

Chapter 5

Fungal Growth Under Simulated Deep-sea Conditions

5.1 Introduction

Hydrostatic pressure, as distinct from high hydrostatic pressure, is present in all biological environments. In air, the pressure is typically close to normal atmospheric pressure at sea level but it decreases with increase in altitude, simultaneously changing the partial pressure of constituent gases. The aquatic environments present the full range of hydrostatic pressure, from micro-pressures generated by a few centimeters of water column to approximately 1100 bar in the deepest ocean trenches. Microbial activity under elevated pressure, compared with that at 1 atmosphere (1 bar) is inhibited. All aspects of activity, including growth, respiration, and specific biochemical processes, appear to be affected at elevated pressures. Since the report of presence of barophilic bacteria by Yayanos in 1979, the research on these organisms started in full vigour.

The adaptation of animals and microorganisms to the high hydrostatic pressure of deep water has been studied at the cellular, kinetic and molecular levels, using orthodox biochemical and physiological preparations and methods, in conjunction with less familiar high pressure techniques and thermodynamic analysis [[Somero, 1992](#)].

Most of the physical parameters for the deep sea, such as salinity (34.8 ± 0.3 ppt), pH (7.8 – 8.0), oxygen concentration ($3 - 5 \text{ mL O}_2 \text{ L}^{-1}$) and temperature (> 50 % of the marine water masses have temperatures of less than 2 - 4°C in the deep sea) are not growth limiting in the deep-sea habitat [[Zobell, 1968](#); [Dietrich, 1978](#); [Gage & Tyler, 1991](#)]. The effect of hydrostatic pressure on growth of fungi is the least

investigated physical parameter but results of [Zobell & Johnson \[1949\]](#) and [Yamasato *et al.* \[1974\]](#) indicate a general and broad tolerance, at least in most yeast species.

It is a surprising fact that although the susceptibility of animals in shallow water to high (i.e. deep sea) pressures was established in 1870s, it was only in the 1970s that comparable pressure experiments were carried out on deep water animals to ascertain their short term tolerance to decompression and to high pressure [[MacDonald *et al.*, 1972](#); [MacDonald & Teal, 1975](#); [Menzies *et al.*, 1974](#)]. The basic requirement for these studies was an instrument, which would be able to stimulate the elevated pressure conditions existing in the deep-sea environments. Different types of instruments were used for this, but the majority of the work was carried on bacteria. Lorenz and Molitoris developed one such equipment, modified from an equipment developed for bacteria [[Jannasch & Wirsen, 1977 a, b](#); [Helmke, 1979](#); [Schade *et al.*, 1980](#), [Bernhardt *et al.*, 1987](#)], to cultivate fungi, especially yeasts, under simulated deep sea condition of elevated hydrostatic pressure. With the help of these instruments developed, a lot of studies for bacteria have been carried out, but fungi have remained neglected.

Elevated pressure either inhibits or favours those biochemical processes which occur with an expansion or reduction in system volume, respectively [[Somero, 1992a](#)]. The upper pressure limit for life is yet unknown. Pressure-inducible genes, which aid in pressure acclimatization, have been proposed to exist in marine bacteria, which experience large vertical changes in the water column [[Bartlett, 1991](#)].

In case of bacteria, the pressure effects on gene expression, membranes, membrane proteins, DNA structure & function, cell division, protein & enzyme

functions have been studied in detail (Table 5.1); while in case of fungi the studies have remained restricted only up to their detection in the deep-sea sediments and ability to grow under elevated pressure and produce extracellular enzymes active under elevated hydrostatic pressure [Raghukumar & Raghukumar, 1998].

Despite the fact that the basis for all pressure effects arise from a single influence, namely the change in system volume that accompanies a physiological or biochemical process, high pressure exerts many effects on living organisms, making it difficult to indicate exact pressure - points in cell growth and viability. In addition, pressure effects also depend on other physicochemical characters, like the nutrients [Marquis, 1982], temperature & pH [Marquis, 1994]. Several workers have studied the ‘wonder microorganism’, *Escherichia coli* extensively for the pressure-sensitive processes. Some of the key pressure-sensitive processes described for *E. coli* have been listed in the table below.

Table 5.1 Pressure-sensitive processes in *Escherichia coli* [Bartlett, 2002]

Process	Pressure affecting/abolishing process (bar)	Reference
Motility	100	Meghnathan & Marquis, 1973
Substrate transport*	260	Landau, 1967
Cell division	200 - 500	Zobell, 1970; Zobell, 1963; Zobell, 1962
Growth	500	Yayanos & Pollard, 1969
DNA replication	500	Yayanos & Pollard, 1969
Translation	600	Yayanos & Pollard, 1969; Gross <i>et al.</i> , 1993
Transcription	770	Yayanos & Pollard, 1969
Viability	1000	Pagan & Mackey, 2000

Such extensive studies are lacking in case of fungi, with exception of yeasts like *Saccharomyces cerevisiae* and a few species of *Rhodotorula*.

5.2 Objectives

The main objectives targeted while studying the growth of fungi under elevated hydrostatic pressure were -

1. To enumerate the germination of spores under elevated hydrostatic pressure.
2. To compare the biomass production by the deep-sea and terrestrial fungi under elevated hydrostatic pressure and low temperature.
3. To try and adapt the cultures not showing growth under elevated hydrostatic pressure, to grow under these conditions.
4. To study the effect of temperature and pressure on the viability of spores.
5. To study the effect of nutrient concentrations on the germination of spores under high hydrostatic pressure.

5.3 Methodology

5.3.1 Growth and spore germination under elevated hydrostatic pressure

All the 181 deep sea fungal isolates obtained using different techniques and nutrient media were examined for spore germination and growth under elevated hydrostatic pressures.

5.3.1.1 Spore germination

The cultures were inoculated onto MEA plates and incubated at 1 bar pressure and 30 °C until sporulating fungal colonies appeared (within 3-5days). 8 mL of autoclaved seawater along with 2 mL 2 %-sterile Tween 80 (added since the spores being hydrophobic, do not mix homogeneously in the solution) was taken in sterile 15 mL centrifuge tubes. Spores were obtained by scraping the surface of the growing fungal colonies with a loop and were suspended in the seawater-tween 80 mixture in the centrifuge tubes. This was mixed vigorously by vortexing the tube for 15 to 20 min. This was used as master stock suspension for all further experiments. To this sterile sea water and tween 80 mixture, 1 mL of spore suspension prepared as described previously was added. The contents were mixed by vigorous vortexing. This is done serially till the appropriate spore count is attained, which is observed after each dilution directly under the microscope by direct examination using Neubauer counting chamber. A drop of conidial suspension was placed on the engraved grid and let to stand for 1-2 min to allow the spores to settle at the bottom. It was covered with a cover slip in such a way that no air bubbles were trapped. The number of spores in the four corners and the middle square were counted. The number of spores mL^{-1} of the suspension was calculated mathematically as follows:

$$\text{Spores } \text{mL}^{-1} = \text{Average number of spores in one large square} \times 10^4 \text{ cm}^{-3}$$

The disadvantage of the above method was that sometimes an accurate spore count could not be done as germinating spores got trapped in the outer grids and hence could not be quantified giving false counts. As an alternative 25 μL of sample

was directly observed under the microscope on a plain slide and the total spores present on a whole slide were counted.

The spore concentration in the suspension was calculated as described above. From this suspension, 100 μL was inoculated in 5 mL of MEB. This was incubated for 20 h. At the end of this incubation period, 50 μL was spread-plated on MEA and the plates were incubated at 1 bar and 30 °C until fungal colonies appeared (within 8–10 days). The percentage of germinating conidia was calculated by counting in a Neubauer chamber and CFU (colony forming units) on the MEA plates. The plates were observed to determine the dilution at which well-isolated colonies were observed. Based on these results, it was found that a spore concentration of not more than 1,00,000 and with an optimum of 50,000 to 70,000 spores mL^{-1} was ideal and this was used as the standard for all further experiments.

The spores were then inoculated in MEB to obtain a concentration of approximately $5 \times 10^4 \text{ mL}^{-1}$, in pouches made of sterilized polypropylene and sealed without trapping any air bubbles. These pouches were suspended in deep-sea culture vessel that was filled with sterile water and pressurized to the desired hydrostatic pressure and incubated at 30° and 5°C. Three replicates were maintained for each treatment. After 7 days of incubation, the deep-sea culture vessels were decompressed gradually (at the rate of 50 bar 15 min^{-1}). The percentage of germinating conidia was counted as described above.

The spores of all the cultures including the deep-sea cultures failed to germinate at low temperature of 5°C; hence all the further experiments involving spore germination under different conditions were carried out at 30°C (5.3.7).

5.3.1.2 Biomass production under elevated hydrostatic pressure

The cultures were grown in malt extract broth for 2-3 days (before the onset of sporulation) and the mycelial biomass was homogenized using sterile glass beads. A known weight of the finely broken mycelial suspension were inoculated in malt extract broth (MEB) and incubated under 200 bar pressure at 30 and 5°C as described above. After 20 days, the contents of the pouches were filtered over pre-weighed filter papers, dried to a constant dry weight and the difference in the initial and final biomass determined as mycelial dry weight.

Fungi grown under elevated hydrostatic pressure at 5° and 30°C were observed under microscope and their morphology under different culture conditions was recorded by photomicrography.

5.3.1.3 Effect of type of inoculum on growth under elevated hydrostatic pressure and low temperature

In case of a sporulating fungus, the spores germinate to give rise to vegetative mycelia, which in turn give rise to new spores. The requirements for the germination of the spores and the growth of mycelia are quite different. Due to this, spore and mycelia may react differently to the high hydrostatic pressure and low temperature. To investigate this, spores and mycelia (before sporulation sets in) of a deep-sea fungus, # A 4634 (*Aspergillus terreus*) were inoculated in sterile MEB in plastic pouches and incubated at following conditions:

- | | |
|-------------------|------------------|
| 1. 1 bar & 30°C | 2. 1 bar & 5°C |
| 3. 200 bar & 30°C | 4. 200 bar & 5°C |

After an incubation period of 20 days at the above conditions, the plastic bags were cut open and the results were recorded in terms of biomass produced.

5.3.2 Screening and acclimatization of fungal isolates for growth under elevated hydrostatic pressure

Many of the deep-sea fungal isolates failed to grow at elevated pressures of 300, 400 and 500 bar when incubated at these pressures directly. Therefore, an experiment was carried out to examine if these could be gradually acclimatized to grow at elevated pressures. The cultures were initially grown at 50 bar / 30°C for 20 days, after which the pressure vessel was decompressed and the bags brought back to 1 bar pressure and checked for growth and viability upon culturing. Cultures that had grown at 50 bar pressure were transferred to fresh pouches under sterile conditions and incubated at 100 bar pressure. This process was continued at 200, 300 and 400 bar pressure. Viability of fungi after exposure to each pressure was tested by growing on MEA at 30°C / 1 bar pressure.

5.3.3 Comparative growth of deep-sea and terrestrial fungi under elevated hydrostatic pressure

A comparison was done between the deep-sea isolates, terrestrial isolates (obtained from IMTECH, Chandigarh) and some shallow water isolates (Lakshadweep lagoon) with respect to the biomass production and conidial germination under

elevated hydrostatic pressure at 30°C and 5°C. The experimental set up was as described below.

Two isolates of *Aspergillus terreus* (MTCC 279 & MTCC 479) and one *Aspergillus sydowii* (MTCC 635) culture isolated from terrestrial environments obtained from Microbial Type Culture Collection (MTCC, Chandigarh, India) and a few representative deep-sea fungi and shallow water fungi from Lakshadweep coral lagoons were used for this study. Two types of inocula were used. In one, conidia from the fungi were inoculated in malt extract broth containing 1 % glucose and incubated under elevated hydrostatic pressure of 200 bar and different temperatures (30 and 5°C) for comparing their germination ability. The number of conidia showing germination were determined microscopically after 5 days of incubation. In the other, fungal mycelia, before sporulation, were broken into fine pieces using sterilized glass beads and inoculated in malt extract broth with 1% glucose. The dry weight of the inoculum was recorded. The cultures were incubated under 200 bar at 30°C and 5°C for 20 days. The biomass as fungal dry weight was determined as described above.

5.3.4 Adaptation of cultures not showing growth under elevated hydrostatic pressure using chemical additive in the growth medium

The fungal cultures which failed to grow at an elevated pressure of 50 bar (lowest hydrostatic pressure used during the screening at which the cultures were able to grow and survive) were tried to adapt to make them grow at elevated pressures using a chemical additive in the form of DMSO (Dimethyl sulfoxide) in the growth medium.

Four deep-sea fungal cultures were used for these studies. The medium used for this was MEB with 1% glucose, 1 % DMSO and 1% TTC (2,3,5-Triphenyl Tetrazolium Chloride). DMSO is known to increase stress tolerance [Komatsu *et al.*, 1991] while TTC was used as a vital stain [Bäckor & Fahselt, 2005]. A control medium was used in the form of MEB with 1% glucose. The culture in the form of fresh mycelia, just before sporulation, were inoculated in the above medium (15 ml each) in plastic bags and incubated under an elevated pressure of 200 bar at 30°C and 5°C. The bags were opened after 20 days of incubation in the above conditions and the results recorded microscopically and in terms of biomass produced.

5.3.5 Effect of elevated pressure on viability of spores

The viability of spores incubated under elevated pressure was checked using five cultures viz. A 4634, NIOCC 20, MTCC 479, A 6137 and A617. The spore suspensions were prepared as described previously and inoculated in MEB to give approximate concentration of 6×10^4 spores mL⁻¹ and incubated at 100, 200, 300 and 500 bar at 30°C. The viability of spores was checked after 10 days of incubation at the above mentioned hydrostatic pressures by spread plating the spore suspension on MEA plates and incubating at 1 bar / 30°C. The results were recorded in terms of CFU mL⁻¹.

5.3.6 Effect of low temperature on viability of spores

As the spores were not showing any germination at low temperature, the viability of spores at low temperature was determined. Four cultures used for this

study were A 4634, NIOCC 20, MTCC 479 and A617. The spore suspensions were prepared as described previously and inoculated in MEB to give approximate concentration of 6×10^4 spores mL^{-1} and incubated at 1 bar and 5°C . The viability of spores was checked randomly up to 16 days for all the cultures by spread plating the spore suspension on MEA plates and incubating at 1 bar/ 30°C . The results were recorded in terms of cfu mL^{-1} .

