

## Chapter 4

Effect of simulated deep-sea  
conditions on growth and protein  
patterns of a few select cultures

## **4.1 Introduction**

Oceans contribute towards major proportion of biosphere with an average depth of ~ 3800 m. Therefore various marine organisms are subjected to such elevated hydrostatic pressure i.e. 38 MPa and low temperature conditions. Since the majority of the biosphere is composed of elevated pressure environments, the effect of such extreme conditions on biological systems needs to be studied. There is a growing interest in understanding microbes and potential applications of them in these extreme environments (Yayanos, 1995; Abe et al, 1999; Abe and Horikoshi, 2001; Bartlett, 2002; Simonato et al, 2006). Evidence of elevated pressure adaptation has been discovered in deep-sea organisms belonging to the lower Eukarya (Atkins, 2000), invertebrates and fishes (Kelly et al, 1999), and even in deep diving marine mammals (Castellini et al, 2002). In general, elevated pressure either inhibits or favors those biochemical processes which occur with an expansion or reduction in system volume, respectively (Somero, 1992). Likewise, mechanisms associated with low-temperature growth of microorganisms involve alterations in cellular membrane fluidity, uptake or synthesis of compatible solutes, structures of macromolecules such as ribosomes and protein synthesis mechanisms (Wemekamp-Kamphuis et al, 2002). Therefore, understanding the connections between high pressure and low temperature can provide valuable insight about the particular signals generated by these stress conditions (Bartlett, 2002).

### **4.1.1 Effect of elevated pressure and low temperature on microbial cells**

Pressures and temperatures of different magnitudes exert different effects on organisms. In general, elevated hydrostatic pressure in the range of several dozen MPa, have been found to be nonlethal, but may affect adversely the organisms which are adapted to atmospheric pressure (Abe et al, 1999; Bartlett, 2002; Abe, 2004). The growth of mesophilic microorganisms have been shown to be inhibited at an elevated pressure of 40-50 MPa. The

cessation of growth is accompanied by morphological changes such as formation of filaments in *Escherichia coli* (ZoBell, 1970) and cell chains or pseudomycelia in the marine yeast *Rhodospiridium sphaerocarpum* (Lorenz and Molitoris, 1992; Lorenz, 1993). Much higher pressures, in the range of 100 MPa may also be used for sterilization purposes (Sonoike et al, 1992; Takahashi, 1992). These effects depend not only on the magnitude but also on the duration of pressure applied in combination with temperature, pH, oxygen supply and composition of culture media (Abe, 2007a). Both low temperature and elevated pressure inhibit an early step of translation (Schwarz and Landau, 1972; Broeze et al, 1978). The cold shock response has been suggested to be an adaptive response to facilitate the expression of genes involved in translation initiation (Jones et al, 1987). Also, elevated pressure, like low temperature incubation, results in the continued synthesis of stringently controlled proteins, involved in transcription and translation despite the growth rate decrease; this behavior also suggests decreased translation capacity (Welch et al, 1993). Finally, both low temperature and elevated pressure decrease membrane fluidity (Chong et al, 1981; MacDonald, 1984), which perturbs a variety of membrane associated processes, including trans-membrane ion and nutrient flux, and DNA replication.

To study the effect of elevated hydrostatic pressure and low temperature some model organisms such as *E. coli*, *Bacillus subtilis*, and the budding yeast *Saccharomyces cerevisiae*, whose complete genomes have been sequenced, are used as powerful genetic tools by several workers (Welch et al, 1993; Abe and Horikoshi, 2000). These microbes can be classified either as piezophilic i.e. high-pressure loving or piezo-tolerant i.e. capable of tolerating elevated pressure but showing better growth at atmospheric pressure. The first pure culture of a piezophilic bacterial isolate, strain CNPT-3 was reported in 1979 (Yayanos et al, 1979). This spirillum-like bacterium showed efficient doubling rate at elevated pressure i.e. 50 MPa, but no growth could be observed at atmospheric pressure conditions even after incubation for several weeks. Numerous piezophilic and piezotolerant bacteria have since been isolated and characterized by the DEEPSTAR group at JAMSTEC, from deep-

sea sediments at depths ranging from 2,500 m-11,000 m (Kato et al, 1995, 1996, 1998). Most of the strains reported were found to be not only piezophilic, but also psychophilic i.e. low temperature loving, showing no growth at temperatures above 20 °C. In addition, high-pressure induction of heat-shock proteins has been confirmed in other studies with *E. coli* (Haskin et al, 1993; Groß et al, 1994). Heat-shock proteins may also be induced in piezophiles upon decompression. A stress protein showing similarity to a heat-shock protein was found to be induced in the deep-sea piezophilic hyperthermophilic bacteria *Thermococcus barophilus* subsequent to shift to atmospheric pressure (Marteinsson et al, 1999). Since high pressure and low temperature are known to show similar effects on protein synthesis and membrane structure, the induction of both pressure-shock and cold-shock proteins may represent an attempt by some bacterial isolates to ameliorate the damaging effects of elevated pressure on membrane integrity, translation processes, and the stability of macromolecules.

#### **4.1.2 Effect of simulated deep-sea conditions on growth and protein patterns of fungi**

In comparison to bacteria, only a few studies have reported the effect of elevated hydrostatic pressure and low temperature conditions on growth patterns of fungi. Raghukumar et al. (2004), reported germination and growth of spores of *A. Sydowii*, isolated from deep-sea sediments of Chagos Trench under an elevated hydrostatic pressure and low temperature conditions. In addition, mycelial inocula of fungi isolated from deep-sea sediments of the Central Indian Basin produced substantial growth at both 30° and 5°C under elevated hydrostatic pressure (Damare et al, 2006). Among yeasts, *Saccharomyces cerevisiae* is a facultative anaerobe and has been used in various studies for analyzing the effects of elevated hydrostatic pressure and low temperature. Being a facultative anaerobe, it has been proved as most suitable organism to cope up with the closed pressure vessel condition where the oxygen concentration is very low during incubations (Abe, 2007a). The

genome of this yeast encodes approximately 6000 genes out of which more than 4800 are non essential ones. Recent large scale phenotypic screening of the *Saccharomyces cerevisiae* gene-deletion library has revealed numerous unexpected genes and metabolic pathways that are involved in tolerance of environmental stresses (Martin and Drubin, 2003; Chasse and Dohlman, 2004). Analyses of genome-wide gene expression profiles of this yeast were done showing no growth at elevated pressure and low temperature conditions. However, after being recovered from the stress conditions the genes responsible for transcription of energy metabolism, cell defense and protein metabolism have been found to be highly upregulated (Iwahashi et al, 2003). Also, the survival of *Saccharomyces cerevisiae* at elevated pressures has been shown to be enhanced by preceding heat-shock treatment (Iwahashi et al, 1991). The heat shock treatments confer enhanced synthesis of heat shock proteins (Hsps) and metabolism of trehalose in this yeast (Singer and Lindquist, 1998). Among many Hsps, Hsp104 plays an essential role in acquired tolerance by unfolding denatured intracellular proteins in an ATP-dependent manner (Sanchez and Lindquist, 1990). In addition, in *S. cerevisiae*, the rate of synthesis of several other proteins, e.g., ubiquitin, some glycolytic enzymes, and a plasma membrane protein, is strongly enhanced upon exposure of cells to stress. A plasmid carrying the *TAT2* gene, which encodes a high-affinity tryptophan permease, enabled the cells of *S. cerevisiae* to grow under conditions of pressure in the range of 15 to 25 MPa. Additionally, cells expressing the Tat2 protein at high levels became endowed with the ability to grow under low-temperature conditions at 10 or 15°C as well as at high pressure (Abe and Horikoshi, 2000). Abe and Horikoshi. (1995) found that hydrostatic pressures of 40–60 MPa promoted acidification of the vacuoles in yeast cells and that expression of the tryptophan permease i.e. TAT2 gene can be the rate limiting factor affecting the growth of tryptophan requiring yeast cells.

Apart from *S. cerevisiae*, there are very few other fungal isolates which have been used as model organisms to study the effect of elevated hydrostatic pressure and low temperature stresses. By applying high throughput

techniques these stress conditions need to be applied for understanding their effects on filamentous and unicellular marine fungi too.

