self-digestion. The synthesis of more than one protease, which was observed mainly in marine strains, could be understood as a strategy to better cope with the fluctuating supply of nutrients as well as to enhance the uptake of proteins in oligotrophic environments. Although references to the number of proteolytic bands developed in SDS-PAGE from the culture supernatants of cold-adapted bacteria are infrequent in the literature, Turckiewicz et al (1999) recently found two different protein bands active against denatured haemoglobin working with an Antarctic marine strain of *Sphingomonas paucimobilis.*

Table 4. pH values for maximal activity of crude proteases from Antarctic strains showing optimum temperature for proteolytic activity at 40 °C.

Strain	pH	Strain	pH
Ele-2*,[1]	8	P96-18*,[3]	7 - 9
Ele-3[1]	8 - 11	P96-29[3]	7 - 9
Prot-8[1]	8 - 9	P96-33[3]	8
Prot-9*,[1]	6 - 11	P96-35*,[3]	7 - 9
Prot-11*,[1]	8	P96-41[3]	7 - 9
814[1]	8 - 9	P96-43[3]	7 - 9
PIEL-1[1]	8 - 9	P96-49[3]	8
P95-1*,[2]	7 - 9	P96-50[3]	9
P95-21*,[2]	7 - 9	P96-51[3]	8
P95-24*,[2]	7 - 10	P96-52[3]	8 - 10
P96-5[3]	8	P96-53[3]	7 - 9
P96-7[3]	9	P96-54[3]	9
P96-12[3]	7		

*Single proteolytic band developed in gelatin-SDS-PAGE.
[1,2,3] Strain isolated at 10 - 13 ºC, 20 ºC and 4 - 6 ºC, respectively.

Given the relatively high cost and complexity of biotechnological processes based on the culture of psychrophiles, which must be kept very cool, psychrotolerant organisms will be more suitable than psychrophiles to support an enzyme production process at moderate temperatures (20 - 25 °C) while producing enzymes with high activity at low temperatures. Such a process would yield an enzyme with considerable activity at temperatures significantly lower than those shown by the enzymes currently in use. On the basis of this concept, we chose 20 °C as the more suitable temperature to cultivate and produce proteases. We consider that this is an adequate temperature to carry out a process in a template climate zone, as it can be maintained without expending costly energy in cooling the reactor.

We also point out the relevance of selecting the proper conditions when designing a screening programme for the selection of proteolytic bacteria in the explored area. Isolation temperature was shown to be an important factor to take into account to direct the screening towards the isolation of bacteria producing the desired type of proteases.

To conclude, the results presented here suggest that the selected psychrotolerant strains are potentially useful for industrial applications, and that further studies will be necessary to optimize the natural capability of these strains to produce and secrete proteases. Moreover, we would like to emphasize the relevance of the Antarctic environment as a source of psychrotolerant microorganisms whose physiological characteristics are often unusual in comparison to the corresponding mesophiles (Shivaji et al. 1989) and which offer new possibilities in biotechnology.

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