5.3.7 Effect of nutrient concentration on spore germination under elevated pressure

The effect of various nutrients and the concentration of the nutrients on the spore germination of the deep-sea and the terrestrial IMTECH cultures were studied. The nutrients were supplied in the form of MEB and sediment extract (described in detail in chapter 8) in different dilutions as 1:10, 1:20, 1:40, 1:60, 1: 80 and 1:100. The sediment extract was prepared from the deep-sea sediments obtained from Central Indian Basin. For preparation of sediment extract, 35 g of sediment was suspended in 100 mL of sterile seawater. To the sediment suspension, EDTA was added (final concentration of 1 %) [<http://www.ar.wroc.pl/~weber/ekstrak2.htm>] to facilitate the extraction of water-soluble nutrients into the seawater. The whole mixture was kept on a rotary shaker overnight. Next day, the suspension was filtered sequentially through sterile GF/F and 0.22μ duraphilic filter papers (Millipore, USA). This filtrate termed sediment extract was used for the further experiment. During the extraction process, antibiotics (penicillin 40,000 U/ 100 mL and ampicillin 0.1 g / 100 mL) were added to the sediment suspension to avoid bacterial growth.

Five cultures used for this were A 4634, NIOCC 20, MTCC 479, A 617 and A 6133. The cultures were inoculated on MEA and allowed to grow and sporulate at 1 bar / 30°C. After the cultures were properly sporulating, the spores were scrapped and suspended in sterile seawater with 2 % tween 80. The mixture was vortexed vigorously to get a homogeneous spore suspension with minimum spore clumps. The spore concentration was estimated by taking the spore count on a haemocytometer. These spores were inoculated in different dilutions of MEB and sediment extract as stated above to give a spore concentration of approximately 5×10^4 spores mL⁻¹. These were incubated at 1 bar for 20 and 24 h and at 500 bar for 10 days. At the end of the incubation period, the spores were examined microscopically for germination and the results recorded in terms of total number of spores showing germination.

Effect of some additives like sugars on the spore germination of these cultures was also determined. The sugars checked were glucose and sucrose. The spores were inoculated in MEB with 10, 20 and 40 % glucose and sucrose and incubated under the same conditions as described above. At the end of the incubation period, the germinated spores were counted microscopically and the results recorded in terms of total number of spores showing germination.

5.4 Results

5.4.1 Biomass production under elevated hydrostatic pressure

A large number of the fungi (109 in total) did not show growth when directly incubated under elevated pressures of 500, 400 and 300 bar. Spore germination was also not seen amongst all the cultures under elevated pressure. All the cultures failed

Table 5.2 Biomass produced by a few deep-sea fungi under different pressure and temperature conditions

Isolate #	Fungi	Biomass produced (mg dry wt.)		
		200 bar and 30°C	200 bar and 5°C	1 bar pressure and 30°C
A 4637	<i>A. terreus</i>	30.0	31.2*	134.5
A 4636	<i>A. terreus</i>	38.4	7.7	132.7
A 4634	<i>A. terreus</i>	18.3	19.6*	156.3
A 4633	<i>A. terreus</i>	20.8	19.8	121.3
A 4630	<i>Aspergillus</i> sp.	25.6	9.0	126.7
A 4628	<i>A. terreus</i>	10.0	13.7*	118.3
A 61 P10	<i>A. terreus</i>	20.6	12.9	125.7
A 61 P4	Unidentified	17.8	27.1*	128.2
A 4625	Unidentified	65.2	13.8	140.2
A 614	<i>A. terreus</i>	8.1	2.9	59.6
A 6137	Unidentified	15.4	6.5	71.6
A 3457	<i>Fusarium</i> sp.	21.1	29.6*	228.0
A 3449	<i>Fusarium</i> sp.	14.2	10.1	125.8
A 3441	Unidentified	31.5	38.9*	178.0
A 348	Non-sporulating	4.7	5.4*	64.3
A 3428	<i>Curvularia</i> sp.	4.5	19.3*	175.7
A 61 P63	Yeast	23.4	9.1	249.2
A 3426	Unidentified	21.0	22.2*	234.6
A 6136	<i>Aspergillus</i> sp.	20.0	9.1	169.2
A 6139	Unidentified	17.5	15.4	171.4
A 61 P64	Yeast	12.2	16.8*	150.7
A 6128	<i>Aspergillus</i> sp.	9.3	9.3	165.7
A 6126	<i>Aspergillus</i> sp.	9.8	8.9	203.4
A 3415	Unidentified	1.7	11.3*	105.3
A 3412	<i>Aspergillus</i> sp.	3.1	1.7	140.0

* Fungi showing better growth at 5°C than at 30°C under 200 bar pressure

to show germination when incubated at low temperature of 5°C at both 1 bar and elevated pressures. From the cultures showing growth at 200 bar, 27 isolates were used for biomass production. Amongst these, a few cultures (Table 5.2) were found to show better growth at 200 bar and 5°C than at 200 bar / 30°C.

5.4.2 Effect of type of inoculum on growth at elevated hydrostatic pressure

The deep sea *Aspergillus terreus* # A4634, showed a lot difference in the biomass produced when the inoculum was in the form of mycelia and spores (Table 5.3). The biomass produced by the culture was more or less similar at all the four incubation conditions when the inoculum was in the form of mycelia (inoculated just before sporulation sets in). When the spores were used as inoculi, there was a drastic reduction in the biomass produced at 5°C at both the pressures, 1 bar and 200 bar (Table 5.3).

Table 5.3 Biomass produced by deep sea *Aspergillus terreus* (# A 4634) at different hydrostatic pressures and temperatures when the inoculum used was in the form of mycelia and spores

	Biomass produced (mg)			
	Mycelial inoculum		Spore inoculum	
	1 bar	200 bar	1 bar	200 bar
30°C	17.6	13.4	15.7	11.6
5°C	12.4	8.9	0.41	0.2

5.4.3 Acclimatization of fungal isolates for growth at elevated hydrostatic pressure

Majority of the 109 fungal isolates that did not grow at 300 bar pressure showed growth at 50 bar pressure (Table 5.4). Decreasing numbers of fungi showed viability and growth when they were gradually subjected to higher pressure. Only 2

strains of *A. terreus* and the yeast # A 344 grew at 300 bar and only the latter grew at 400 bar pressure.

Table 5.4 Number of fungi surviving and showing active growth after exposure to sequential increase of hydrostatic pressure

Incubation pressure	Total fungi tested – 109	
	Surviving	Actively Growing
50 bar	106 (97 %)	74 (68 %)
100 bar	81 (74 %)	42 (39 %)
200 bar	22 (20 %)	16 (15 %)
300 bar	11 (10 %)	3 (3 %)
400 bar	3 (3 %)	1 (0.9 %)

All the incubations were done at room temperature. The numbers within bracket represent percentage values.

Most of the fungi used for this experiment were fungi isolated at 1 bar pressure from sediments that were enriched with 1/5 diluted malt extract broth and incubated at 300 bar pressure at 5°C for 20 days. Fungi that showed active growth were included in the numbers that show survival.

5.4.4 Comparative growth of deep-sea and terrestrial fungi under elevated hydrostatic pressure

The terrestrial cultures obtained from IMTECH also showed growth at 200 bar pressure at both 30°C and 5°C, but the biomass produced by these was less than the similar deep sea isolate of *Aspergillus terreus* (Table 5.5).

Table 5.5 Comparison of biomass produced by the deep-sea isolates, the terrestrial species obtained from Microbial Type Culture Collection (MTCC), Chandigarh, India and some shallow water cultures obtained from Lakshadweep coral reef slope

Culture	Biomass produced by the fungi (mg)			
	1 bar / 30°C	200 bars / 30°C	1 bar / 5°C	200 bars / 5°C
<i>Aspergillus terreus</i> MTCC # 279	12.7	6.2	4.7	6.6
<i>Aspergillus terreus</i> MTCC # 479	10.6	7.7	4.3	10.4
<i>Aspergillus sydowii</i> MTCC # 635	12.6	7.6	9.3	6.3
Shallow water non-sporulating form 1	16.1	0	5.0	9.3
Shallow water non-sporulating form 2	17.1	7.7	10.1	5.7
Shallow water non-sporulating form 3	13.3	4.9	6.7	3.4
Deep-sea isolates				
<i>A. terreus</i> (# A 4634)	11.2 (0.2)*	8.9 (0.8)	5 (0.3)	9.6 (1.1)
<i>Aspergillus</i> sp (#A 61 P4)	4.6 (1.0)	3.0 (0.3)	2.5 (0.6)	2.0 (0.2)
Unidentified (#A 3415)	10.5 (2.3)	8.0 (0.8)	6.2 (16.0)	5.0 (0.2)
<i>Aspergillus</i> sp (# A 6128)	10.0 (3.0)	3.3 (1.0)	1.9 (0.3)	1.6 (0.2)
<i>Cladosporium</i> sp (#A 6136)	3.7 (0.2)	0.9 (0.1)	1.4 (0.2)	1.5 (0.2)
Non-sporulating (# A 3428)	1.9 (0.3)	0.4 (0.3)	0.7 (0.2)	0.6 (0.2)
Orange yeast (#A 61P63)	1.9 (0.2)	1.3 (0.2)	1.5 (0.3)	1.3 (0.1)
Off-white yeast (#A 344)	6.6 (0.7)	1.0 (0.7)	3.3 (0.4)	1.7 (0.8)

Mycelia, before sporulation used as the inoculum; figures within brackets represent standard deviation

5.4.5 Germination of spores of deep sea and terrestrial cultures at elevated hydrostatic pressure and low temperature

The deep sea *Aspergillus terreus* (A 4634) showed germination at elevated pressure of 200 bar at 30°C (Table 5.6), but failed to germinate at 5°C (Fig. 5.1). Out of the remaining 3 terrestrial isolates, two cultures showed negligible (1 % and 0.5 %) germination and the third one failed to show germination. All 3 cultures failed to show germination at 5°C under 1 bar and 200 bar pressure.

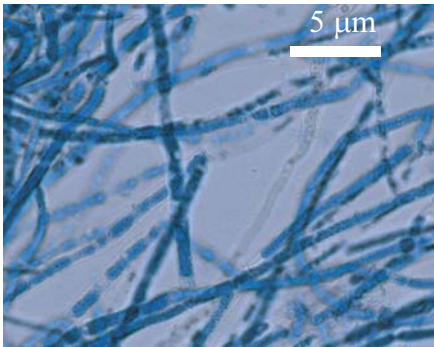
Table 5.6 Germination of spores of the deep-sea fungus # A4634 and the terrestrial species from Microbial Type Culture Collection (MTCC), Chandigarh, India

Culture	% germination			
	200 bar / 30°C	200 bar / 5°C	1 bar / 30°C	1 bar / 5°C
<i>Aspergillus terreus</i> MTCC 279	1		92	
<i>Aspergillus terreus</i> MTCC 479	0.5		94	
<i>Aspergillus sydowii</i> MTCC 635	0	No germination	91	No Germination
<i>Aspergillus terreus</i> - deep sea isolate A 4634	66		94	

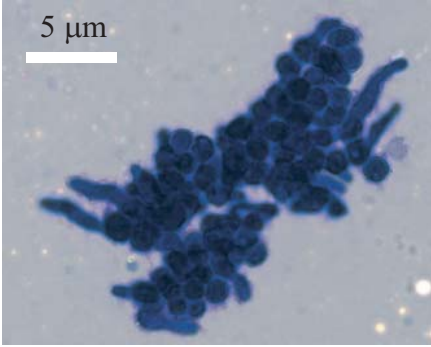
5.4.6 Effect of additive in the growth medium on growth of deep sea cultures at elevated hydrostatic pressure

The addition of DMSO did not show significant difference in the biomass production, under different conditions of growth in all the isolates tested (Table 5.7). Generally, with increasing hydrostatic pressure, there was a decrease in biomass production in all the isolates. Only the different growth conditions influenced the biomass production significantly (Table 5.8).

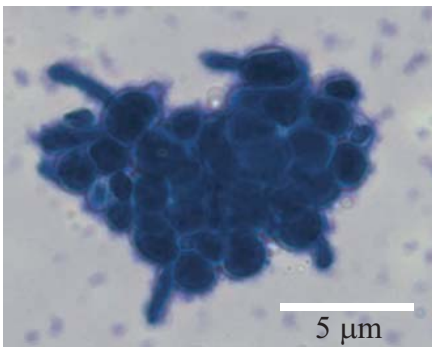
Fig. 5.1 Germination of spores of deep sea *Aspergillus terreus* at different hydrostatic pressures and temperatures.



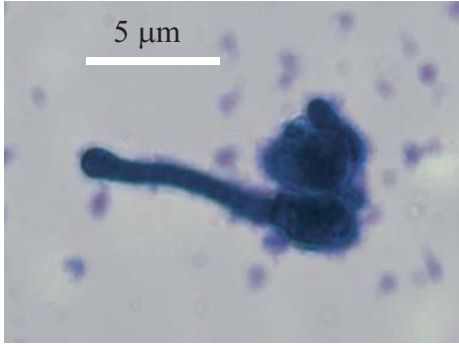
30°C and 1 bar



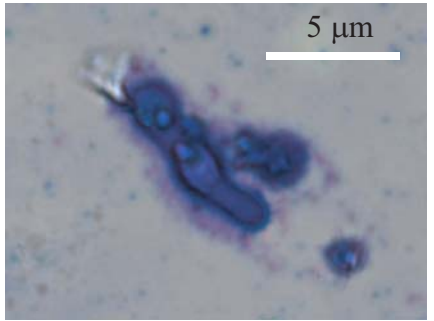
30°C and 50 bar



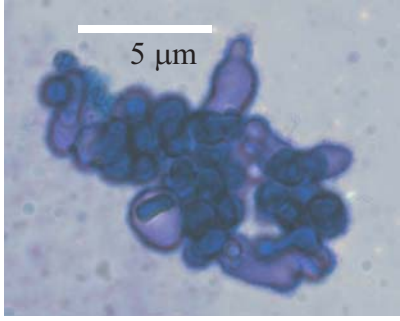
30°C and 100 bar



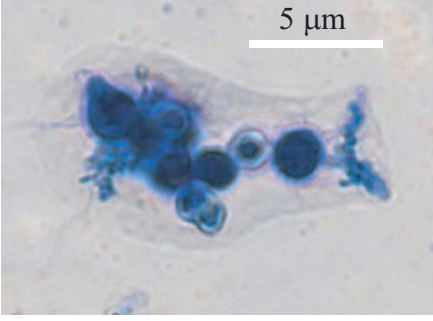
30°C and 200 bar



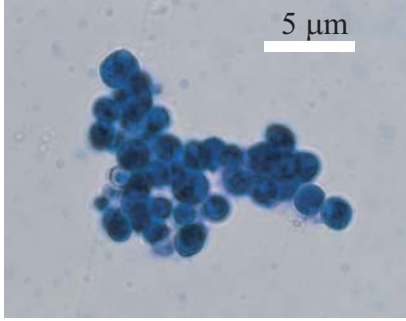
5°C and 1 bar



5°C and 50 bar



5°C and 100 bar



5°C and 200 bar

**Table 5.7 Biomass (mg mL⁻¹) produced by fungal isolates
in the presence of DMSO**

Incubation conditions	A 6136		A 6126		A 6127		A 6134	
	MEB	MEB + DMSO	MEB	MEB + DMSO	MEB	MEB + DMSO	MEB	MEB + DMSO
200 bar / 30°C	0.33	0.23	0.06	0.10	0.03	0.17	0.05	0.05
200 bar / 5°C	0.23	0.63	0.21	0.13	0.11	0.44	0.13	0.03
1 bar / 30°C	2.49	4.12	4.41	3.97	8.58	4.39	7.55	7.47
1 bar / 5°C	0.29	0.41	0.22	0.17	0.28	0.31	0.21	0.37