## **Objective**

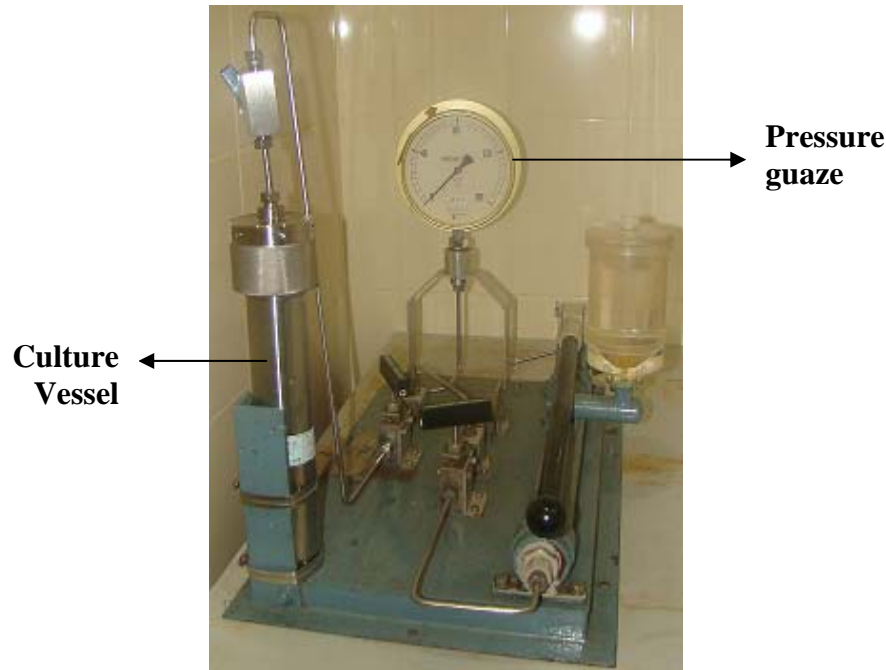
The objective of this chapter is to analyze the effect of elevated hydrostatic pressure and low temperature conditions on spore germination, growth properties and protein patterns of deep-sea fungi isolated from sediments of the Central Indian Basin. The hydrostatic pressure and temperature at the sampling site of the Central Indian Basin at a depth of ~5000 m is ~50 MPa and 3°C respectively.

## **4.2 Materials and methods**

### **4.2.1 Growth of mycelial fungi and yeasts under simulated deep-sea conditions**

Growth and production of biomass of deep-sea filamentous fungi and yeast was compared under simulated conditions of elevated hydrostatic pressure and low temperature. For raising mycelial inoculum, cultures of 16 deep-sea filamentous fungi isolated during the cruise #ABP26 (Table 2.5) were grown in MEB for 3 days at 0.1 MPa and 30°C. Vegetative mycelium prior to the onset of sporulation was homogenized with sterile glass beads. A known weight of finely broken mycelial suspension was inoculated in 5 ml of MEB in pouches made with sterilized gas permeable polypropylene sheets and sealed without trapping any air bubbles. The pouches were suspended in a deep-sea culture vessel (Tsurumi & Seiki Co., Japan) (Fig. 4.1) and incubated at 0.1 MPa/30°C, 20 MPa/30°C, 0.1 MPa/5°C and 20 MPa/5°C for comparing the effect of different pressure and temperature conditions on growth. After 20 days, the contents of the pouches were filtered over pre-weighed filter papers, dried to a constant dry weight and the difference between the initial and final biomass determined as mycelial dry weight (Raghukumar and Raghukumar, 1998). Three terrestrial filamentous fungi were also grown and incubated

under different conditions of elevated hydrostatic pressure and low temperature as mentioned above and were compared with deep-sea isolates. The yeasts isolated during the cruise #ABP26 (Table 2.5) were similarly grown in YPD broth and the biomass was lyophilized and weighed. The biomass of mycelial fungi and yeasts were also compared in media made with sea water (35 ppt) and distilled water.



**Fig. 4.1 Deep-sea culture vessel.**

#### **4.2.2 Comparison of growth and oxido-reductase production of deep-sea fungi with their counterparts from mangroves**

Two of the filamentous fungi i.e. NIOCC#F53 and #F61 isolated during cruise #ABP38 (Table 2.6), showed affiliation with *Cerrena* and *Trametes* species respectively. These two species have also been isolated previously from degrading wood samples of mangroves in the lab and are known as efficient producer of oxido-reductase enzymes (D'Souza-Ticlo et al, 2006; Verma et al, 2010). To compare the growth of deep-sea isolates with their counterparts from mangroves all the four cultures i.e. mangrove isolates: NIOCC#2a, NIOCC#DV2, and deep-sea isolates: NIOCC#F53, NIOCC#F61, were grown on MEA plates prepared with 100% seawater. All these four cultures were also

grown on Boyd and Kohlmeyer (B&K) agar medium prepared with 50% seawater (Appendix 6.1h) containing 4 mM guaiacol, a substrate for oxidoreductase enzyme. The production of an intense brown color under and around the fungal colony in guaiacol-supplemented medium indicated presence of laccase activity (D'Souza-Ticlo et al, 2006). To compare the growth under simulated deep-sea conditions, the two deep-sea and two mangrove fungi were grown in MEB for 3 days at 0.1 MPa and 30°C. Vegetative mycelium prior to the onset of sporulation was homogenized with sterile glass beads. A known weight of finely broken mycelial suspension was inoculated in 5 ml of MEB in pouches made with sterilized gas permeable polypropylene sheets and sealed without trapping any air bubbles. The pouches were suspended in a deep-sea culture vessel and incubated at 0.1 MPa/30°C, 20 MPa/30°C, 0.1 MPa/5°C and 20 MPa/5°C for comparing the effect of different pressure and temperature conditions on growth. After 20 days, the contents of the pouches were filtered over pre-weighed filter papers, dried to a constant dry weight and the difference between the initial and final biomass determined as mycelial dry weight (Raghukumar and Raghukumar, 1998).

#### **4.2.3 Spore germination under simulated deep-sea conditions**

Four sporulating deep-sea fungi i.e. NIOCC#F2, #F5, #F6 and #F13, two terrestrial fungi i.e. MTCC#IM2, #IM3 and one fungal isolate, NIOCC#85 from Lakshadweep sample were compared for their spore germination properties at elevated hydrostatic pressure and low temperature conditions. All these fungi were grown on MEA agar plates at 0.1 MPa pressure and 30°C, and the spores were collected by gently flooding the plates with sterile seawater. The spore suspension was appropriately diluted with sea water containing 2% Tween 80 and vortexed for 5 min. One ml of this spore suspension was added to five ml of the MEB medium (Damare and Raghukumar, 2008) prepared with seawater and packed in polypropylene pouches. These pouches were suspended in a deep-sea culture vessel and



incubated at 0.1 MPa/30°C, 20 MPa/30°C, 30 MPa/30°C, 0.1 MPa/5°C, 20 MPa/5°C and 30 MPa/5°C. The percentage of germination was calculated by counting germinated spores in 20 microscope fields.

#### **4.2.4 Synchronous effect of different hydrostatic pressures and salinities on spore germination of four sporulating fungi**

Four sporulating deep-sea fungi i.e. NIOCC#F2, #F5, #F6 and #F13 were grown on MEA agar plates at 0.1 MPa pressure and 30°C, and the spores were collected by gently flooding the plates with sterile seawater. The spore suspension was appropriately diluted with sea water containing 2% Tween 80 and vortexed for 5 min. One ml of this spore suspension was added to five ml of the sediment extract medium (Damare and Raghukumar, 2008) prepared with three different salinities, 1.7, 17 and 34 ppt and packed in pouches. These pouches were incubated at 20, 30, 40 and 50 MPa pressure at 30°C for 15 days. The germination percentage was calculated by counting germinated spores in 20 microscope fields.

#### **4.2.5 Growth and viability studies of Psychrotolerant and Mesophilic yeast isolates**

Among twelve yeast cultures isolated during cruise #ABP26, four were selected for growth studies under elevated hydrostatic pressure and low temperature conditions. Out of these four, two psychro-tolerant yeasts, NIOCC#PY12 and #PY13 identified to be *Cryptococcus* sp. and two mesophilic yeasts, NIOCC#Y1 and #Y3 were *Rhodotorula cassicola* and *Rhodospiridium toruloides* respectively (Table 2.7). These yeast isolates were maintained on MEA plates by repeated sub-culturing every 20 days. Psychrotolerant yeasts were maintained at 5°C. Yeast cultures were inoculated into 20 ml YPD medium and incubated at 28°C overnight at 150 rpm on a Rotary Shaker Incubator. One ml from the above grown culture was inoculated in fresh twenty ml YPD medium and incubated as described above.

Fresh YPD medium was inoculated with the above grown culture to get an initial O.D. of 0.1 at a wavelength of 600 nm. Cultures were diluted and O.D. was taken at 12 h intervals at 600 nm. Growth curves were constructed for these yeast isolates at three temperatures i.e. 5, 15 and 28°C.

To check the viability under elevated pressure and low temperature conditions, all these four deep-sea yeast and one terrestrial yeast i.e. MTCC#1716, were grown in the 20 ml YPD broth in an incubator shaker at 150 rpm for a period of four days as initial inoculum. One ml of the above grown culture was further inoculated into hundred ml of the YPD medium and incubated for another four days until the absorbance at 600 nm reached a value of 1.0. These cultures were packed in sterile plastic bags and were sealed at both the ends with sealing machine. The packed bags were placed in a pressure vessel and it was filled completely with distilled water. The pressure vessels were incubated at following temperature and pressure conditions for a period of 24 h: 1) 0.1 MPa/30°C, 2) 0.1 MPa/5°C, 3) 30 MPa/30°C, 4) 30 MPa/5°C, 5) 50 MPa/30°C, 6) 50 MPa/5°C. The pressure vessels were opened after the 24 h shock and the cultures were diluted 100 times i.e.  $10^{-2}$  dilution with sterile seawater. Twenty five  $\mu$ l of the above diluted cultures was spread plated on MEA plates in duplicates. The plates were further incubated at room temperature for 48 h until colonies appeared. The viability was determined by calculation of colony forming units (CFU) as follows:

$$\text{CFU/ml} = \text{No. of colonies} * \text{volume plated} / \text{Dilution factor}$$

#### **4.2.6 Growth curve of NIOCC#PY13 at different conditions of temperature, pressure and nutrient concentrations**

Based on growth and viability studies as mentioned above, NIOCC#PY13 was selected for further studies Growth curve was constructed for the psychrotolerant yeast #PY13 by estimating its growth at different conditions of temperatures, 5, 15 and 28°C, pressures, 20 and 30 MPA and YPD concentrations, 5, 50 and 100%. This isolate was grown to get the starting O.D. of 0.1 at 600 nm as described above. Four ml of this medium was packed

in sterile polypropylene plastic bags and the ends were sealed with a sealing machine (Quickseal, Sevana, India). These bags were placed in four pressure vessels in triplicates. Absorbance at a wavelength of 600 nm was measured at 48 h interval to monitor the growth. Each time one pressure vessel was used.

#### **4.2.7 Intracellular Protein profile of NIOCC#PY13 after giving shocks of different elevated pressure and low temperatures**

The psychro-tolerant yeast NIOCC #PY13 was grown in YPD medium at 0.1 MPa/30°C in an incubator shaker at 150 rpm for 48 h. One ml of the above grown culture was inoculated to fresh YPD medium to get an absorbance of 0.1 at a wavelength of 600 nm. Ten ml of the above diluted culture was packed in sterile plastic bags and incubated at the following pressure and temperature conditions for 24 h in the pressure vessels:

- |                |                 |
|----------------|-----------------|
| 1. 5°C         | 6. 30 MPa/30°C  |
| 2. 30°C        | 7. 40 MPa/5°C   |
| 3. 20 MPa/5°C  | 8. 40 MPa/30°C  |
| 4. 20 MPa/30°C | 9. 50 MPa/5°C   |
| 5. 30 MPa/5°C  | 10. 50 MPa/30°C |

After 24 h shock, the pressure vessels were depressurized and the biomass harvested, washed with sterile distilled water and centrifuged at 3000 g for 5 min. The pellet obtained was used for preparing the total cell protein extracts. Protein extraction was done by Yeast buster protein extraction reagent protocol (Merck, Novagen, U.S.A) ([Appendix 6.2a](#)). Protein concentration was estimated spectrophotometrically using Bradford reagent (Sigma, St. Louis, Montana, U.S.A), ([Appendix 6.2b](#)). Ten µl protein of each extract (containing equal concentration of protein) was loaded on to 12% resolving gel for electrophoresis ([Appendix 6.2c](#)). Electrophoresis was carried out at a constant voltage of 80V. Thereafter the gels were stained by silver staining method ([Chevallet et al, 2006](#)) ([Appendix 6.2d](#)). The stained gels were photographed using Gel documentation system (AlphaImager 2200, U.S.A).

#### **4.2.8 Statistical analyses**

The statistical analyses were carried out in STATISTICA 5.0 for the preparation of 3D scatter plots and Microsoft Excel program software (1-way ANOVA) for growth curves experiments.

### **4.3 Results**

#### **4.3.1 Biomass production and spore germination of fungi under simulated deep-sea conditions**

All of the deep-sea fungi, filamentous as well as unicellular yeasts showed growth under 20 MPa pressure and 5°C temperature (Figs. 4.2 and 4.3). In contrast, terrestrial isolates did not show considerable growth at elevated pressure and low temperature conditions (Fig 4.4). One-way analysis of variance (ANOVA) revealed that biomass produced was significantly different at different hydrostatic pressure and temperature conditions for deep-sea fungi (see the legend for Figs. 4.2 and 4.3). Also the deep-sea isolates showed better growth in media prepared with sea water than with distilled water (Figs. 4.5 and 4.6). A one-way ANOVA clearly demonstrated a distinct difference in growth of fungi in seawater versus distilled water (see the legend for Figs. 4.5 and 4.6). The results of ANOVA were significant at 0.01%. However, none of the cultures showed absolute requirement of seawater for growth. All of the mycelial fungi showed sporulation in seawater medium.

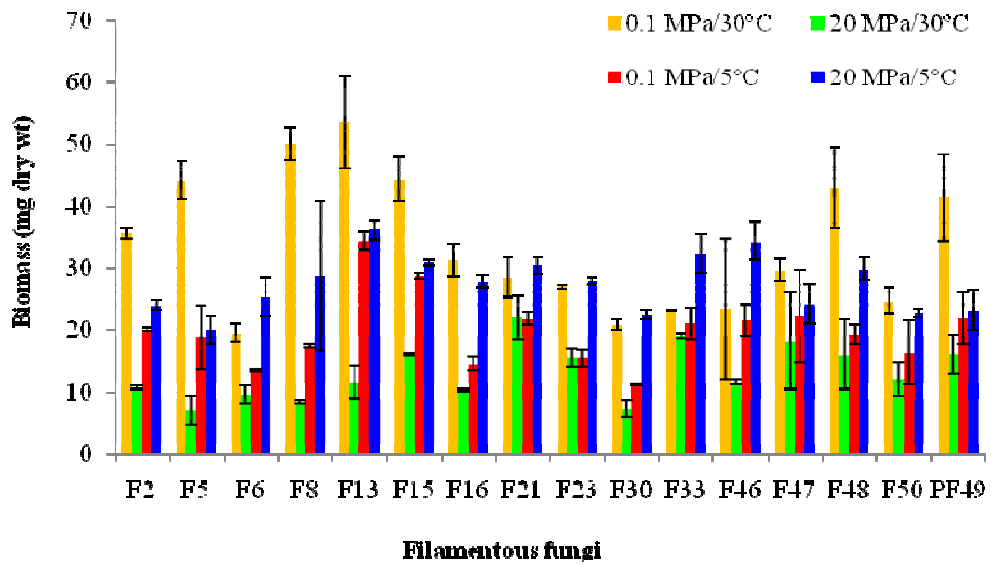


Fig. 4.2 Biomass (mg dry wt/20 ml) of mycelial fungi after 20 days incubation in malt extract broth (MEB) under different pressure and temperature conditions (SD values<10%)

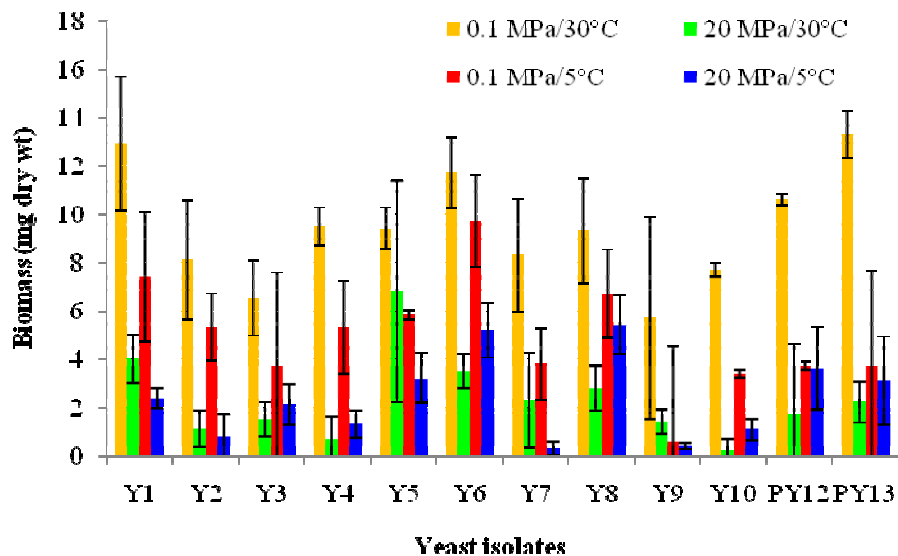


Fig. 4.3 Biomass (mg dry wt/20 ml) of yeasts after 20 days incubation in yeast extract, peptone, dextrose (YPD) broth under different pressure and temperature conditions (SD values<10%)

Results of 1-way ANOVA performed for significance of different growth conditions on production of biomass (Fig. 4.2 & 4.3):

	Fungi	Yeast
F critical	2.8	2.8
F value	25.5	30.5
P value	9.06-E-11***	7.83E-11***
Degrees of freedom	63	47

\*\*\* significant at >0.01 %. F value greater than F-critical value indicates significant difference

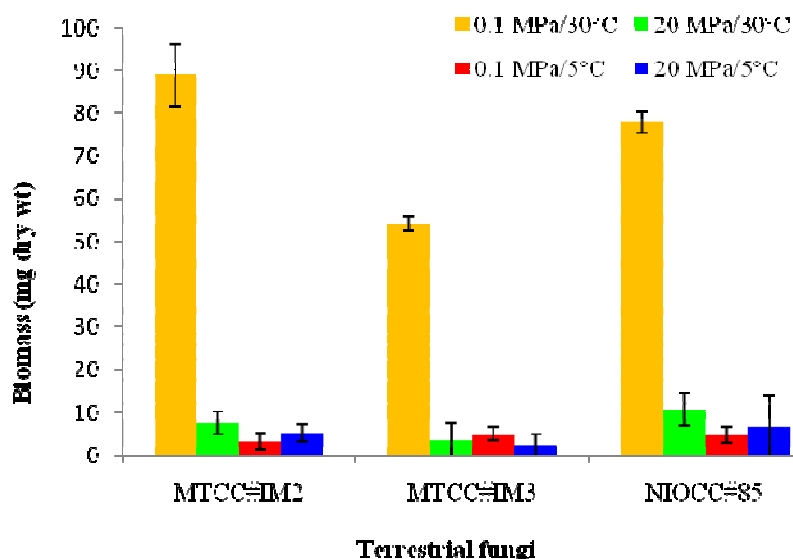


Fig. 4.4 Biomass (mg dry wt/20 ml) of terrestrial fungi after 20 days incubation in MEB under different pressure and temperature conditions (SD values <10%)