**Table 5.8 Statistical interpretation of the effect of DMSO on biomass production
by the deep-sea isolates under different incubation conditions of
pressure and temperature**

Culture		Df	F critical	F - value	P - value
A 6136	Growth conditions	3	9.277	14.621	0.027
	Presence of DMSO	1	10.128	1.76	0.277
A 6126	Growth conditions	3	9.27663	366.271	0.00024
	Presence of DMSO	1	10.128	1.574	0.2985
A 6127	Growth conditions	3	9.2766	8.2427	0.0584
	Presence of DMSO	1	10.128	0.715	0.4599
A 6134	Growth conditions	3	9.2766	3892.303	0.000006
	Presence of DMSO	1	10.128	0.0072	0.9379

* F-value greater than F critical indicates statistical significance in the parameters

5.4.7 Effect of elevated hydrostatic pressure on germination of spores

The elevated pressure decreased the germination rate of all the deep-sea as well as terrestrial isolates, except in few cases. The deep-sea isolate # NIOCC 20 showed highest germination rate at all the pressure used, closely followed by # A 617 (Table 5.9). Surprisingly, the terrestrial *A. terreus* (MTCC 479) showed better germination rate than the deep-sea *A. terreus* (# A 4634). In the same experiment, the

Table 5.9 Germination of different fungal isolates under elevated hydrostatic pressure

Incubation pressure (bar)	Germination of spores (%)				
	A 4634	NIOCC 20	A 6137	A 617	MTCC 479
100	10	28.5	15.7	22.1	21
200	9.2	25.1	4.2	20	19
300	4.6	16.4	12.8	11.5	4.5
500	3	7.8	4.2	5.2	6.4

* The germination results were recorded by taking haemocytometer counts after 10 days of incubation at the above-mentioned pressures at 30°C.

viability of spores decreased with increasing pressure. The deep-sea isolates showed a lot of variability amongst themselves. The spores of the deep-sea isolate # A 4634 showed highest viability at all the pressures tested (Table 5.10). The viability decreased gradually from 92.3 to 12.8 % at 100 and 500 bar respectively in case of # A 4634, while in case of other deep-sea and terrestrial isolates, the viability was not much affected by the incubation pressure, but was on the lower side.

Table 5.10 Viability of spores incubated under elevated hydrostatic pressure

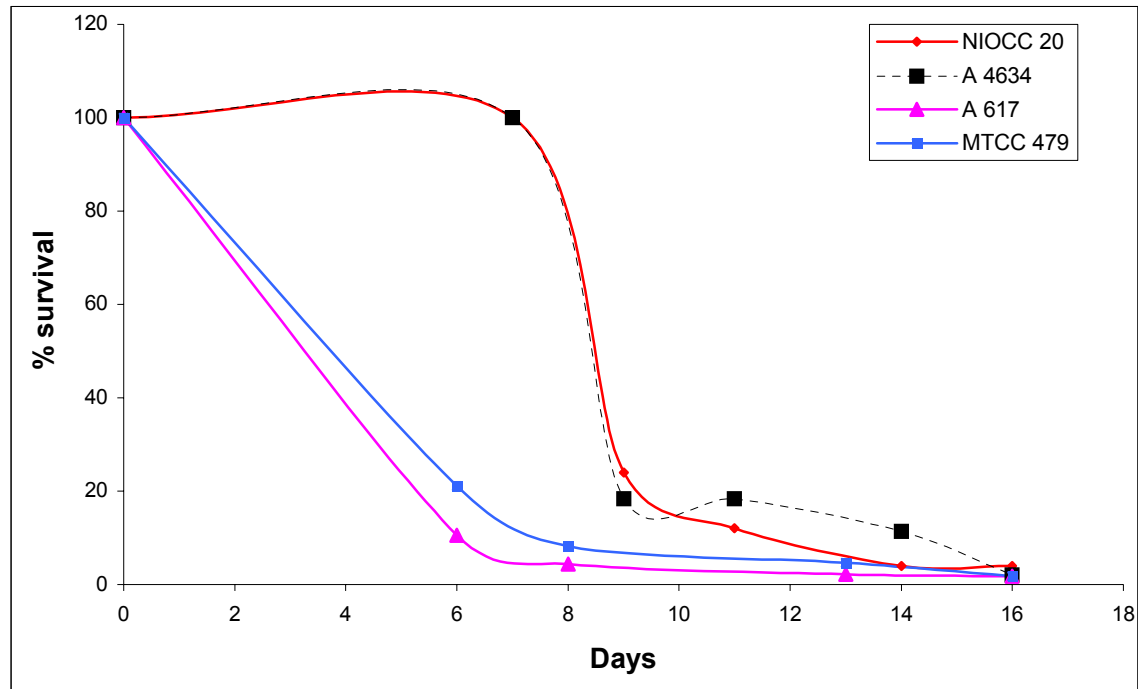
Incubation pressure (bar)	Viability of spores (%) based on CFU mL ⁻¹ on MEA				
	A 4634	NIOCC 20	A 6137	A 617	MTCC 479
100	92.3	14.2	9.6	13.2	14.3
200	86.9	14.2	5.4	16.8	25.2
300	62	12.1	7	14.7	21.8
500	12.8	7.1	11.5	7.4	24

* After the incubation period of 10 days at the above-mentioned hydrostatic pressures at 30°C, the aliquots were spread plated on MEA plates and the CFU counts recorded after an incubation of 2 to 3 days at 30°C.

5.4.8 Effect of low temperature on viability of spores

The viability of spores decreased over a period of time at 5°C. The deep-sea cultures A 4634 and NIOCC 20 showed 100 % viability up to 8 days while the terrestrial culture MTCC 479 and the other deep sea isolate A 617, showed a reduction

Fig. 5.2 Viability of spores incubated at low temperature over a temporal scale



in viability by more than 80 % on 6th day itself (Fig 5.2). There was a drastic drop in viability for NIOCC 20 and A 4634 by 7th to 9th day by 80 % after which the viability went on decreasing gradually, to 4 and 2 % respectively on the 16th day. The other two cultures showed more or less similar viability by the 16th day (1.7 and 1.8 %).

5.4.9 Effect of nutrient concentration on germination of spores

The spore germination was not affected by the nutrient concentrations in the sediment extract at 1 bar/ 30°C. There was a random increase or decrease in the germination percentage of the spores of both the deep sea as well as the terrestrial isolate (Table 5.11).

Table 5.11 Effect of nutrient concentration on spore germination – Sediment extract dilutions

Culture #	Dilutions					
	1:10	1:20	1:40	1:60	1:80	1:100
NIOCC 20	100	90	100	64	99	91
A 4634	80	71	69	67	82	59
A 617	63	73	50	73	84	77
MTCC 479	62.5	57	48	60	69	93

* The spores were incubated at 1 bar / 30°C for 24 h and the % germination of spores calculated (mentioned in the columns). There was no germination at 500 bar / 30°C after 10 days of incubation, but the spores were showing swelling, a sign of initiation of germination.

5.5 Discussion

Zobell & Johnson [1949] and Yamasato *et al.* [1974] have shown that yeasts isolated from the terrestrial and marine environments grow at elevated hydrostatic pressure, with an upper limit of approximately 500 bar. However, they grew the cultures in closed glass tubes, and only the oxygen dissolved in the medium was available for the growth of the cells. This was also true for other methods used for bacteria such as cultivation in thick-walled silicone tubing [Kosmoswosky, 1990; Groß *et al.*, 1994] and in plastic syringes [Yayanos *et al.*, 1981; Alongi, 1990]. Helmke [1980] demonstrated for actinomycetes that such cultivation techniques do not produce sufficient biomass for growth assessment. To overcome this limitation, Helmke cultured the actinomycete cells in polypropylene bags in a fluorocarbon-filled pressure vessel. Berger & Tam [1970] cultured aerobic bacteria in plastic bags but used water, which had less oxygen solubility, and due to no exchange of oxygen, the system becomes anaerobic very rapidly. In the present study also, polypropylene bags were used which resulted in a good yield of biomass, which was used for subsequent studies as reported in the later chapters.

Most of the isolates obtained were described to be terrestrial cultures based on their morphology. These could have blown from land by the winds as discussed in the previous chapter. These take some time to adapt to the conditions prevailing in the deep sea, and only a few sturdy forms are able to adapt and lead a normal life, the rest either die or remain in dormant but viable state. This could be the reason why the cultures did not grow when incubated directly under elevated pressure, but were showing growth when subjected to gradual increase in the hydrostatic pressure. This is similar to what might be occurring in the deep sea when the fungal hyphae or spores

are transported to the deeper parts, at a rate depending on the particle to which it is associated. Thus it can be said that slower the descent, more the chances for adaptation and thereby survival of the fungus. This is in concurrence with the hypothesis stated by [Turner](#) and [Raghukumar et al.](#) as discussed in the previous chapter.

The results indicated that the fungi should be able to grow up to 5°C at a depth of at least 3000 m (equivalent to pressure of 300 bar). But again as previously reported [[Jannasch, 1987](#); [Yayanos & Delong, 1987](#)], there was no correlation found between isolation depths of fungi and the maximum or optimum growth pressure for them.

Low temperature is known to decrease the metabolic activities and all the cellular processes. Reduced growth and low metabolic rates under deep-sea conditions are well studied in bacteria [[Wirsen & Jannasch, 1975](#)]. The spores were unable to germinate at low temperature although they were able to germinate at elevated hydrostatic pressures, thus showing that it is temperature, which is the limiting factor and not the high hydrostatic pressure. The viability of spores and thus in effect culture was also found to be affected by the pressure as well as by the low temperature explaining the low recovery of fungi from the deep - sea sediments as compared to Lakshadweep coralline sediments.

Although DMSO has been shown to increase the chances of growth under high hydrostatic pressure in case of *S. cerevisiae* [[Komatsu et al., 1991](#)], that was not the case here. DMSO did not seem to affect the biomass produced under elevated pressure. Even the spore germination did not show much effect in the presence of DMSO. This showed that DMSO may be effective osmoprotectant for yeast cells as reported previously, but not in the present studies. [Komatsu et al. \[1991\]](#) have shown

that addition of DMSO helps in structurization of water molecules which prevents yeast cells from hydrostatic pressure injury.

The spore germination doesn't seem to be affected by the concentration of the nutrients as recorded in the experiments in these studies. Thus, in the deep-sea sediments, sudden increase or decrease of nutrients (Feast & Famine) in the vicinity may not affect the germination of spores. This could be also due to the fact that the metabolic activities of the spores are much lower than those of mature hyphae, hence less requirement of nutrients [[Hawker & Madelin, 1976](#)].

To answer the questions about how cells respond to changes in hydrostatic pressure, a new research field called baro-(piezo-) physiology was developed. In this field, intermolecular interactions among various biological molecules, aspects of cellular metabolism and chemical reactions with large volume changes are the different topics of interest. In the next chapter we will deal with two of these aspects viz. stress proteins and an osmoprotectant, trehalose.

Chapter 6

Adaptation of Fungi to Deep-sea Conditions

6.1 Introduction

The deep-sea microbial community is very complex as described in the previous chapters. Fast sinking particles from surface water contain surface bacteria, cyanobacteria, phytodetritus, etc. These particles can reach a depth of 4500 m in only 4 to 6 weeks [Turley, 1995]. Thus, there must be numerous '1-bar-adapted' organisms as well as high-pressure adapted organisms at the bottom of the sea. Additionally, large number of non-peizophiles, the growth rate of which are reduced by elevated hydrostatic pressure, are present in the deep-sea sediments. Therefore to understand the complete diversity of microbial life under deep-sea conditions, we must consider the survival strategies not only of peizophilic organisms but also of 1 - bar - adapted organisms.

Hydrostatic pressure in the range of 300-500 bar usually inhibits the growth of various organisms. The basis of all pressure effects arises from the single fact, that is, the change in the system volume that accompanies a biological reaction. When a reaction is accompanied by a volume increase, it is inhibited by increasing pressure. When a reaction is accompanied by a volume decrease, increasing pressure enhances it. For example, dissociation of ribosome subunits is significantly enhanced by increasing pressure because solvation of charged groups is accompanied by a volume decrease [Abe & Horikoshi, 2001].

DNA synthesis is one of the most pressure-sensitive cellular processes, with the initiation of DNA replication rather than polymerization itself being particularly sensitive. By contrast, RNA synthesis is relatively pressure resistant and occurs at pressures up to 680 bar [Yayanos & Pollard, 1969]. Protein synthesis, however, is highly sensitive to elevated pressure. Ribosomes associated with both mRNA and

tRNA show perfect stability at elevated pressures up to 1000 bar. However, uncharged ribosomes (ribosomes without mRNA or tRNA) become dissociated at pressures greater than 600 bar [Gross, 1993]. Hence, the destabilization of uncharged ribosomes is one of the factors limiting cell growth under conditions of high hydrostatic pressure. In this sense, piezophiles which can grow at pressures > 600 bar, are likely to have undergone critical changes in their ribosomal proteins.

In *Escherichia coli*, a pressure of 546 bar induces a unique stress response that results in higher levels of heat-shock proteins (Hsps) or cold-shock proteins (Csps), as well as many proteins that are produced only in response to high pressure [Welch *et al.*, 1993]. DnaK and GroEL, which are molecular chaperones [Craig & Gross, 1991; Hartl, 1996], are induced at 546 bar. In the yeast *Saccharomyces cerevisiae*, an extreme pressure of 1500 bar causes severe damage to membrane integrity and protein structure, resulting in cell death [Kobori *et al.*, 1995]. It has been found that a short period of heat shock treatment allows the cells to survive at this lethal level of pressure [Iwahashi *et al.*, 1991]. A molecular chaperone, Hsp 104, and intracellular trehalose are known to play a role in cell survival at extremely high pressures [Iwahashi *et al.*, 1997].

Studies aimed at ascertaining what happens intracellularly as hydrostatic pressure is applied to living cells can provide an insight into the dynamics of metabolic events that occur in response to pressure. In one such study, the intracellular pH in *S. cerevisiae* was measured under high hydrostatic pressure using pH sensitive fluorescent probes [Abe, 1995 & 1997]. Hydrostatic pressure promotes the acidification of vacuoles in a manner dependent on the magnitude of the pressure

applied, up to 600 bar. Pressure-induced vacuole acidification is caused by the production of carbon dioxide [Abe, 1997].

Because the effect of pressure on membrane structure is considerable, the function of membrane proteins must also be affected by increasing pressure. *Photobacterium fundum* SS9 has been investigated genetically in great detail, particularly the synthesis of the outer membrane proteins OmpH and OmpL. OmpH is most abundant when SS9 is grown at its pressure optimum of 280 bar, whereas OmpL is produced in the greatest quantity at 1 bar [Bartlett *et al.*, 1989; Welch & Bartlett, 1996]. OmpH is thought to function as a nutrient transporter in nutrient-limited environments such as the deep sea.