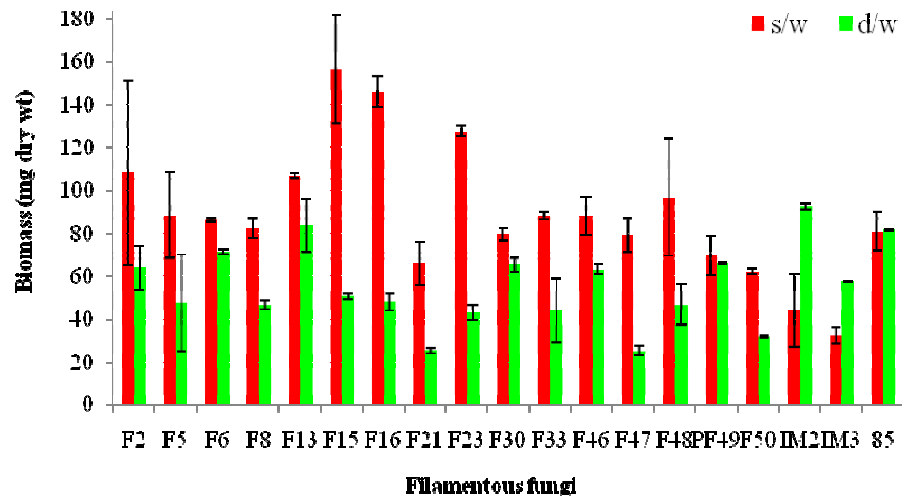


Fig. 4.5 Biomass (mg dry wt/10 ml) of mycelial fungi after 6 days incubation in MEB prepared in sea water (SW) and distilled water (DW) (SD values<10%)

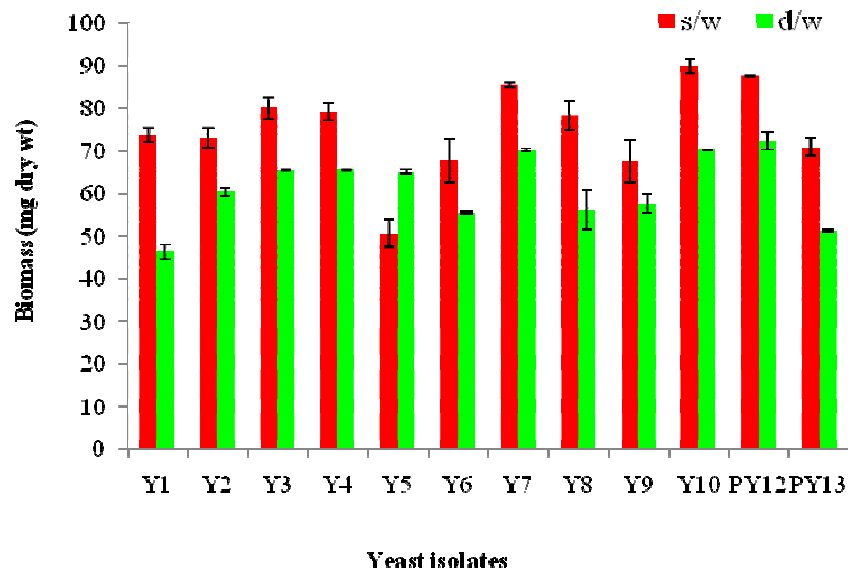


Fig. 4.6 Biomass (mg dry wt/10 ml) of yeasts after 6 days incubation in yeast extract peptone and dextrose (YPD) broth prepared in sea water (SW) and distilled water (DW) (SD values<10%)

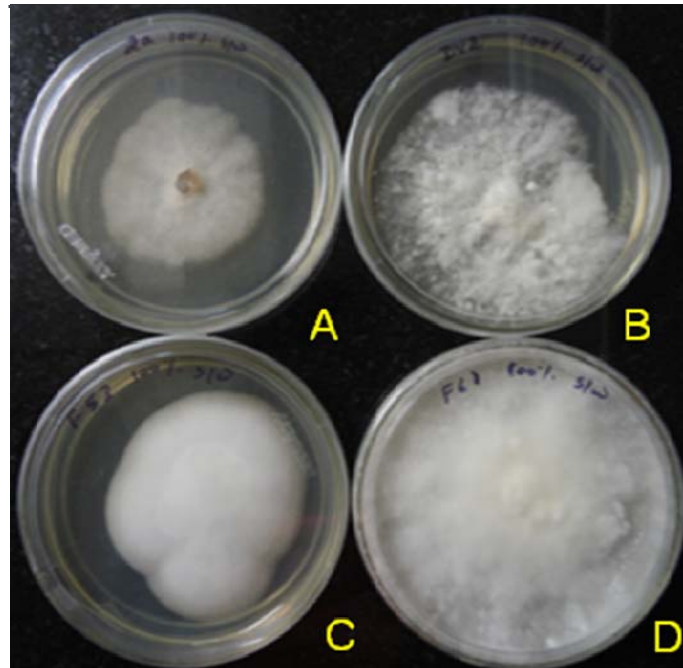
Results of 1-Way analysis of variance (ANOVA) performed for the significance of salinity effect on filamentous fungi and yeasts (data in Fig. 4.5 & 4.6)

	<b>Fungi</b>	<b>Yeast</b>
F critical	4.2	4.3
F value	30.8	13.1
P value	4.9E-06***	0.002***
Degrees of freedom	31	23

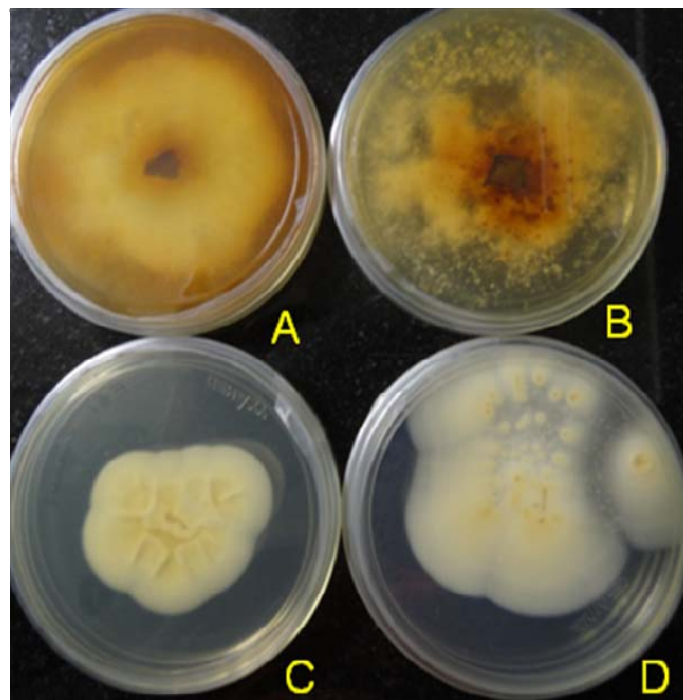
\*\*\* significant at >0.01 %. F value greater than F-critical value indicates significant difference

Two deep-sea isolates i.e. *Cerrena* and *Trametes* sp. showed better growth on MEA plates prepared with 100% seawater than their counterpart isolates from mangroves (Fig 4.7a). Also, these deep-sea isolates did not show production of oxido-reductase enzyme i.e. laccase on the media plates supplemented with guaiacol whereas mangrove isolates showed brown color, indicating laccase production (Fig 4.7b). Under simulated deep-sea conditions of elevated hydrostatic pressure and low temperature (20 MPa/5°C) deep-sea isolates showed better production of biomass than the mangrove isolates (Fig 4.8).





4.7 a) Growth in media with 100% seawater

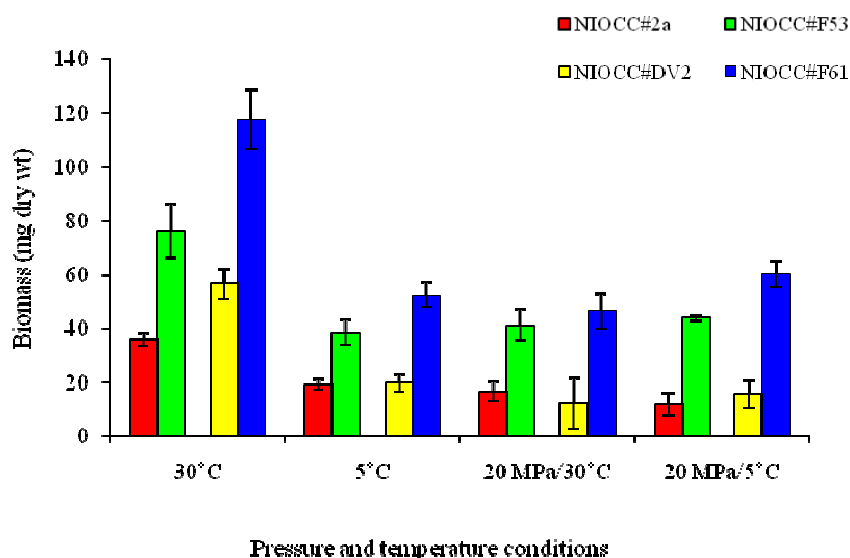


4.7 b) Laccase production

**Fig. 4.7** Growth characteristics (a) and oxido-reductase production (b) of the deep-sea cultures in comparison to their counterparts, isolated from mangroves.

A. *Cerrena* sp., B. *Trametes* sp. (Mangroves)

C. *Cerrena* sp., D. *Trametes* sp. (Deep-sea)



**Fig. 4.8 Biomass (mg dry wt/20 ml) of mycelial fungi after 20 days incubation in malt extract broth under different pressure and temperature conditions**

**NIOCC#2a and #DV2: Mangroves isolates**

**NIOCC#F53 and #F61: Deep-sea isolates**

Spores of four fungi from deep-sea showed germination of spores (Fig. 4.9) under 20 MPa/30°C whereas only one isolate i.e. NIOCC#F13 could germinate at 30 MPa/30°C (Table 4.1). All the terrestrial isolates failed to germinate at elevated pressure even at room temperature. None of the fungi showed spore germination at 5°C both at atmospheric as well as elevated pressure conditions (Table 4.1). In addition, spores from these four sporulating deep-sea fungi showed germination of spores under 20-50 MPa pressure at 30°C and no dependence on salinity (Fig. 4.10). A one-way ANOVA showed statistically significant effect of hydrostatic pressure on spore germination but salinity did not show such effect (see the legend for Fig. 4.10). With increasing hydrostatic pressure, a decrease in spore germination was observed. The four fungi appeared to be euryhaline because the spores germinated from 1.7 to 34 ppt salinity.

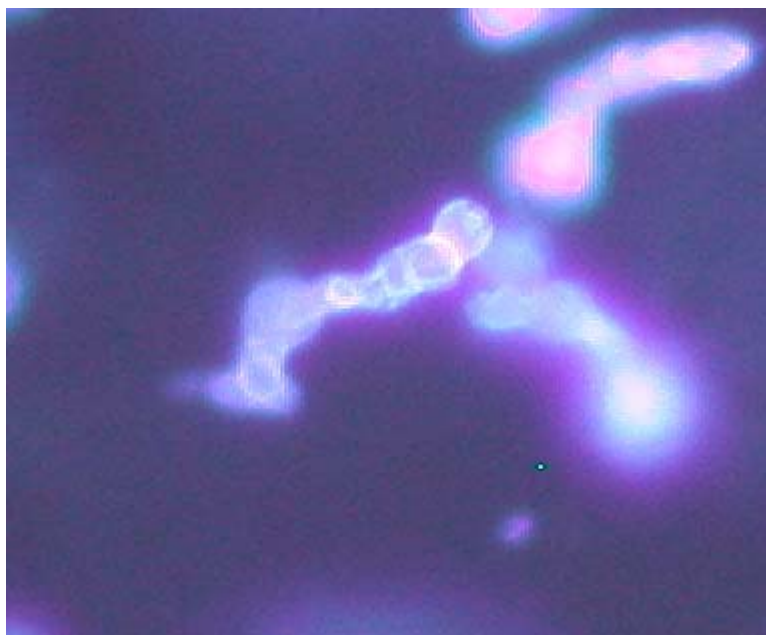
**Table 4.1 Percentage Germination of spores**

Culture no.	0.1 MPa /30 <sup>0</sup> C	20 MPa /30 <sup>0</sup> C	30 MPa /30 <sup>0</sup> C	0.1 MPa /5 <sup>0</sup> C	30 MPa /5 <sup>0</sup> C	20 MPa /5 <sup>0</sup> C
NIOCC#F2	100	76.9	0	No germination		
NIOCC#F5	100	100	0			
NIOCC#F6	100	100	0			
NIOCC#F13	100	76.24	31.25			
MTCC#IM2	100	0	0			
MTCC#IM3	100	0	0			
NIOCC#85	100	0	0			

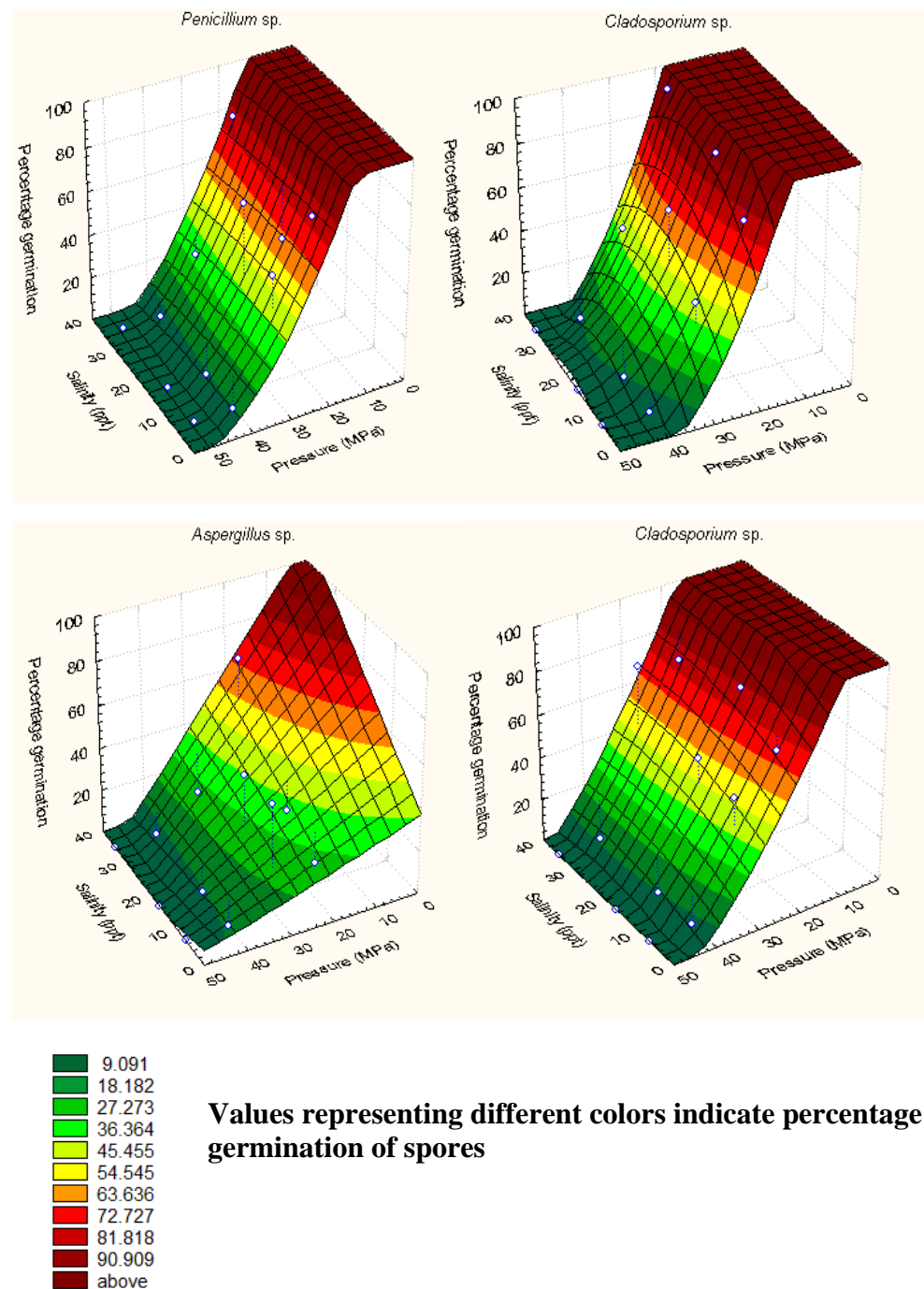
NIOCC#F2, #F5, #F6, #F13: Deep-sea isolates

MTCC#IM2, #IM3: Terrestrial isolates

NIOCC#85: Isolate from shallow water from a lagoon in the lakshadweep island



**Fig 4.9 Spores of NIOCC#F6 (*Aspergillus sp.*) germinated under 20 MPa pressure for 15 days and stained with calcofluor**



**Fig. 4.10 Synchronous effect of different hydrostatic pressure (20, 30, 40 and 50 MPa) and salinity (1.7, 17 and 34 ppt) on spore germination of four fungi after incubation in Malt Extrat Broth for 15 days (SD values<10%).**

Results of 1-Way ANOVA performed to understand the effect of varying pressure and salinity on % spore germination for the culture NIOCC#F2

	<b>Pressure/% germination</b>	<b>Salinity/% germination</b>
F critical	4.3	4.3
F value	82.6	1.3
P value	6.7E-09***	0.3
Degrees of freedom	23	23

\*\*\* significant at >0.01 %. F value greater than F-critical value indicates significant difference

Results of 1-way ANOVA performed to understand the effect of varying pressure and salinity on % spore germination for the culture NIOCC#F5