The significance of nutrient availability was genetically documented in *S. cerevisiae*, the growth of which is inhibited by high pressure. Under high-pressure conditions, the uptake of tryptophan via the high-affinity tryptophan permease TAT2 is impaired and the expression of TAT2 is down regulated, leading to growth arrest [Abe & Horikoshi, 2000]. These results are consistent with those of transport studies in marine bacteria, which indicate that the rate of uptake of many substrates, including glutamate and acetate is low at a pressure up to 180 bar. Interestingly, the addition of excess tryptophan or over expression of TAT2 protein enables *S. cerevisiae* cells to grow at 250 bar [Abe & Horikoshi, 2000]. These results suggest that the uptake of tryptophan is one of the most pressure-sensitive processes in living yeast cells.

There is evidence that the composition and structure of the lipid bilayer of the cell membrane, respiratory proteins and a membrane-localized signaling system contribute significantly to baro- (peizo-) adaptation. However the main difficulties in

understanding pressure regulated metabolic processes in living cells are that --- 1) the effects of pressure on living organisms are usually analyzed after decompression and; 2) it is still difficult to predict the orientation of a given metabolic pathway at elevated pressure. Therefore direct measurements under high-pressure conditions are required to investigate the association and/or dissociation of proteins, metabolic flux and electron transfer through respiratory system [[Abe et al., 1999](#)].

The fungal stress response often involves the production of various protective molecules [[Jennings, 1993](#)] and these form complexes with essential enzymes keeping them functional.

Due to all these facts, there is a great interest in elucidating the mechanisms of high-pressure adaptation in peizophiles. However, even non-peizophiles respond to changes in hydrostatic pressure. These organisms may well be able to place themselves in a state of suspended animation without significant loss of viability until the pressure is reduced and growth conditions become more favourable.

Exposure of both prokaryotic and eukaryotic cells and/or tissues to a variety of physiological stresses results in the rapid synthesis of a specific class of proteins. This phenomenon is known as ‘stress response’ and the newly formed transient proteins are termed as ‘stress proteins’. These proteins play a significant role in cells and tissues in manifestation of adaptation and may serve as defense mechanism against a variety of stress conditions. As the adaptation of other kinds of stress or shock condition is often mediated by the production of such specific stress proteins (e.g. heat shock, cold shock or antifreeze proteins), a suitable approach to high-pressure response would be to look for alterations in the protein patterns of microorganisms grown at different hydrostatic pressures, ranging from atmospheric pressure to the limit of viability.

Stress proteins were first described in cells from *Drosophila melanogaster* in response to exposures to high temperatures [Ritossa, 1962], and so the term ‘Heat Shock Proteins’ (Hsp) was coined. Since then a range of environmental stresses have been shown to induce heat shock proteins, and the term ‘Stress Proteins’ has subsequently been used to describe these proteins. The environmental stresses, which induce these proteins, include trace metals [Sanders *et al.*, 1991], changes in osmolarity [Kultz, 1996], hypoxia/ anoxia [Mestril *et al.*, 1994; Myrmel *et al.*, 1994] and exposure to UV radiation [Nepple & Bachofen, 1997]. These proteins are among the most highly conserved proteins in existence [Lindquist & Craig, 1988]. Either the stress proteins themselves or their close relatives present in all organisms at normal growth conditions play vital roles in normal cell function also.

In all organisms, the induction of Hsps is remarkably rapid and intense showing that it is an emergency response [Daniels *et al.*, 1984; Kimpel & Key, 1985; Li & Laszlo, 1985] and there is a striking relationship between the induction condition and the organism’s environment. For example, there are many dimorphic pathogens that cycle between relatively cool temperatures in one phase of their life cycle and the warmer temperature of their mammalian hosts in another phase. This change in temperature is accompanied by the strong induction of Hsps in both prokaryotic and eukaryotic pathogens.

In recent years, major focus on the role of the stress proteins has been mostly in -

1. Cell biology in regulatory concerns of protein folding-unfolding and transport.
2. Immunobiology of the involvement in the immune response during tissue or cell damage and infectious diseases

3. Pathophysiology and medicine on the effect of stress response in human disease.

At present, the stress proteins are classified into six different classes as –

1. **Hsp 110 kDa and above** – Most of the eukaryotes produce proteins of greater than 100 kDa in response to high temperatures. This is a constitutively expressed protein in eukaryote. Its synthesis increases about 5-fold after stress conditioning. The synthesis of the protective heat shock produced in response to high temperature is regulated by the 110 kDa heat shock transcription factor (hstf).
2. **Hsp 90** – This group is one of the most abundant cytoplasmic proteins in most of cells even under normal conditions. This is found in association with several cellular proteins. Association with Hsp 90 either enhances or inhibits the normal function of these proteins. The normal abundance of Hsp 90 may limit the extent to which its synthesis may be induced on exposure to stress.
3. **Hsp 70** – The gene HSP 70 encodes the abundant heat inducible 70 kDa Hsp. HSP 70 of most eucaryotes is a member of a multigene family whose genes are expressed under a variety of physiological conditions. The Hsp 70 family accounts for a majority of the translational activities in cells in response to the environmental changes.
4. **Hsp 60 (or chaperonin)** – The major bacterial hsps belong to this class. It is also found in the mitochondria and chloroplasts of the eukaryotic cells. These may be associated transiently with the newly synthesized unfolded peptides. This class of proteins is also required in bacteriophage assembly and host DNA

replication at normal conditions. This is also called as Gro-EL in *E. coli* and in other related bacteria.

5. **Small Hsps** – The small Hsps are a very diverse group. These are 15 – 30 kDa, low molecular weight (LMW), stress proteins. These proteins are more species specific than the larger stress proteins and less conserved, with significant variation occurring within the same class of organism [Mansfield & Key, 1987]. The small Hsps share the property of being induced at specific stages in development at normal temperatures. These proteins are not synthesized under normal conditions. Their synthesis is regulated during development and differentiation is modified by environment [Ayme & Tissiers, 1985; Ingolia, 1982].
6. **Ubiquitin** – This is highly conserved 76-residue protein, which is found in all eukaryotic cells, and is induced by heat. Its synthesis is increased with increase in temperature. It has a complementary role to Hsp70 in resolubilizing and stabilizing proteins by targeting denatured proteins for degradation and removal.

Cold shock proteins (Csps) – These are relatively new types of stress proteins, first reported in *Escherichia coli*, viz. Csp A. Both eukaryotes and prokaryotes exhibit a cold shock response upon an abrupt temperature downshift [Thieringer *et al.*, 1998]. Membrane fluidity decreases with decreased temperature, affecting the membrane-associated cellular functions. Cold shock also causes stabilization of secondary structures in RNA and DNA resulting in reduced efficiency of translation, transcription and DNA replication [Phadtare *et al.*, 1999]. These deleterious effects are overcome by induction of cold shock proteins. Cold-shock-inducible proteins are the

key factors needed for cellular adaptation to lower temperature. Identification of cellular thermosensors and elucidation of the functions of the cold-shock proteins is essential for understanding cold-shock response and adaptation.

Stress proteins are studied by a variety of molecular and cell biology techniques like-

1. **Polyacrylamide gel electrophoresis** - The classical way to visualize the total protein expressed by a cell is two-dimensional IEF/SDS-PAGE as developed by O'Farrell [O'Farrell, 1975]. This method can, under favourable conditions, resolve more than 1000 proteins in one gel and needs only minimal amounts of cell extract. Proteins are separated according to their isoelectric point by isoelectric focussing in the first dimension and in the second dimension, it separates out according to molecular weight of the proteins. The technique has been successfully applied to investigate the pressure response of bacteria like *Escherichia coli* and *Thermotoga maritima*, filamentous fungi like *Asteromyces cruciatus* and *Dendryphiella salina*, and marine yeasts *Debaromyces hansenii*, *Rhodosporidium sphaerocarpum* and *Rhodotorula rubra* [Groß *et al.*, 1994].
2. **Northern blotting** – In this, the total RNA is extracted from the tissue/cells. This is subjected to agarose-formaldehyde or glyoxylic gel electrophoresis, and the RNA separated on nylon or nitrocellulose. After fixation, membrane is hybridized with desired labelled cDNA probes. Visualization can be done by radiography.

3. **PCR** – Since stress proteins are gene-induced products, the study of the structure of individual stress genes in living organisms is very important. Molecular cloning allows such study.
4. **Western blotting** – Western blotting describes the transfer of proteins from an electrophoresis gel to membrane (usually nitrocellulose), and immunoblotting (or immunoassay), the subsequent probing (detection) of these proteins using immunological techniques (i.e. the use of antibodies). Western blotting may be performed with chromophores or radio-labelled samples, either to compare the protein detected by immunoblotting with those labeled with radio-isotope, or because a particular sample is only available as a radiolabelled protein.

Trehalose as a stress protectant

One of the protective compounds known to be accumulated during stress is the sugar, trehalose (α -D-glucopyranosyl (1-1) - α -D-glucopyranoside) [d'Enfert & Fontaine, 1997]. Trehalose is a disaccharide composed of two glucose molecules bound by an alpha, alpha-1, 1 linkage. Since the reducing end of a glucosyl residue is connected with the other, trehalose has no reducing power. It is widely distributed in nature. It is known to be one of the sources of energy in most living organisms, including bacteria, fungi, insects, plants, and invertebrates. It is known to protect organisms against various stresses such as dryness, freezing, pressure, etc. Trehalose plays a key role in stabilizing membranes and other macromolecular assemblies under extreme environmental conditions. Apart from trehalose, other stress protectants reported in microorganisms are osmolytes like glycine betaine, ectoine, sucrose, mannitol and glycerol [DasSarma & Arora, 2001]. Organic osmolytes occur at high

levels in marine invertebrates. These are mostly free amino acids such as taurine, which are compatible with cell macromolecules and methylamines such as trimethylamine oxide (TMAO), which may have nonosmotic role as protein stabilizer, and present in higher amounts in many deep sea animals [Yin *et al.*, 2000]

Trehalose has been detected in a variety of organisms and is known to serve numerous functions [Crowe *et al.*, 1992]. In fungi, the role of trehalose has been subject to great debate but large amounts of evidence have established the disaccharide as a protectant of cellular structures during stress [Thevelian, 1996]. In yeast, the investigations of arguments for trehalose as a stress protectant are numerous. The survival of *S. cerevisiae* grown in 3M NaCl correlated significantly with intracellular levels of trehalose, while mutants unable to produce trehalose were more sensitive to the severe salt stress treatment [Hounsa *et al.*, 1998]. The accumulation of trehalose has also been seen in cells of *S. cerevisiae* grown media lacking in carbon, nitrogen, phosphorus or sulphur and in cells subjected to desiccation or freezing [Thevelian, 1996].

Whether trehalose is accumulated in all fungal species during stress and whether this accumulation is universal or relates to specific stress factors remains to be established. The regulated trehalose hydrolyzing enzymes have mainly been investigated in zygomycetes and yeasts even though higher fungi may be capable of regulating the level of cytosolic trehalose.

6.2 Objectives

The main objectives kept in mind while studying the adaptive response to pressure and temperature stress by the deep-sea fungi were –

1. To compare the difference in whole cell protein profiles of the cultures when grown at different temperatures and hydrostatic pressures.
2. To investigate the response of the cultures to hydrostatic pressure when exposed for different time durations
3. To estimate accumulation of trehalose by the cultures in response to exposure to different temperatures.

6.3 Methodology

6.3.1 Response of one bar grown cultures to elevated hydrostatic pressure and low temperature

The cultures used for these studies were those of 2 filamentous fungi (one deep sea *Aspergillus terreus* and one *Aspergillus terreus* from IMTECH) and one deep-sea yeast. The response of these cultures with respect to proteins produced under the stress of pressure and temperature was studied.

The studies were carried out in 2 different parts -----

1. Effect of elevated pressure when exposed for different time periods
2. Effect of different pressures for a fixed time period.

6.3.1.1 Effect of pressure for different time periods

The cultures were grown in malt extract broth (MEB) prepared in seawater for 2 to 3 days. Just before sporulation, the mycelia were homogenized and inoculated in MEB with 1% glucose in plastic bags and incubated under hydrostatic pressure of 300 bar at 30°C and 5°C, and at atmospheric pressure at 30°C and 5°C. After 6, 12, 24 and 48 hours of incubation at the above conditions, one bag of each was harvested and lyophilized for protein extraction. The second set of bags at the 300 bar was further incubated at atmospheric pressure at the respective temperatures, while the bags at

atmospheric pressure were continued to be incubated at the same conditions. After 6 days of incubation, the bags were opened, cultures harvested and lyophilized for protein extraction later.

6.3.1.2 Effect of different pressures for a fixed period (48 hours)

The cultures were grown in malt extract broth (MEB) prepared in seawater for 2 to 3 days. Just before sporulation, the mycelia were homogenized and inoculated in MEB with 1% glucose in plastic bags and incubated at 50, 100, 200, 300, 400 and 500 bar for 48 hours. After 48 hours of incubation at the above conditions, one bag of each was harvested and lyophilized for further protein extraction. The second set of bags at the 50 bar was further incubated at atmospheric pressure at the respective temperatures, while the bags at atmospheric pressure were continued to be incubated at the same conditions. After 6 days of incubation, the bags were opened, cultures harvested and lyophilized for further protein extraction.

From the biomass harvested in the above experiments, the total protein was extracted using ProteoPrep™ Sample Extraction Kit as described below. The total protein concentration in the extract was determined using Folin Phenol method and electrophoresed using SDS-PAGE and 2-D PAGE.

6.3.2 Protein extraction methods

6.3.2.1 Whole cell protein extraction

The protein was extracted from the lyophilized biomass using ProteoPrep Sample Extraction Kit (PROT-TOT, Sigma) ([Appendix A – III](#)). The protocol described by the supplier was used which is described herein. 100 mg of lyophilized,

powdered biomass was suspended in 2 mL of Reagent 4 (Cellular and Organelle Membrane Solubilizing reagent) and sonicated. During sonication care was taken that the temperature of the mixture did not rise above 30°C. The temperature should also not fall below 15°C since the urea and thiourea will precipitate out of the solution. The sonicated suspension was then centrifuged at 15,000 g for 30 minutes at 15°C. The supernatant was transferred into a clean tube and the cell debris was discarded. The supernatant was then reduced by adding tributyl phosphine to a final concentration of 5 mM and incubated at room temperature for 1 hour. After reduction, the sample was alkylated by adding iodoacetamide to a final concentration of 15 mM and incubating for 1.5 hours at room temperature. The final reduced and alkylated sample was centrifuged at 20,000 g for 5 minutes to pellet any insoluble material. This was the final protein extract. The total protein concentration of the samples was measured by reading absorbance at 280 nm.

6.3.2.2 Urea buffer extraction method

Urea sample buffer extraction method was followed. The lyophilized and powdered biomass (approx. 100 mg) was mixed with urea buffer ([Appendix A - IV](#)). The mixture was mixed vigorously and boiled for 2 minutes. Then the mixture was vortexed and again boiled for 1 minute. The mixture was cooled to room temperature, centrifuged and the supernatant collected. Total protein estimation in the supernatants was carried out using Folin phenol method [[Lowry et al., 1951](#)] ([Appendix A - V](#)). Ten µg protein of each extract was loaded on to 12 % resolving gel for electrophoresis [[Laemmli, 1970](#)] ([Appendix A - VI](#)). The electrophoresis was carried out at a constant 60V. After electrophoresis, the gels were stained as per silver staining

method [Heukeshovan & Dernick, 1985]. The gels were first fixed in a fixative solution for a minimum period of 60 minutes. The fixed gels were then dehydrated in methanol. The gels were rehydrated in 0.02 % $\text{Na}_2\text{S}_2\text{O}_3$. After the gels were rehydrated, a rinse with distilled water was carried out. The gels were then transferred to silver stain solution. After an incubation period of 30 minutes, the gels were rinsed thoroughly with distilled water to remove the residual unreacted silver stain. The developer was added to the gels and gently mixed till the protein bands appeared. After the intensity of the bands was sufficient enough for recording, the developing reaction was stopped by discarding the developer and adding the fixative solution once again (Appendix A - VII). The stained gels were photographed using Gel documentation system.

6.3.3 Intracellular protein profiles of cultures grown in different conditions of hydrostatic pressure and temperature

Two filamentous fungi, *Aspergillus terreus* from the deep-sea sediment (#A 4634) and from Microbial Type Culture Collection (MTCC 479), Chandigarh, India and one yeast isolate (# A344) obtained from the deep-sea sediments were used for this experiment. The cultures were inoculated in MEB and incubated under 1 bar / 30°C for 48 hours; in case of the yeast, they were incubated in a shaker water bath. After sufficient growth in MEB, these cultures were homogenized with glass beads and inoculated in MEB with 1 % glucose in sterile plastic pouches and incubated under following conditions -

1. 1 bar / 30°C
2. 1 bar / 5°C
4. 100 bar / 5°C
5. 500 bar / 30°C

3. 100 bar / 30°C

6. 500 bar / 5°C

The inoculum was harvested, lyophilized and kept as control.

After 20 days of incubation under the above conditions, the pressure vessels were depressurized as described previously and the biomass harvested, washed with sterile distilled water, lyophilized and used for preparing the total cell protein extracts. The proteins were extracted using the urea buffer extraction method. The total protein content in the extracts was measured using Folin phenol method. Ten µg protein of each extract was loaded on to 12 % resolving gel for electrophoresis [Laemmli, 1970] (Appendix A - VI). The electrophoresis was carried out at a constant 60V. After electrophoresis, the gels were stained by silver staining method described above. The stained gels were photographed using Gel documentation system (AlphaImager 2200, U.S.A).

6.3.4 Western blotting

The whole cell proteins extracted using the Sigma total protein extraction kit were used to detect the type of proteins produced in response to the stress of pressure and temperature by the fungal cultures. After electrophoresis, the proteins on the polyacrylamide gels were electroblotted onto nitrocellulose membranes using the electroblotting apparatus (Bio-Rad, USA). The blotting was carried out at 500V for 3 h in the transfer buffer (Appendix A - VIII). After transferring the proteins on to the membrane, the membrane was stained with Ponceau R in order to stain the marker and check for the presence and location of the proteins in the samples on the gel. After marking the positions with a lead pencil, the membrane was washed with TBST buffer (Appendix A - VIII) to remove the Ponceau stain. Then the proteins on the membrane

were blocked with blocking agent (5% milk) for 60 min. Again the membrane was washed with TBST buffer (10 min x 3 times). Primary antibody (anti Hsp 25 and anti Hsp 70) was then added on to the membrane and allowed to react for 60 minutes. The excess antibody washed away using TBST buffer (5 min x 3 times). Then the secondary antibody (goat anti-rabbit IgG – ALP, Sigma Chemicals, Cat. A 3687) was added on to the membrane and allowed a reaction time of 60 minutes. The excess secondary antibody was washed using TBST buffer (5 min x 3 times). The substrate for alkaline phosphatase, conjugated to secondary antibody, BCIP/NBT was added and the membrane gently shaken till purple bands appear. The excess substrate was immediately washed with distilled water and membrane allowed to dry, and the results recorded. The two anti – hsps used (anti-Hsp 25 & anti-Hsp 70) were from Stressgen, Canada (Cat. SPA – 810C & SPA 811 respectively).

6.3.5 Trehalose accumulation by yeast cells in response to elevated hydrostatic pressure and low temperature

Three yeast cultures were used for these studies. One of them was a filamentous yeast (A 344) and the other two were the orange yeasts (A61P35 & BC7E1) isolated from the deep-sea sediments.

To raise the inoculum, the cultures were inoculated in 20 mL sterile MEB and incubated at 30°C on a shaker for 48 hours. From these flasks, the cultures were inoculated to 100 mL MEB and incubated for a further 48 hours at 30°C on a shaker. These growing cultures were then added to sterile plastic pouches to be incubated under following conditions –

1. 100 bar / 30°C
2. 200 bar / 30°C
3. 300 bar / 30°C
4. 500 bar / 30°C
5. 1 bar / 30°C
6. 1 bar / 15°C
7. 1 bar / 5°C

One part of the culture, used for adding to the pouches was harvested by centrifugation, washed with distilled water and lyophilized for trehalose extraction.

The cultures were incubated at the above-mentioned conditions for a period of 18 hours, at the end of which, the cultures were harvested by centrifugation, washed with distilled water and lyophilized for trehalose extraction and estimation.

6.3.5.1 Trehalose extraction

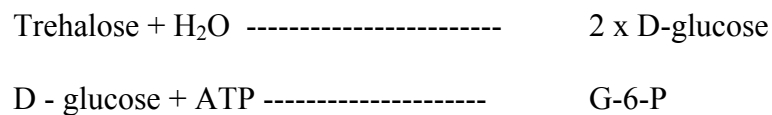
Trehalose Assay Kit, K-TREH, manufactured by Megazyme International Limited (Ireland) was used for the estimations. The assay is specific for trehalose, being linear over a range of 4 to 80 µg of trehalose per reaction. Before the estimation, it is essential to extract trehalose and remove the free glucose. Accurately, 100 mg of lyophilized, powdered biomass was weighed and suspended in 1 mL of hot water (~80°C). The contents were placed in a water bath at 80°C with intermittent mixing with vortexing, for 15 minutes, till the sample was completely dispersed. The final volume was made up to 1 mL again, wherever there was reduction in the volume. The contents were allowed to cool to room temperature. The samples were then centrifuged and the supernatant stored at 4°C till assayed.

Being a total cell extract, it may be having reducing sugars, which need to be removed before the trehalose content is estimated. For this, 0.2 mL aliquot of the solution was dispensed in a glass test tube and 0.2 mL of alkaline borohydride solution

was added to it. This was mixed vigorously and incubated at 40°C for 30 minutes to effect complete removal of reducing sugars to sugar alcohols. At the end of the incubation period, 0.5 mL of 200 mM acetic acid was added to the tube with vigorous stirring on a vortex mixer to remove the excess borohydride solution and adjust the pH to approx. 4.5. After 5 minutes, 0.2 mL of 2M imidazole buffer pH 7 was added to the mixture to adjust the pH to approximately 7. This was the final sample solution, which was used for the trehalose estimations.

6.3.5.2 Trehalose estimations

The samples treated as above were used for the trehalose estimations. Trehalose is hydrolyzed to D-glucose by trehalase, and the D-glucose released is phosphorylated by the enzyme hexokinase (HK) and ATP to glucose-6-diphosphate (G-6-P) with the simultaneous formation of ADP



In the presence of the enzyme glucose-6-phosphate dehydrogenase (G6P-DH), G-6-P is oxidized by NADP^+ to gluconate-6-phosphate with the formation of reduced NADPH.



The amount of NADPH formed in this reaction is stoichiometric with the amount of D-glucose and thus twice the amount of trehalose. It is the amount of NADPH that is measured by the increase in absorbance at 340 nm. For the estimations, following steps were followed,

Pipette into cuvettes	Blank	Sample
Distilled water (at ~ 25°C)	2.2 mL	2.0 mL
Sample solution	-	0.20 mL
Imidazole buffer	0.20 mL	0.20 mL
NADP ⁺ /ATP	0.10 mL	0.10 mL
HK/G-6-PDH	0.02 mL	0.02 mL
Mix, read the absorbances of the solutions (A ₁) after approximately 5 minutes and start the reactions by addition of:		
Trehalase suspension	0.02 mL	0.02 mL
Mix, read the absorbances of the solutions (A ₂) at the end of the reaction (approximately 5 minutes). If the reaction has not stopped after 5 minutes, continue to read the absorbances at 2 minutes intervals until the absorbances remain the same over 2 minutes		

The absorbance difference (A₂ – A₁) for both blank and sample were calculated. The absorbance difference of blank was subtracted from the absorbance difference of the sample, which is ΔA_{trehalose}. The concentration of trehalose was calculated as:

$$C = \frac{V \times MW}{\epsilon \times d \times v \times 2} \times \frac{1.1}{0.2} \times \Delta A_{\text{trehalose}}$$

where, V = final volume (mL)

MW = molecular weight of trehalose (g/mol)

ε = extinction coefficient of NADPH at 340 nm
= 6300 [l x mol⁻¹ x cm⁻¹]

d = light path [cm]

v = sample volume [mL]

2 = 2 molecules of D-glucose released from each molecule of trehalose hydrolyzed

1.1 / 0.2 = 0.2 mL of the sample extract is treated with borohydride and the final volume after treatment and neutralization is 1.1 mL
 For trehalose, this can be solved as,

$$C = \frac{2.54 \times 342.3}{6300 \times 1 \times 0.2 \times 2} \times \frac{1.1}{0.2} \times \Delta A_{\text{trehalose}}$$

$$= 1.8976 \times \Delta A_{\text{trehalose}}$$

From this, the content of trehalose in terms of g / 100 g of biomass was calculated based on the amount of biomass used for the extraction.

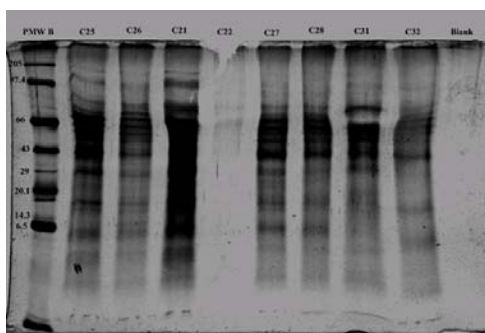
6.4 Results

6.4.1 Protein profiles of normal and stressed deep-sea culture

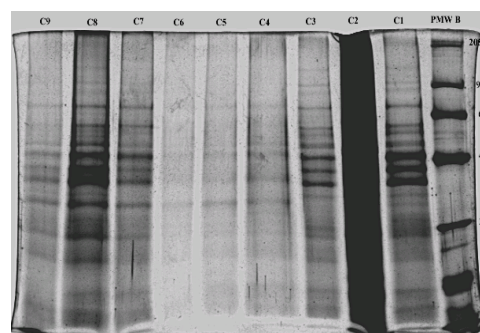
The protein profile for the deep-sea yeast culture # A 344, grown under elevated pressure for 48 h, and given a pressure shock of 48 h and again grown at 1 bar pressure for 96 h, was obtained after electrophoresing the protein extracts. There were subtle differences visible to naked eye in the protein profiles (Fig. 6.1) obtained from cultures grown under different conditions; hence the profiles were reconstructed to get a diagrammatic representation. This was done using the AlphaImager FC 2200 software and the Band Scoring analysis method therein (Fig. 6.2a - 6.5b). The protein profiles of the deep-sea yeasts are given here.

50 bar: The number of proteins detected was more under the elevated pressure of 50 bar at both 30°C and 5°C, and at 1 bar/5°C than at 1 bar/30°C (Fig. 6.2a). Quite a large number of low molecular weight proteins were detected in the 48 hours old culture grown at 50 bar/30°C (lane 4; positions 2, 4, 6, 7, 8 & 11), 50 bar/5°C (lane 3;

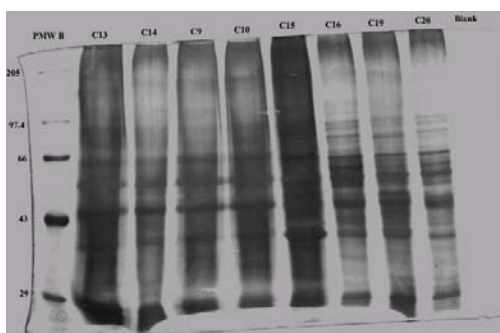
Fig. 6.1 Protein profiles (SDS PAGE) of deep sea yeast # A 344 grown at different hydrostatic pressure and given a pressure shock during growth



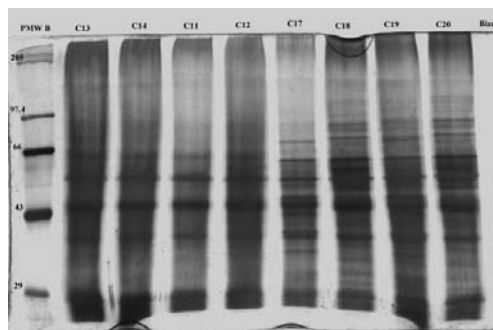
Corresponding to Fig. 6.2a & 6.2b



Corresponding to Fig.6.3a & 6.3b



Corresponding to Fig.6.4a & 6.4b



Corresponding to Fig.6.5a & 6.5b

Lane No.	Gel I	Lane No.	Gel II	Lane No.	Gel III	Lane No.	Gel IV
1	3 to 205 kDa	1	200 bar / 30°C – 48 h	1	3 to 205 kDa	1	3 to 205 kDa
2	1 bar / 30°C – 48 h	2	100 bar / 5°C – 144 h	2	1 bar / 30°C – 48 h	2	1 bar / 30°C – 48 h
3	1 bar / 5°C – 48 h	3	100 bar / 30°C – 144 h	3	1 bar / 5°C – 48 h	3	1 bar / 5°C – 48 h
4	50 bar / 30°C – 48 h	4	100 bar / 30°C – 48 h & 1 bar / 30°C - 96 h	4	200 bar / 30°C – 48 h	4	300 bar / 30°C – 48 h
5	50 bar / 5°C – 48 h	5	100 bar / 5°C – 48 h & 1 bar / 5°C - 96 h	5	200 bar / 5°C – 48 h	5	300 bar / 5°C – 48 h
6	50 bar / 30°C – 48 h & 1 bar / 30°C - 96 h	6	1 bar / 5°C – 48 h	6	200 bar / 30°C – 48 h & 1 bar / 30°C - 96 h	6	300 bar / 30°C – 48 h & 1 bar / 30°C - 96 h
7	50 bar / 5°C – 48 h & 1 bar / 5°C - 96 h	7	1 bar / 30°C – 48 h	7	200 bar / 30°C – 48 h & 1 bar / 30°C - 96 h	7	300 bar / 30°C – 48 h & 1 bar / 30°C - 96 h
8	1 bar / 30°C – 144 h	8	100 bar / 5°C – 48 h	8	200 bar / 30°C – 144 h	8	300 bar / 30°C – 144 h
9	1 bar / 5°C – 144 h	9	100 bar / 30°C – 48 h	9	200 bar / 5°C – 144 h	9	300 bar / 5°C – 144 h
10.	Extraction Reagent Blank	10	3 to 205 kDa	10.	Extraction Reagent Blank	10.	Extraction Reagent Blank

*In all the above gels, the differences in the protein profile were not easily visible to the naked eye, hence to understand the picture better, the profiles were reconstructed to get diagrammatic representations using Alpha Imager FC 2200 software and the Band Scoring analysis method therein. In Fig. 6.2a to 6.8b, the protein bands are labeled serially in each lane from top to down and these serial numbers do not correspond to the numbers in the different lanes. The molecular weight is denoted by the colours of the protein band.