	<b>Pressure vs% germination</b>	<b>Salinity vs% germination</b>
F critical	4.3	4.3
F value	78.3	1.9
P value	1.06E-08***	0.2
Degrees of freedom	23	23

\*\*\* significant at >0.01 %. F value greater than F-critical value indicates significant difference

Results of 1-way ANOVA performed to understand the effect of varying pressure and salinity on % spore germination for the culture NIOCC#F6

	<b>Pressure vs% germination</b>	<b>Salinity vs% germination</b>
F critical	4.3	4.3
F value	92.2	0.02
P value	2.5E-09***	0.9
Degrees of freedom	23	23

\*\*\* significant at >0.01 %. F value greater than F-critical value indicates significant difference

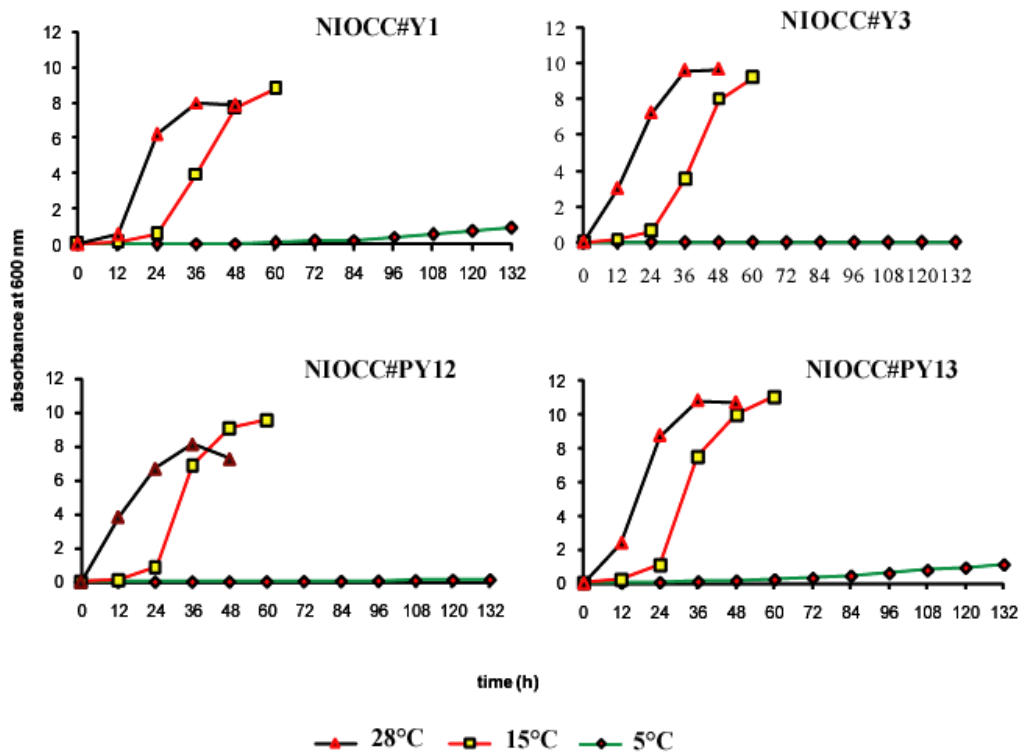
Results of 1-Way ANOVA performed to understand the effect of varying pressure and salinity on % spore germination for the culture NIOCC#F13

	<b>Pressure vs% germination</b>	<b>Salinity vs% germination</b>
F critical	4.3	4.3
F value	80.5	2.0
P value	8.4E-09***	0.2
Degrees of freedom	23	23

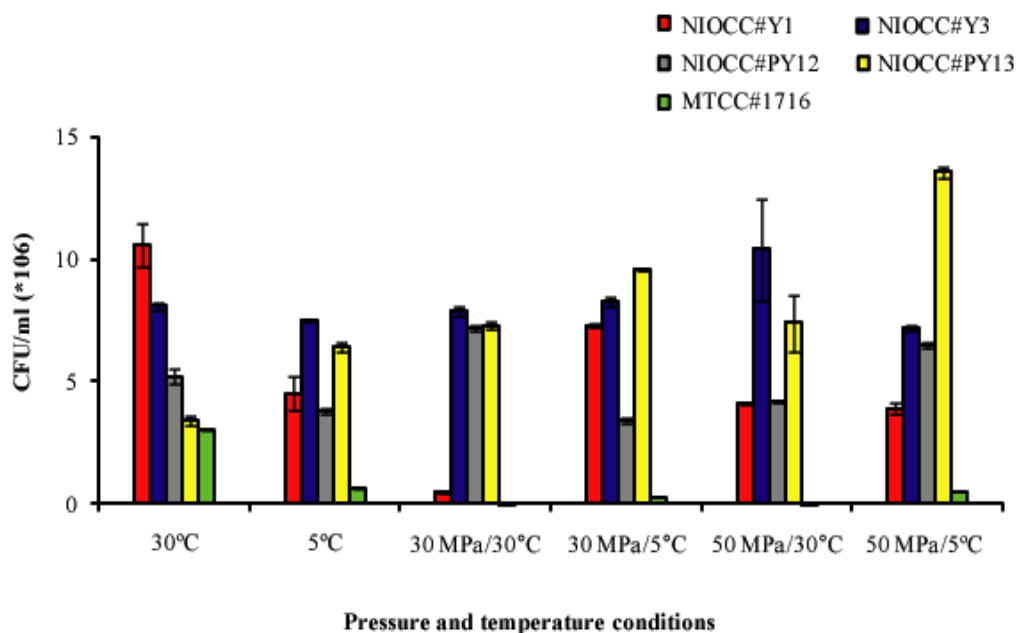
\*\*\* significant at >0.01 %. F value greater than F-critical value indicates significant difference

### 4.3.2 Growth and viability patterns of yeast isolates under simulated deep-sea conditions

All the four yeast isolates showed growth at 15 and 28°C. Out of these only two yeasts i.e. NIOCC#Y1 and #PY13 showed some growth at 5°C (Fig. 4.11). The psychrotolerant yeast #PY13 showed higher viability at 50 MPa/5°C than 30°C (Fig. 4.12). Other three deep-sea isolates were also found to be viable under simulated deep-sea conditions. The terrestrial yeast MTCC#1716 showed very less viability under all the elevated pressure and low temperature conditions (Fig. 4.12).



**Fig. 4.11** Growth curves of four yeast cultures at different temperatures (SD values <10%)

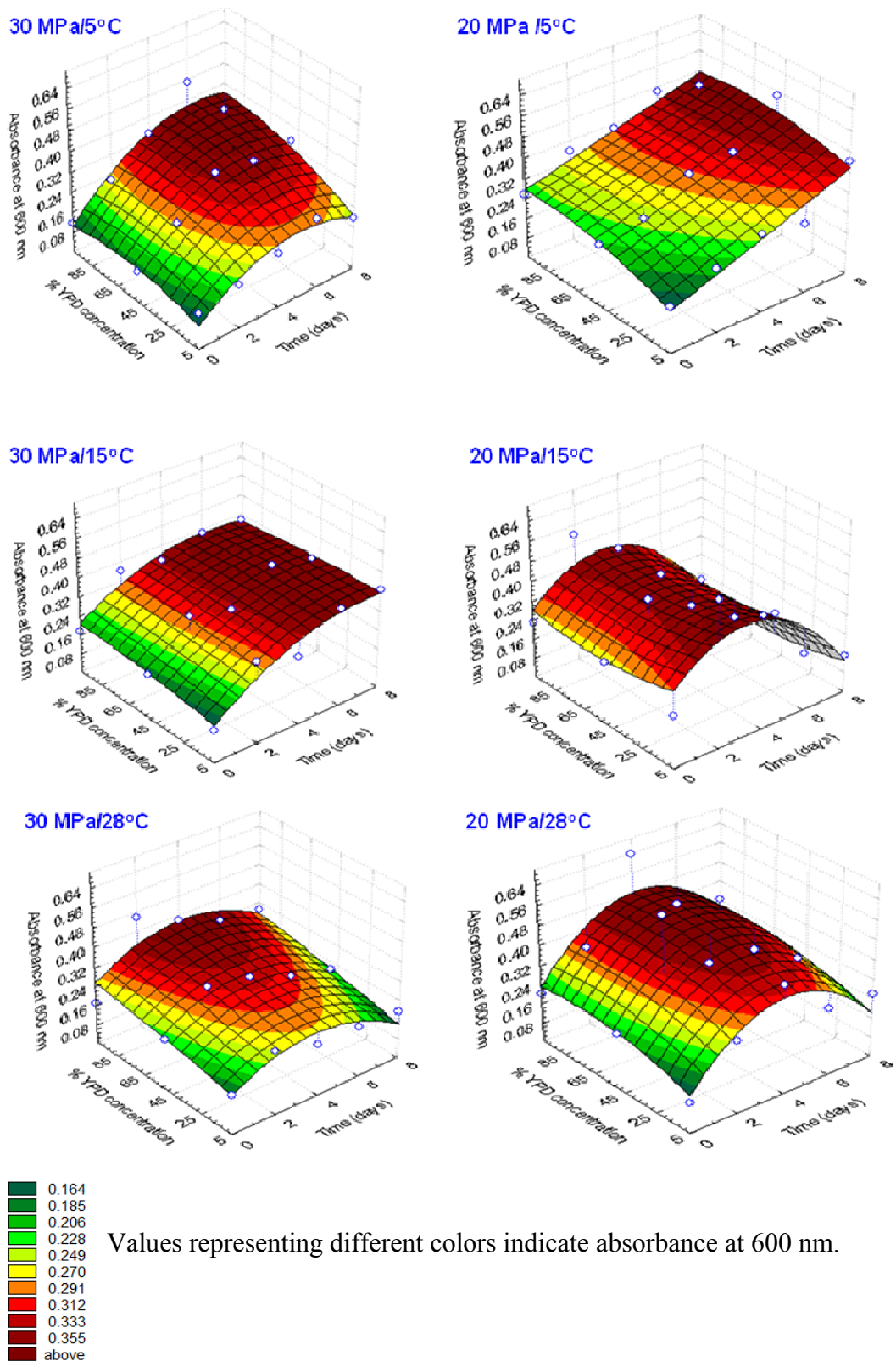


**Fig. 4.12 Viability of yeast cultures subjected to different pressure and temperature shocks for a period of 24 h**

**NIOCC#Y1; NIOCC#Y3; NIOCC#PY12; NIOCC#PY13: Deep-sea Yeasts and MTCC#1716: a terrestrial yeast**

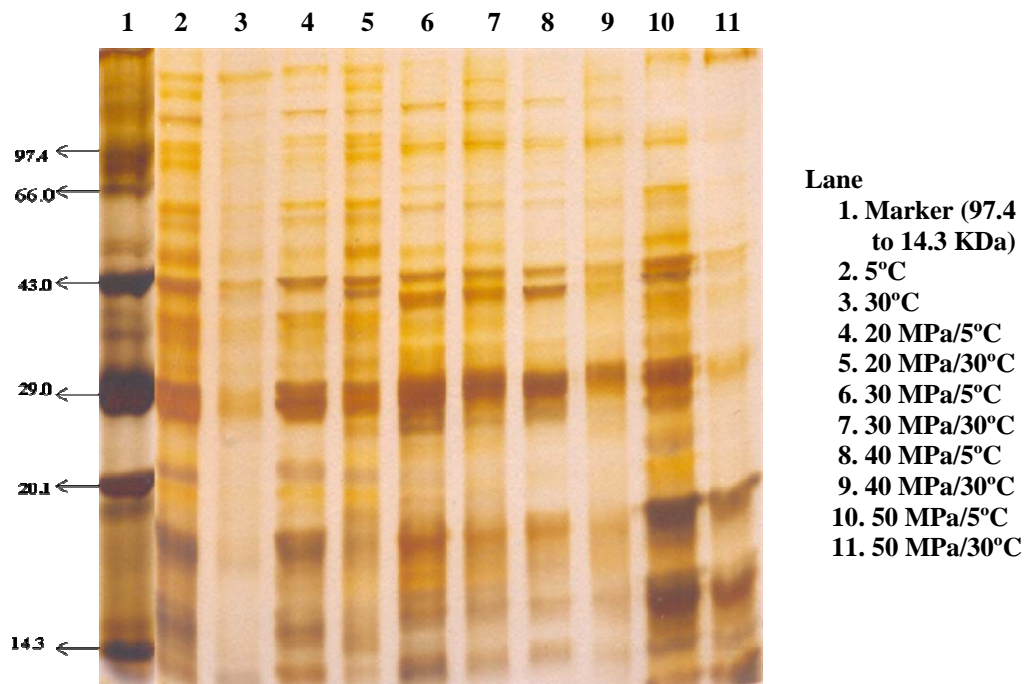
### 4.3.3 Effect of different pressure, temperature and nutrient concentrations on growth of NIOCC#PY13

Low temperature was found to be the major factor affecting the growth of the psychro-tolerant yeast #PY13 than the elevated pressure. The yeast showed better growth at higher nutrient concentration when grown at 5°C (Fig. 4.13). However at 15 and 28°C, the culture was found to grow to the same degree with all the nutrient concentrations. Time taken to achieve maximum growth was highest at 5°C i.e. ~ 8<sup>th</sup> day whereas at higher temperatures the growth was maximum on 4<sup>th</sup> day (Fig. 4.13). Best growth was observed at 15°C under all the pressure and nutrient concentrations.



**Fig. 4.13** Surface plot graph showing effect of varying nutrient concentrations, temperature and pressure on growth of NIOCC#PY13





**Fig. 4.14 SDS-PAGE for total cellular protein of the yeast NIOCC#PY13 after subjecting them to shocks of different temperature and pressure conditions for a period of 24 h**

#### **4.3.4 Protein profiles at different pressure and temperature conditions**

Protein profile was found to be different under different pressure and temperature conditions. More number of protein bands were observed at 5°C than 30°C (Fig. 4.14). The protein concentration was also high at lower temperatures. A combination of 50 MPa and 5°C was found to produce maximum number of protein bands. Elevated hydrostatic and low temperature shocks induced production of more number of low molecular weight protein molecules (Fig. 4.14)

## **4.4 Discussion**

### **4.4.1 Effect of elevated hydrostatic pressure and low temperature on growth and spore germination of fungi**

Although most of the fungi isolated from deep-sea sediments of the CIB were terrestrial species, they seem to have adapted to growth in the deep-sea and evolved into distinct physiological species. This was inferred from the fact that all the cultures showed growth at 20 MPa pressure and 5°C. All the filamentous fungi and a few yeasts showed better biomass production at 20 MPa/5°C than at 20 MPa/30°C (Figs. 4.2 and 4.3). These results were concordant with the earlier reports by Damare et al. (2006), where deep-sea fungi belonging to terrestrial sporulating species also showed growth at 20 MPa/5°C. However no correlation has been reported between isolation depths and optimal pressure for growth of these deep-sea fungi (Jannasch, 1987; Yayanos & Delong, 1987). In contrast, three terrestrial sporulating fungi obtained from culture collection belonging to *Aspergillus* sp. did not show increase in biomass at elevated hydrostatic pressure and low temperature conditions (Fig. 4.4) suggesting the adaptation of deep-sea isolates for growth under such extreme conditions. This study supports the hypothesis put forth by Raghukumar and Raghukumar. (1998) and Damare et al. (2006), that terrestrial fungi blown to the sea surface and sinking to the deep-sea sediments have adapted to the alien environmental conditions. These forms may take some time to adapt to the extreme conditions existing in the deep-sea resulting in the adaptation of most sturdy forms whereas other forms either die or remain in dormant but viable state (Raghukumar and Raghukumar, 1998).

Biomass production for deep-sea fungal isolates was much better in seawater medium than in medium prepared with distilled water in filamentous as well as unicellular fungi (Figs. 4.5 and 4.6). However, none of these fungi showed absolute requirement for sea water or NaCl for growth as has been shown for a few deep-sea actinomycetes (Jensen et al, 2005). The fact that the culturable fungi from the deep-sea sediments showed homology to ITS and

18S sequences to those in GenBank does not necessarily imply that they are identical species. It is possible that they are physiologically distinct as was reported for *Aspergillus sydowii*, the fungal pathogen of seafan (Alker et al, 2001). Although being a terrestrial fungus, it was metabolically distinct from nonpathogenic terrestrial strains. This hypothesis is further supported by the comparative studies between two deep-sea isolates i.e. *Cerrena* and *Trametes* sp. and their counterparts from mangroves. These two white rot fungi have been isolated earlier from various terrestrial environments and reported as an efficient source of lignin modifying enzymes (Kim et al, 2002; Elisashvili et al, 2002; Michniewicz et al, 2006). Among these lignin modifying enzymes, laccase is an extracellular enzyme belonging to the copper-containing polyphenol oxidases capable of oxidizing phenols and aromatic amines (Thurston, 1994). Laccase has been found to be produced by many white-rot fungi but also occurs in molds and yeasts as well as in plants and bacteria (Thurston, 1994). Fungal laccases have been reported to take part in the formation of fruit bodies and rhizomorphs, in pigmentation of spores, in sex differentiation and as virulent factors of plant pathogenic fungi. Laccases of soil saprophytic fungi are also involved in the humification of organic residues (Eggert et al, 1996; Gianfreda et al, 1999; Zavarzina et al, 2004). The laccases of ligninolytic basidiomycetes, the white-rot fungi, where laccases are an essential component of ligninolytic complex, are the most abundant and the most extensively studied (Leonowicz et al, 2001; Rabinovich et al, 2004).

The deep-sea fungi belonging to *Cerrena* and *Trametes* sp. showed better growth on the MEA media prepared with 100% seawater than the mangrove isolates (Fig. 4.7a). Also these two deep-sea isolates did not show any production of laccase enzyme when grown on specific substrates whereas mangrove isolates showed good production of laccase (Fig.4.7b). The adaptation strategies of these fungi under extreme conditions of pressure and temperature are further supported by their better growth under these conditions compared to their counterparts isolated from mangrove (Fig. 4.8). The absence of lignin modifying enzyme production and better growth under simulated deep-sea conditions of these fungi suggests their different physiological

properties than their terrestrial and mangrove counterparts. The gene responsible for the transcription of the above enzyme may be present in non-functional form in deep-sea isolates which can be further supported by their analysis by using molecular approaches such as PCR amplification and sequencing. Recently more interest is growing in using marine fungi as efficient producers of bioactive compounds for bioremediation (Maneerat et al, 2006; Bhadury et al, 2006). Therefore, transformation and expression of such useful lignin modifying enzyme transcribing genes in marine fungi can prove to be beneficial for efficient bioremediation processes.