Fig. 6.2 a & b Protein profile of deep sea yeast # A344 grown at 50 bar pressure

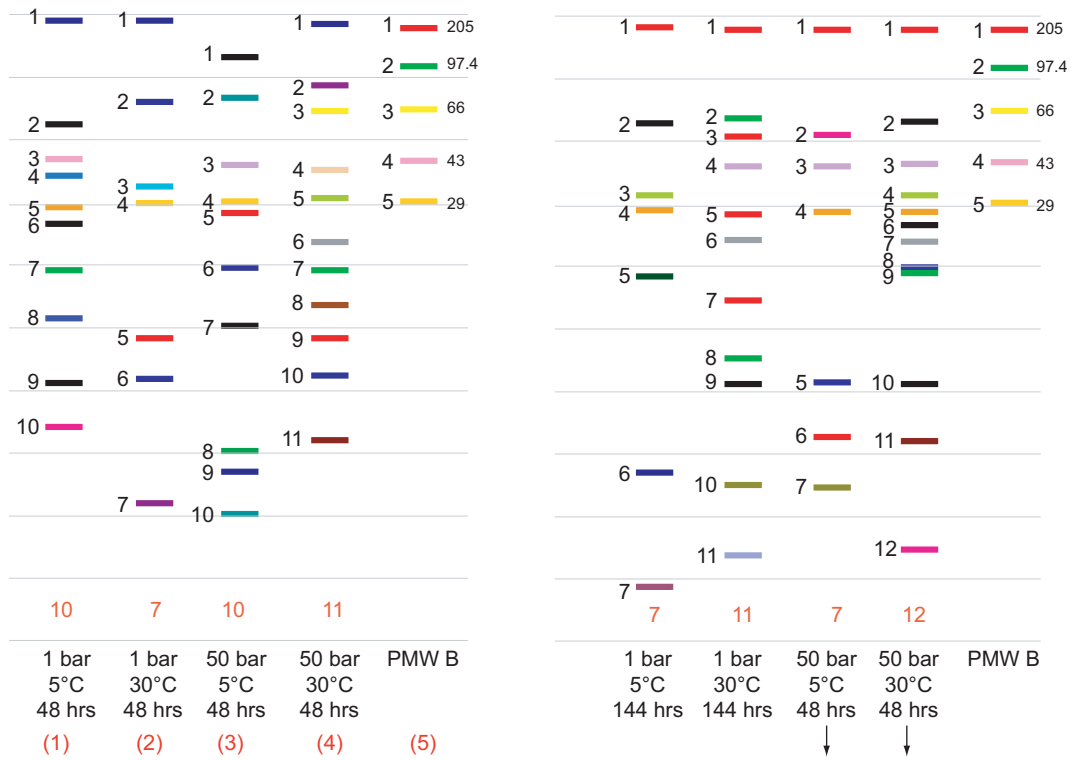


Fig. 6.2a

Fig. 6.2b

Fig. 6.3 a & b Protein profile of deep sea yeast # A344 grown at 100 bar pressure

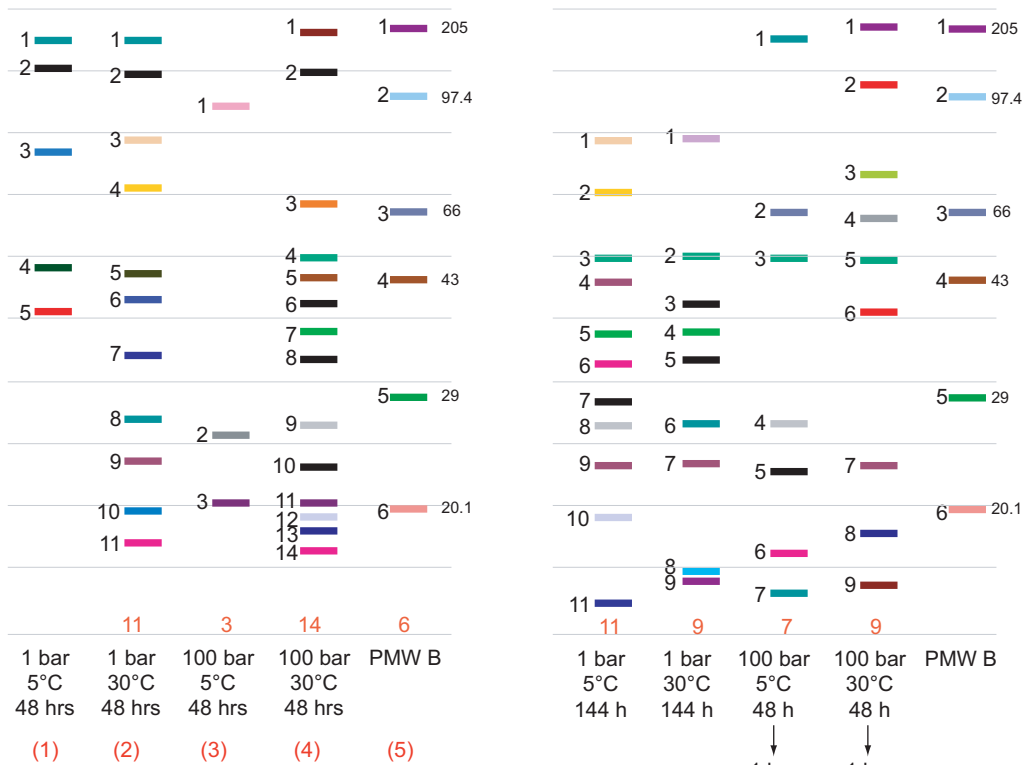


Fig. 6.3a

Fig. 6.3b

positions 1, 3, 6, 7, 8, 9) and 1 bar/5°C (lane 1; positions 3, 4, 6, 7, 8 & 10) as compared to 1 bar/30°C (lane 2; Fig. 6.2a). The culture when grown at 50 bar for 48 h at 30°C and again allowed to grow at 1 bar/30°C for 96 h showed newly expressed proteins (with reference to 1 bar/30°C) at positions 4, 6, 8, 9 & 11 (lane 4; Fig. 6.2b) as compared to the culture grown at 1 bar/30°C for 144 h (lane 2), the total duration of the incubation. Similar pressure shock given at 5°C and then grown at 1 bar/5°C, showed new proteins at positions 3, 5 & 6 (lane 3; Fig. 6.2b) as compared to the culture grown at 1 bar/5°C for 144 h (lane 1). The proteins at positions 4, 6, 7, 8, 9 & 11 of the 1 bar/ 30°C (144 h, lane 2) grown culture were not seen in the culture grown at 1 bar/5°C for 144 h (lane 1; Fig. 6.2b).

100 bar: The culture grown at 100 bar / 30°C for 48 h showed large number of new proteins expressed at positions 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 13 & 14 (lane 4; Fig.6.3a). Almost same situation was seen in the 1 bar grown culture, with the culture grown at 30°C (lane 2) showing more number of proteins than the one grown at 5°C (lane 1; Fig. 6.3a). The culture when given a shock of 100 bar at 30°C for 48 h and allowed to grow at 1 bar/30°C for 96 h, showed new proteins at positions 2, 3, 4 & 8 (lane 4) as compared to the culture grown at 1 bar/30°C for 144 h (lane 2; Fig. 6.3b). The culture when given a shock of 100 bar at 5°C and allowed to grow at 1 bar/ 5°C for 96 h (lane 3; Fig. 6.3b) showed a completely new protein of high molecular weight at position 1 as compared to the culture grown at 1 bar/5°C for 144 h (lane 1; Fig. 6.3b), while the rest of the proteins were detected in more or less same region with probable shifts; while the proteins seen at positions 4, 7 & 9 at 1 bar/5°C (lane 1) were not seen in the 100 bar shocked culture (lane 3; Fig. 6.3b).

200 bar: The 48 h old culture at 1 bar/ 30°C (lane 2; Fig. 6.4a) showed maximum number of detectable proteins as compared to the 1 bar/ 5°C (lane 1) and the pressurized cultures at 200 bar (lane 3). The proteins at positions 1, 3, 6, 8, 10 & 13 at 1 bar/30°C (lane 2; Fig. 6.4a) were not seen at 200 bar/ 30°C, while the proteins at positions 2, 3, 6, 8, 9 & 10 at 200 bar/5°C (lane 3) were newly expressed as compared to protein profile of culture at 1 bar/ 5°C (lane 1) after 48 h growth.

A lot of variability in the protein profile was seen in cultures given pressure shock of 200 bar for 48 h at 30°C and 5°C (lanes 3 & 4; Fig. 6.4b). Although the proteins expressed were appearing as new, there were only minor difference in the molecular weights. The proteins at positions 2, 7, 11, & 13 (Fig. 6.4b) at 1 bar/30°C (lane 2) for 144 hours were not seen in the 200 bar shocked culture (lane 4), while the proteins at positions 6 & 7 of pressure shocked culture were newly expressed as compared to culture grown at 1 bar/30°C for 144 h. The culture when given shock of 200 bar at 5°C expressed many new proteins (lane 3, positions 1, 2, 3, 4, 11 & 14; Fig.6.4b) as compared to the culture grown at 1 bar/ 5°C for 144 h (lane 1).

300 bar: Many of the proteins detected in the cultures grown at 1 bar and 300 bar for 48 h were found to be similar (Fig. 4a). The proteins at positions 1, 2, 7 & 9 (lane 4; Fig. 6.5a) at 300 bar were newly expressed as compared to the culture grown at 1 bar (lane 2), while the protein at position 3 at 1 bar was not detected at 300 bar (lane 1 & 3 respectively). In case of 5°C also, there was similarity in the protein profile of cultures grown at 1 bar and 300 bar. Most of the proteins were nearly similar, with minor shifts in the molecular weight.

Fig. 6.4 a & b Protein profile of deep sea yeast # A344 grown at 200 bar pressure

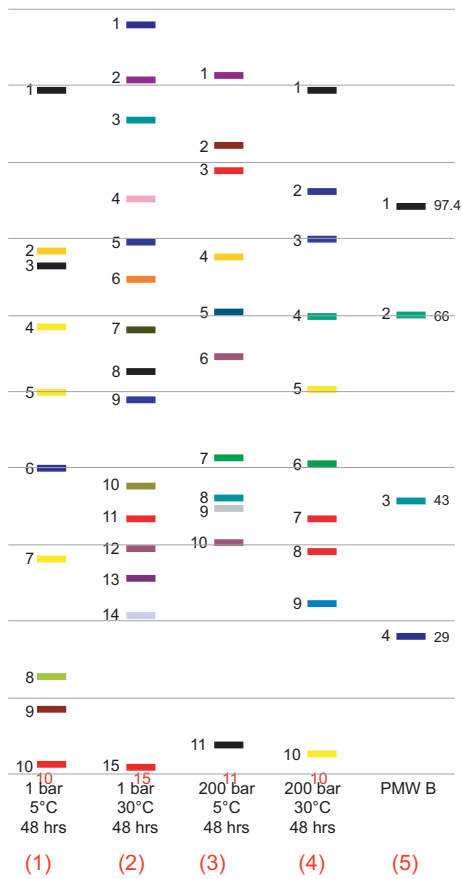


Fig. 6.4a

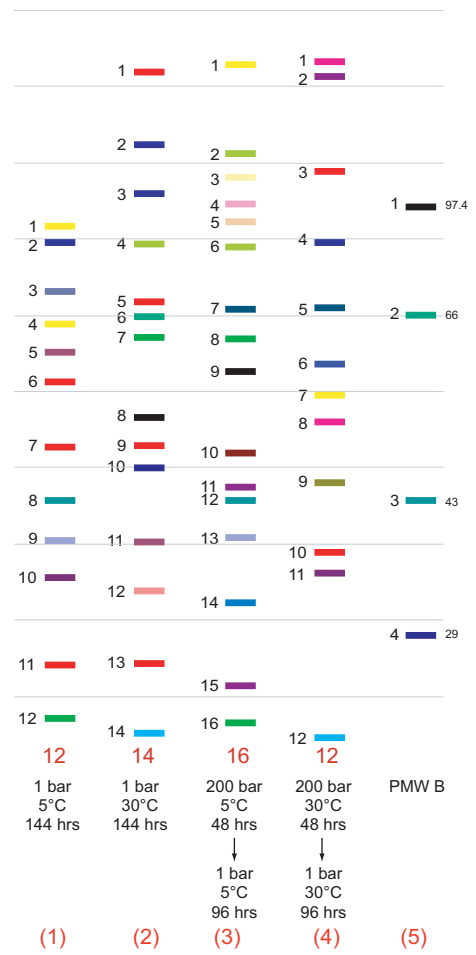


Fig. 6.4b

Fig. 6.5 a & b Protein profile of deep sea yeast # A344 grown at 300 bar pressure



Fig. 6.5a

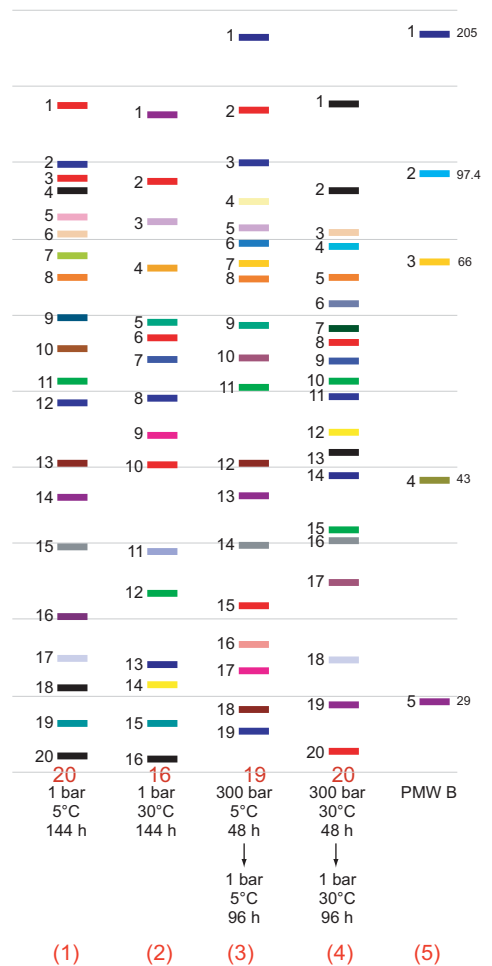


Fig. 6.5b

The 300 bar shocked cultures showed maximum number of detectable proteins in all the experiments conducted. Although the number of proteins detected was quite high, there were not many newly expressed proteins, but only minor shifts with respect to molecular weight. The proteins at positions 4, 6, 14, 15 & 16 (Fig. 6.5b) in 300 bar shocked cultures (lane 4) were newly expressed as compared to 1 bar/30°C grown culture (lane 2). In case of 300 bar shocked culture at 5°C (lane 3), the proteins at positions 6, 7, 9 & 16 were not detected in culture grown at 1 bar/5°C for 144 h (lane 1), while the proteins at positions 3, 4, 12 & 18 (lane 1; Fig. 6.5b) in culture grown at 1 bar/5°C were not detected in the pressure shocked culture.