Spores of four deep-sea fungi i.e. NIOCC#F2, F5, F6 and F13 germinated under elevated pressure conditions whereas spores of other terrestrial fungi failed to germinate under elevated pressure even at 30°C. However spores from none of these fungi germinated at 5°C under elevated hydrostatic pressure or even at 0.1 MPa at this temperature. It has been demonstrated that low temperature and not the elevated hydrostatic pressure is the limiting factor for spore germination of deep-sea adapted fungi (Damare et al, 2006, 2008). The probable reason for no germination at 5°C may be the reduced metabolic activities and cellular processes at low temperature (Wirsen and Jannasch, 1975). Three terrestrial cultures (obtained from a culture collection centre) showed slight mycelial growth under 20 MPa pressure at 5°C as well as at 30°C (data not shown) but their spores failed completely to germinate under these conditions (Table 4.1). Therefore, as reported by Damare et al. (2008) the effect of hydrostatic pressure and low temperature is different on mycelial fragments and fungal spores. Further there was no significant effect of different salinities on spore germination of the above four deep-sea fungi when incubated under a range of 20-50 MPa pressure at 30°C. In contrast, an elevated salinity was previously shown to increase the maximum hydrostatic pressure for growth in the psychrophiles *Moritella marina* (Palmer and Albright, 1970; Urakawa et al, 1998) and *Streptococcus faecalis*, reclassified as *Enterococcus faecalis* (Schleifer and Kilpper-Balz, 1984; Marquis and ZoBell, 1971) and to decrease the piezo-sensitivity of *Escherichia coli* to an extremely high hydrostatic pressure of 270 MPa

(Hauben et al, 1998). However, the results obtained in the present study support the hypothesis proposed by earlier workers that “feast and famine” condition of deep-sea environment does not seem to have effect on spore germination (Hawker and Madelin, 1976; Damare, 2006). The spores having lower metabolic rates than the mature mycelium under elevated hydrostatic pressure and low temperature do not germinate even after supply of higher concentration of nutrients.

#### **4.4.2 Effect of simulated deep-sea conditions on growth, viability and protein pattern of psychro-tolerant yeast NIOCC#PY13**

The yeast NIOCC#PY13 showed comparatively higher growth at 5°C than other three yeast isolates suggesting the psychro-tolerant nature of #PY13 and therefore better adaptation to grow at lower temperatures compared to their mesophilic counterparts. In general, cold adaptability of microorganisms requires structural flexibility, which favors greater complementarity at low energy cost. It provides a rational explanation of the high specific activity of some cold-adapted enzymes (Gerday et al, 2000). Isolation and characterization of DNA-dependent RNA polymerase (Uma et al, 1999), ribonuclease (Reddy et al, 1994) and alkaline phosphatase (Chattopadhyay et al, 1995) from different Antarctic strains have been reported earlier. Other cold-active enzymes have been reported from different cold-tolerant bacteria, including protein-tyrosine phosphatase (Tsuruta et al, 2004),  $\alpha$ -amylase,  $\beta$ -galactosidase (Groudieva et al, 2004) and aminopeptidase (Huston et al, 2004). Following a downshift of temperature from 37°C to 18°C, several genes were shown to be transcriptionally upregulated in a strain of *Bacillus subtilis*, using DNA microarray analysis. Among them were the genes, which encode enzymes involved in the degradation of the branched-chain amino acids (Kaan et al, 2002).

Also the viability of the yeast #PY13 was high when subjected to shock at 50 MPa/5°C than at 30°C and ambient pressure conditions (Fig. 4.12). However viability of other mesophilic and terrestrial yeasts was found

to decrease under extreme conditions of deep-sea. In addition, the temperature was found to be the major factor affecting growth of #PY13 when grown at different concentrations of nutrients and elevated pressure. The growth was maximum at 15°C both under 20 and 30 MPa pressure and was not found to be affected by change in nutrient concentrations. However, at 5°C the maximum growth was attained at a higher concentration of nutrients (Fig. 4.13). These results suggest this yeast are adapted to the elevated hydrostatic pressure and low temperature conditions of deep-sea, provided there are sufficient nutrients to increase its metabolic rates. The degree of microbial inactivation by pressure has been reported to depend on the type of microorganism, pH, composition of the media, as well as on the parameters of the process (Patterson et al, 1995; Kalchayanand et al, 1998; Alpas et al, 2000). The viability and growth of microorganisms have been shown by numerous studies to depend strongly on the osmotic pressure (Scott, 1957; Esener et al, 1981) and temperature (Walton and Pringle, 1980; Hottiger et al, 1987; Suutari et al, 1990) of the growth medium. Beney et al. (2001) reported increase of survival of *S. cerevisiae* cells by exposure to an osmotic stress of 100 MPa by a factor of 2.5 after shifting the cells from 25 to 11°C. Same results were obtained in the present study also where a combination of 50 MPa pressure and 5°C enhanced the viability of #PY13 than atmospheric pressure and 30°C.

Protein profile of the yeast #PY13 obtained under different pressure and temperature shock indicated more number of proteins produced at elevated pressure and low temperature conditions (Fig. 4.14). Likewise, a subset of the DAN/TIR cell wall mannoprotein genes, which were well-documented to be anaerobic and cold-inducible genes (Abramova et al, 2001a, 2001b), was dramatically up-regulated by high pressure and low temperature (Abe, 2007b). The moderately barophilic deep-sea bacterium *Photobacterium* SS9 modulates the abundance of several outer membrane proteins in response to hydrostatic pressure (Chi and Bartlett, 1993). One outer membrane porin protein, designated OmpH, which facilitates uptake of larger substrates, has been found to be induced 10-100-fold in mid-log cells of SS9, grown at the

pressure optima of 28 MPa, as compared with mid-log cells grown at atmospheric pressure (Bartlett et al, 1996). Elevated pressure have also been reported to up-regulate a heat shock protein, *HSP12* (Fernandes et al, 2004) which codes for a protein related to cell wall flexibility (Motshwene et al, 2004), suggesting that pressure directly affects cell wall integrity and the cell responds by production of Hsp12p. It has been confirmed that the cell wall protein Hsp12p is highly hydrophilic and acts on the cell wall as a plasticizer in plastic polymers, interrupting the hydrogen bonding and ionic interactions between adjacent polysaccharide polymers that otherwise result in a stable inflexible structure (Sales et al, 2000; Motshwene et al, 2004).

Also, the maintenance of appropriate membrane fluidity is thought to be one of the key factors for survival and growth under elevated pressure conditions, as suggested by the results of studies with prokaryotes (DeLong and Yayanos, 1985; Abe et al, 1999; Allen et al, 1999). Abramova et al. (2001a) speculated that cells exhibited reduced membrane fluidity under hypoxia as a possible outcome of anaerobiosis and at low temperature as a result of reduced lateral diffusion and increased microviscosity. In addition, yeast cells expressing a substantial level of Tat2 i.e. tryptophan permease, became endowed with the ability to grow at low temperatures as well as at high hydrostatic pressures (Abe and Horikoshi, 2000). The induction of more proteins at elevated hydrostatic pressure and low temperature in the present study suggest enhanced expression of some of the genes transcribing essential proteins required for growth under such extreme conditions. These proteins need to be identified using advanced level techniques such as 2D-Mass spectrometry in order to understand their contribution towards survival of cells under deep-sea conditions. Mass spectrometry is an important emerging method for the characterization of proteins after running on 1D or 2D PAGE (Poly acrylamide gel electrophoresis). The protein to be analyzed is ionized first. The two primary methods for ionization of whole proteins are electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI). MALDI-TOF MS is a relatively novel technique in which a co-precipitate of an UV-light absorbing matrix and a biomolecule is irradiated by

a nanosecond laser pulse (Karas et al, 1987). Most of the laser energy is absorbed by the matrix, which prevents unwanted fragmentation of the biomolecule. The ionized biomolecules are accelerated in an electric field and enter the flight tube. During the flight in this tube, different molecules are separated according to their mass to charge ratio and reach the detector at different times. In this way each molecule yields a distinct signal. The method is used for detection and characterization of other biomolecules also such as peptides, oligosaccharides and oligonucleotides, with molecular masses between 400 and 350,000 Da. It is a very sensitive method, which allows the detection of low i.e.  $10^{-15}$  to  $10^{-18}$  mole quantities of sample with an accuracy of 0.1 - 0.01 %.

In conclusion, elevated hydrostatic pressure and low temperature is generally assumed to have adverse effects on biological systems, but it can serve as a useful parameter to elucidate dynamic structural changes associated with any reaction. Elevated pressure changes the rate of reactions in a manner separable from those due to low temperature. Accordingly, combined analyses using pressure and temperature offer new insights into cellular functions such as the assembly of macromolecules, membrane trafficking, or membrane protein functions. Understanding the connections between different environmental conditions in living cells may contribute to information about the particular signals generated by stresses and may shed light on sensory transduction and biochemical adaptation in organisms.