6.4.2 Western blotting

The proteins from the cultures grown under 1 bar pressure and then given a shock for different time intervals did not show any positive signals with both the anti - Hsps, anti Hsp 25 & anti Hsp 70, used.

6.4.3 Protein profiles of deep-sea and terrestrial cultures grown under elevated pressure

The proteins extracted by the urea buffer extraction method were electrophoresed to obtain the protein profiles of the cultures grown under different conditions of pressure and temperature. The SDS-PAGE profile of the protein extracts of the deep-sea and terrestrial isolates grown under pressure followed the similar pattern that the proteins being not expressed under elevated pressure as compared to 1 bar grown cultures.

At all the conditions, the terrestrial culture of *Aspergillus terreus* (MTCC 479) showed more number of proteins than the deep sea isolate (# A 4634). When the detectable proteins expressed under 1, 100, 500 bar at 30°C by the deep-sea isolate #A4634 were compared, maximum number of proteins were detected at 1 bar/ 30°C (lane 2; Fig. 6.6). The proteins at positions 2, 4, 5 & 7 (Fig. 6.6) at 1 bar/30°C were not seen at 100 & 500 bar (lanes 3 & 4, respectively). At 100 bar/30°C (lane 3), new proteins appeared at positions 2, 3 & 4 (Fig. 6.6), while at 500 bar/30°C (lane 4) new proteins appeared at positions 2 & 4 (Fig. 6.6). The number of proteins detected at 5°C was more than at 30°C for all the 3 conditions of pressure (lane 5, 6 & 7). Even here, maximum numbers of proteins were detected at 1 bar (lane 5) as compared to 100 and 500 bar pressure (lanes 6 & 7 respectively). The proteins at positions 4 & 11 at 1 bar/5°C (lane 5; Fig. 6.6) were not detected at 100 & 500 bar. Quite a few proteins were eliminated in the higher molecular weight range, i.e. more than 40 kDa at 500 bar. The high molecular weight proteins at positions 3, 4, 5 at 100 bar (lane 6; Fig. 6.6) disappeared at 500 bar (lane 7), whereas the low molecular weight proteins at positions 5, 6 & 7 (Fig. 6.6) at 500 bar (lane 7) were not present at 100 bar (lane 6).

In case of the terrestrial isolate MTCC 479, the number of proteins detected at 30°C was more than those detected at 5°C (Fig. 6.7), unlike the deep-sea isolate (Fig. 6.6). The number of proteins in the lower molecular weight was much higher at 1 bar/30°C (lane 1) as compared to 100 & 500 bar (lane 3 & 4). The proteins at positions 4, 6, 7 & 8 detected at 1 bar/ 30°C (lane 1; Fig. 6.7) were not detected at 100 bar (lane 3), while the proteins at positions 4, 5, 6, 7, 8 & 9 (Fig. 6.7) were not detected at 500 bar (lane 4). At 100 bar/5°C (lane 6), proteins at positions 2 & 3 were newly expressed

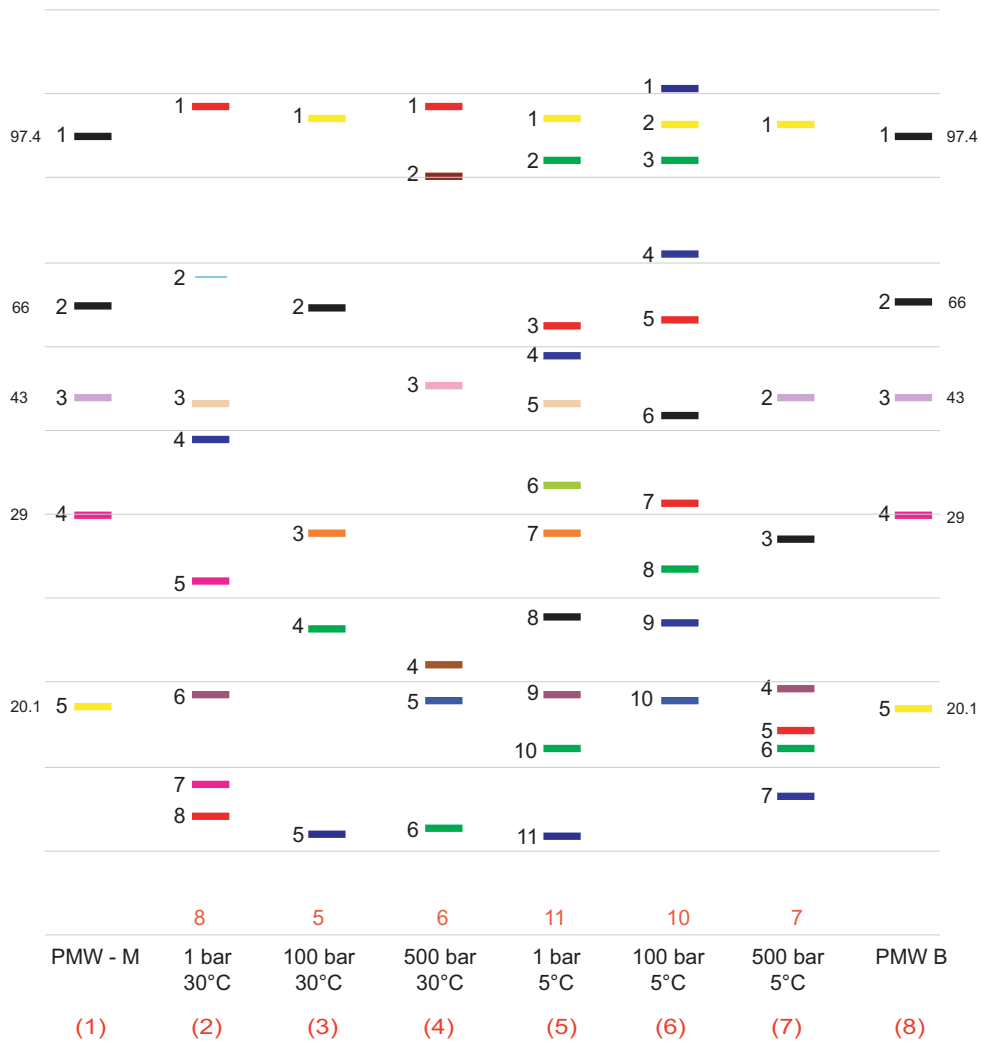


Fig. 6.6 Protein profile of deep sea *Aspergillus terreus* # A4634 grown at different hydrostatic pressures and temperatures

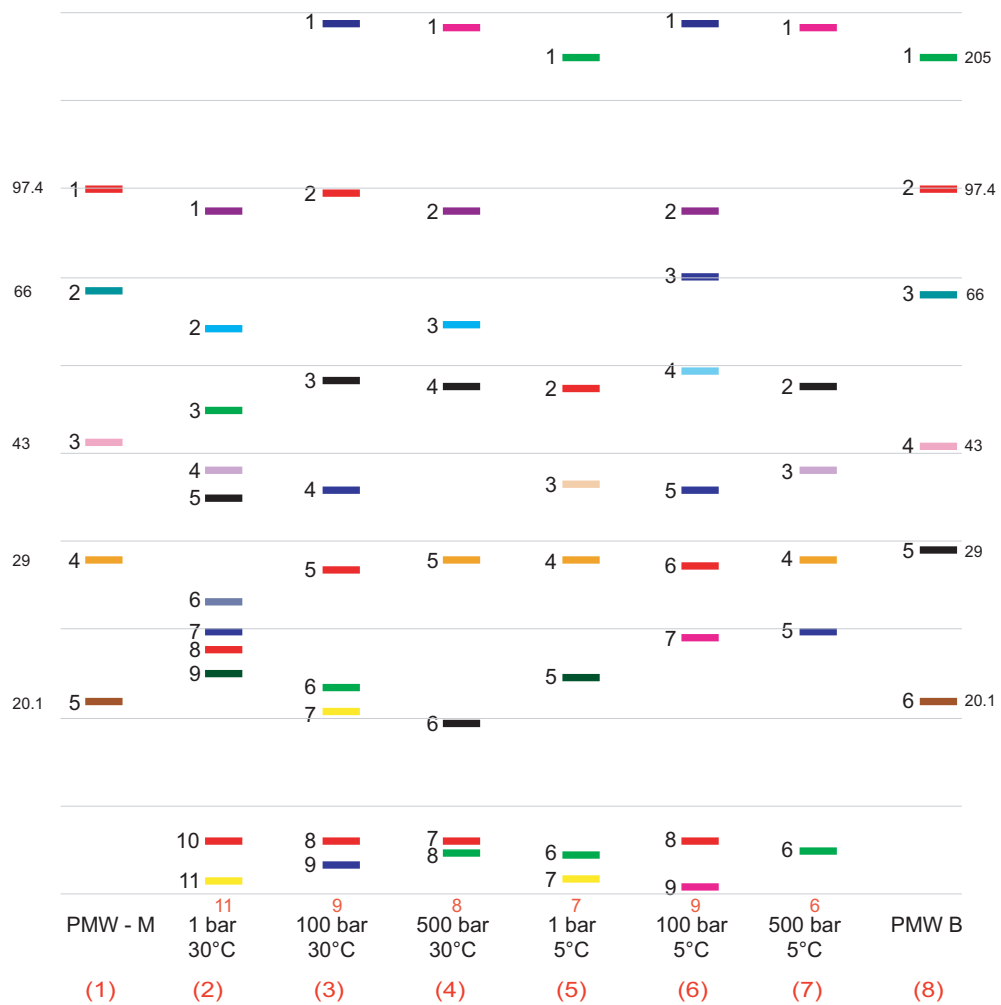


Fig. 6.7 Protein profile of deep sea *Aspergillus terreus* # MTCC 479 grown at different hydrostatic pressures and temperatures

as compared to 1 bar (lane 5) and 500 bar (lane 7), while the rest of the proteins were more or less similar with slight shift in their positions.

When the deep sea yeast # A 344 was grown at 1 bar/30°C, 300 bar/30°C and 300 bar/5°C, there was a huge dissimilarity in protein expression as detected by SDS electrophoresis (Figs. 6.8 a - c). At each interval of time, new proteins were expressed, which is a common pattern observed in any growing culture. The culture grown at 1 bar / 30°C for 6 h (Fig. 6.8a, lane 1) showed totally different proteins, except at position 5 which was also seen in the 24 h old culture (lane 3). The 48 h old culture showed maximum number of proteins (lane 4) as compared to the 6 h, 12 h and 24 h old cultures (Fig. 6.8a, lanes 1, 2 & 3 respectively). When the culture was grown under 300 bar at 30°C (Fig. 6.8b), similar trend was seen with new set of proteins being expressed at different time intervals. But here, more number of proteins was seen in the 6 h old culture (lane 1, Fig. 6.8b) than the 12 h, 24 h and 48 h old cultures (Fig. 6.8b, lanes 2, 3 & 4 respectively). When the culture was grown at 300 bar/ 5°C (Fig. 6.8c), the number of proteins detected at all the time intervals was almost the same, indicating that the culture is probably taking more time than what was used in our experiment to show appearance of new proteins as compared to the 300 bar/ 30°C grown (Fig. 6.8b) or 1 bar / 30°C culture (Fig. 6.8a). This is again in accordance with earlier observations that it is the temperature which is really retarding the growth of the fungus more than the elevated hydrostatic pressure. In case of 300 bar/30°C (Fig. 6.8b), the change in protein profile is seen immediately by 12 h, which is changed considerably by 24 and 48 h.

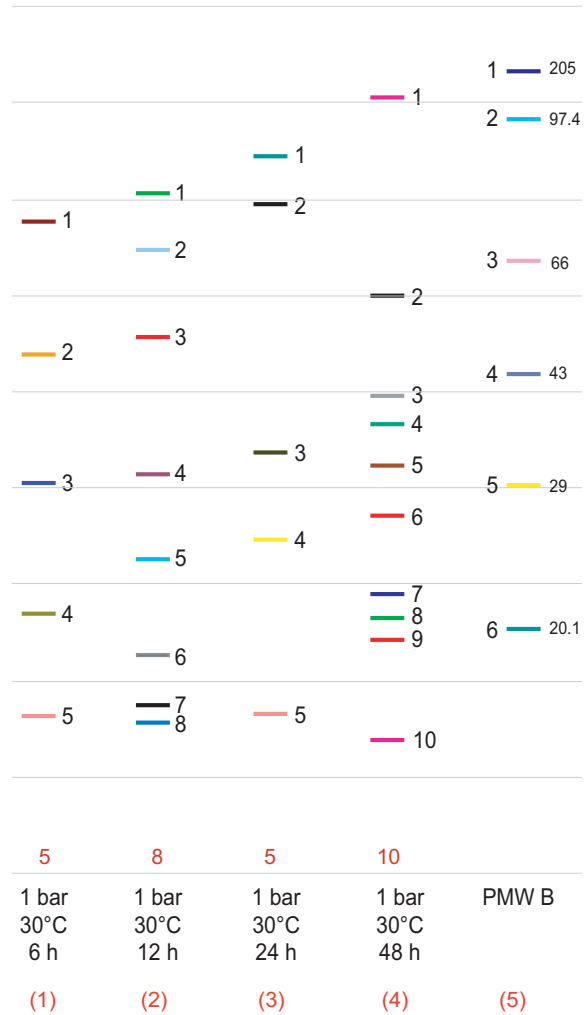


Fig. 6.8a Protein profile at 1 bar / 30°C at different time intervals by the deep sea yeast # A344

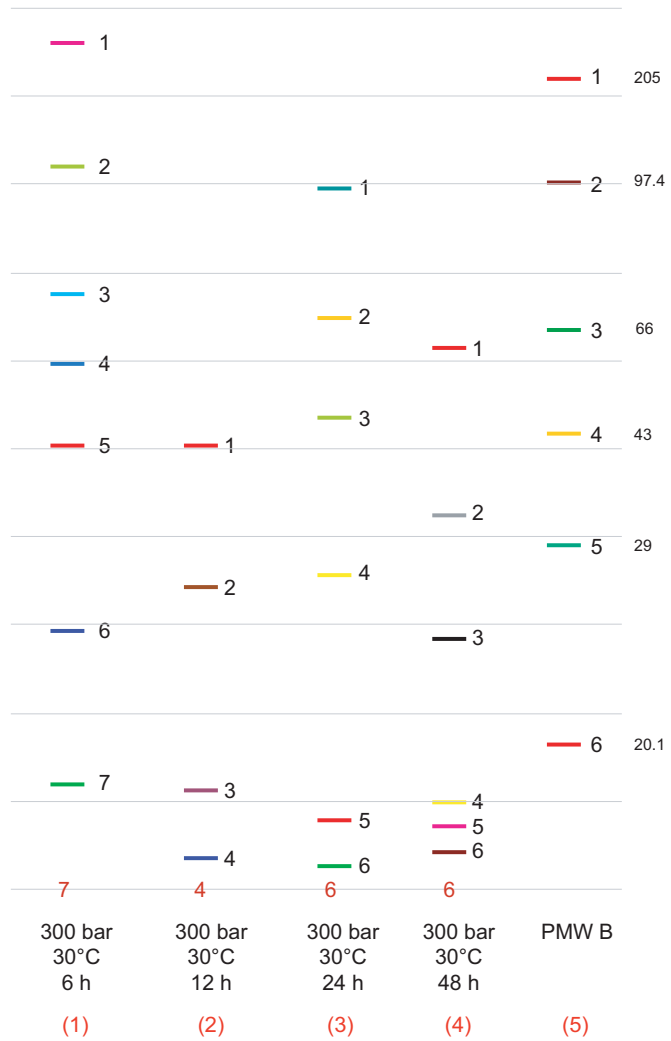


Fig. 6.8b Protein profile at different time intervals at 300 bar / 30°C by the deep sea yeast # A344



Fig. 6.8c Protein profile at different time intervals at 300 bar / 5°C by the deep sea yeast # A344

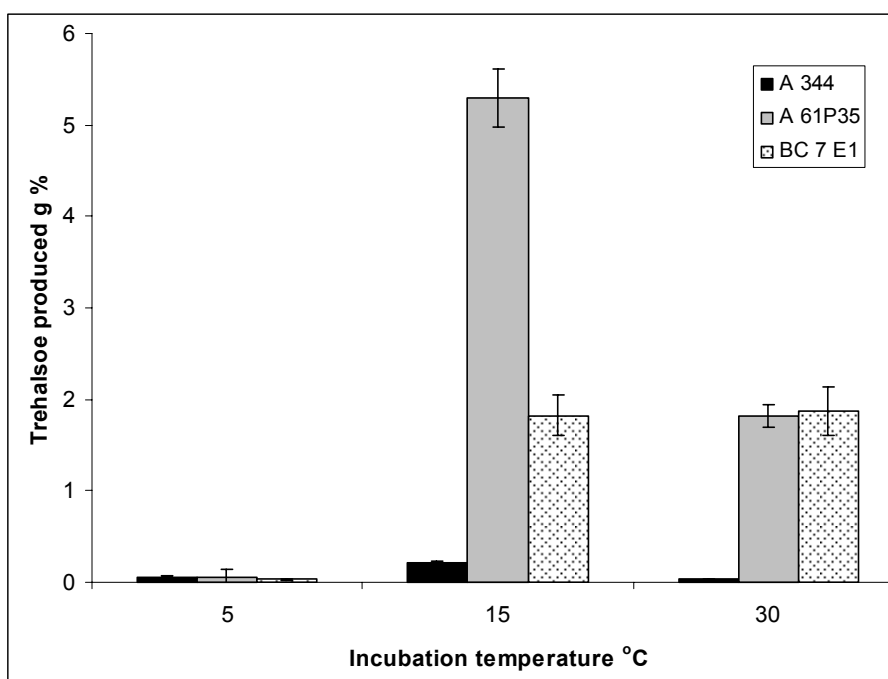
6.4.4 Trehalose accumulation

All the deep sea yeast cultures checked were showing trehalose production. The culture A 61P35 showed maximum trehalose accumulation at 15°C (Table 6.1 & Fig. 6.9). All the cultures showed least trehalose production at 5°C.

Table 6.1 & Fig. 6.9 Trehalose production by the deep-sea yeasts at different temperatures

Culture	Growth condition (°C)	Trehalose produced (g %)
A 344	5	0.06 ± 0.01
	15	0.22 ± 0.09
	30	2.92 ± 0.01
A 61P35	5	0.03 ± 0.01
	15	5.30 ± 0.32
	30	1.35 ± 0.22
BC7E1	5	0.03 ± 0.01
	15	1.82 ± 0.13
	30	1.87 ± 0.27

Fig. 6.9

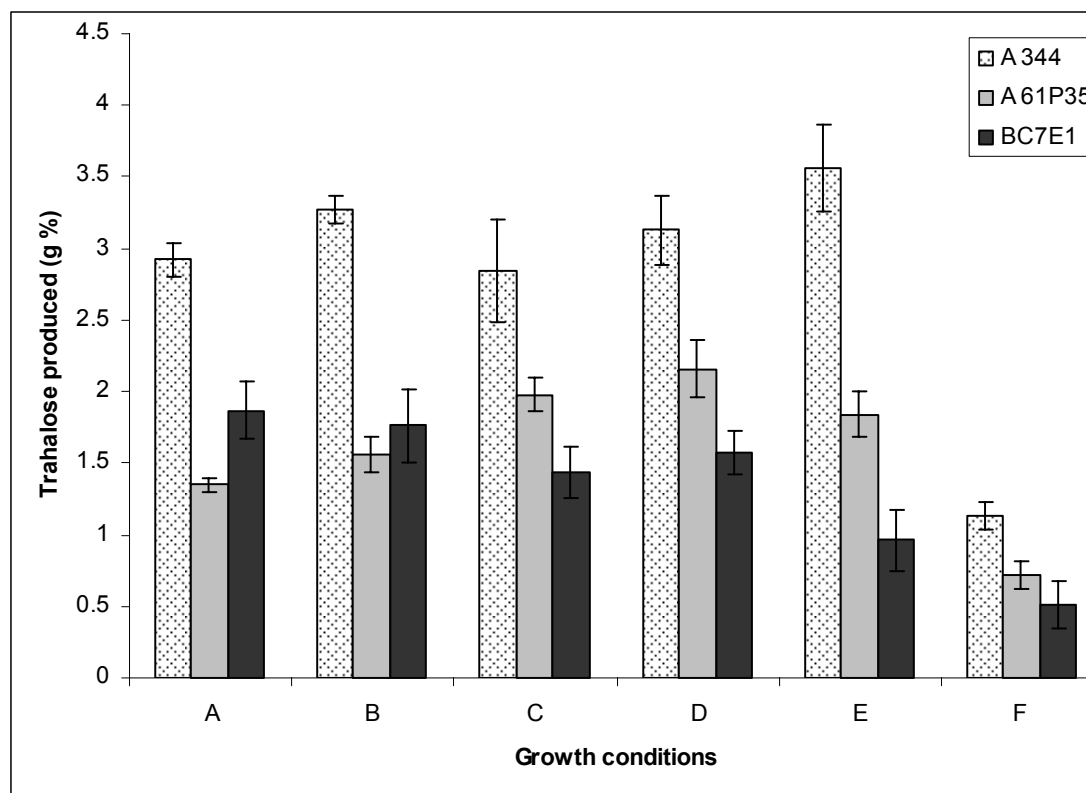


The deep sea yeasts (A 344, A 61P35 & BC7E1) when incubated under elevated pressure did not show any drastic increase or decrease in trehalose accumulation (Table 6.2 & Fig. 6.10). The off-white, filamentous yeast, A 344, showed highest trehalose accumulation at 500 bar / 30°C, while the orange yeast BC7E1 showed least at the same condition. In case of A 61P35, there was slight increase in trehalose accumulation from 1 bar to 300 bar, but again there was decrease at 500 bar, while in case of the other two yeasts used for the experiment, there was random increase or decrease in the trehalose levels without any direct correlation to the growth conditions.

Table 6.2 & Fig. 6.10 Trehalose production by the deep-sea yeasts at different hydrostatic pressures

Growth conditions (18 h)	Trehalose accumulation (g %)		
	A 344	A 61P35	BC7E1
1 bar / 30°C	2.92 ± 0.12	1.35 ± 0.05	1.87 ± 0.2
100 bar / 30°C	3.27 ± 0.1	1.56 ± 0.13	1.76 ± 0.25
200 bar / 30°C	2.84 ± 0.36	1.98 ± 0.12	1.43 ± 0.18
300 bar / 30°C	3.13 ± 0.24	2.16 ± 0.2	1.57 ± 0.15
500 bar / 30°C	3.56 ± 0.3	1.84 ± 0.16	0.96 ± 0.22
Control (Inoculum) 1 bar / 30°C (48 h)	1.13 ± 0.1	0.72 ± 0.1	0.51 ± 0.16

Fig. 6.10



- A 1 bar/30°C
- B 100 bar/30°C
- C 200 bar/30°C
- D 300 bar/30°C
- E 500 bar/30°C
- F Control (Inoculum) 1 bar / 30°C (48 h)

6.5 Discussion

In the present study, the growth of the cultures at high pressure and low temperature was not inhibited indicating the versatility and tolerance of cultures to extreme growth conditions. However, more number of proteins were seen in the cultures grown at 1 bar rather than at the elevated pressures. This could be pointer to the fact that all these cultures were isolated from the deep sea and the elevated

pressure conditions being the native conditions existing, the atmospheric pressure may be acting as a stress, thereby expressing more number of proteins. But this was not supported by other growth experiments to satisfaction. This needs to be studied to the genetic level as in case of bacteria [Bartlett, 2002; Horikoshi, 1998]. This might also be indicating that the metabolic rates are very low at the elevated pressure and low temperature, correspondingly giving less number of proteins or this could be due to biochemical adaptation of cells by transition to a specific surviving state, which is characterized by deceleration of vital activity (DVA), a process related to decreased metabolism [Feofilova, 2003].

The first attempt to characterize the pressure response of an unicellular organism at the protein level targeted the methanogenic archaeon *Methanococcus thermolithotrophicus* [Jaenicke *et al.*, 1988], partly because the negative reaction volume of the methane-forming reaction suggested that this organism might thrive at elevated pressures. In spite of the positive results of this study, further characterization of the detected pressure-induced proteins was hampered by the extremely difficult handling of this organism. Four Pressure-induced-proteins (PIPs) were observed in case of *Rhodotorula rubra* at 450 bar. The profiles obtained at 20, 40 and 45 MPa, revealed no change in protein expression as compared to samples grown at atmospheric pressure. However, at 400 to 500 bar, approaching threshold of complete growth inhibition, significant alterations occur [Groß *et al.*, 1994]. Cultivation of cultures at temperatures from 5 to 30°C showed no inhibition of growth. The pressure shock induces heat and cold shock proteins [Welch *et al.*, 1993], and ribosomes appear to play a role as sensors for these two stress conditions [VanBogelen, 1990], strongly suggesting a similar role for ribosomes in response to hydrostatic pressure.

It has been reported previously [Julseth & Innis, 1990] that stress protein production can occur both during the absence and presence of cell growth. Further, time needed for induction of proteins subjected to shock at low temperature is more than the time needed for induction at higher temperature because of the decreased rate of overall protein synthesis at lower temperature. We also had similar observations (Fig. 6.8 a - c), with more number proteins observed at 30°C grown cells than 5°C grown cells under high pressure.

The filamentous fungi did not give very good protein profiles. The main problem for the filamentous fungi was the amount of biomass produced. The biomass produced was too little to carry out the liquid nitrogen crushing to obtain detectable amount of proteins. It was observed during the standardization of protein extraction methods using, 1 bar-grown culture, that maximum amount of protein was obtained using the liquid nitrogen crushing.

Both the anti hsp antibodies checked did not give positive signals with the proteins present in the sample indicating their novel nature. To get a positive confirmation, these proteins need to be gel purified, and polyclonal or monoclonal antibodies will have to be raised against them. Other approach to study these proteins would be to purify the proteins and do the amino acid sequencing to find out if the proteins are really new.

It has been shown in previous studies with bacteria [Niven *et al.*, 1999] that effect on ribosomal structure observed after cold shock was similar to the effect observed after high pressure treatment, suggesting that there is overlap in the responses to these two types of stress. This was also reported in case of *Listeria monocytogenes* LO28 [Wemekemp-Kamphuis *et al.*, 2002]. In the present studies also

the cultures showed similar response to pressure and low temperature (5°C), with many proteins being induced (Fig. 6.1 to 6.8) in both deep sea and terrestrial isolates. The maximum trehalose accumulation was observed at 15°C or 30°C. Trehalose, being a stress protectant is known to be produced under the stress of low temperature, but the results obtained here were on similar lines of the growth and the protein profiles. More trehalose accumulation at mesophilic temperatures is a further proof of these yeasts being deep-sea inhabitants. Similarly, high hydrostatic pressure also doesn't seem to induce or repress trehalose production (Table 6.2 & Fig. 6.10), except in case of one of the cultures, A 61P35. But even this is also not very high to be called as a response against the increasing pressure, and thereby playing a role in the defense of the cell. A possible role of trehalose in the defense of the yeast *Saccharomyces cerevisiae* against osmotic stress was suggested by Piper [1993]. In case of a thermophilic fungus *Chaetomium thermophilum* var. *copriphilum* [Jepsen & Jensen, 2004], it was shown that cytosolic trehalose accumulation was observed only in case of temperature stress and not osmotic stress caused due to increased concentration of sodium chloride. In the present studies also there was no increase in trehalose accumulation when the cells were exposed to the stress of decreasing temperature or increasing hydrostatic pressure, suggesting that trehalose alone may not be playing a role as stress protectant. Other compatible solutes that accumulate in cells are amino acids and polyols, e.g. glycine betaine, ectoine, sucrose, mannitol and glycerol [DasSarma & Arora, 2001]. Halotolerant yeasts and green algae accumulate polyols, while many halophilic and halotolerant bacteria accumulate glycine betaine and ectoine. Compatible solute accumulation may occur by biosynthesis, *de novo* or from storage material, or by uptake from the medium. A major exception is for the

halobacteria and some other extreme halophiles, which accumulate KCl equal to the external concentration of NaCl [DasSarma & Arora, 2001]. The role of glycine betaine is very well documented in case of prokaryotes, *Listeria monocytogenes* [Mendum & Smith, 2002]. TMAO (Trimethylene N-oxide) is another such protectant found in deep-sea animals [Yancey *et al*, 2002] which is a universal protein stabilizer. Further, TMAO works as a protein stabilizer for organisms that cannot produce it, when provided externally.