

Chapter 7

Proteases  
of  
Deep-sea Fungi

## 7.1 Introduction

The deep-sea environment is a source of unique microorganism with great potential for biotechnological exploitation. Very few studies concerning the isolation and characterization of deep-sea microorganisms have been carried out, and investigations in this field may lead to many new discoveries. Microorganisms living in the deep sea have special features that allow them to live in this extreme environment, and it seems likely that further studies of these organisms will provide important insights into the origin of life and its evolution.

Fungi from marine habitats have received much attention in recent years for the production of useful secondary metabolites [Liberra & Lindequist, 1995; Yu *et al.*, 2003; Jensen & Fenical, 2002]. Research on marine fungi and biotechnologically useful enzymes produced by them, however, has been restricted to those isolated from coastal habitats and to lignocellulose degrading enzymes for application in bioremediation and paper industries [Raghukumar, 2000; 2002]. Fungi and their enzymes from the deep-sea environment have received scant attention. The deep-sea inhabitants being present in extreme conditions produce extracellular enzymes, which are active under such conditions. Amongst the extracellular enzymes produced, proteases are important class of enzymes, which occupy a pivotal position with respect to their physiological role. Proteolytic enzymes play an important role in remineralisation processes in the sea, mainly because proteins and peptides constitute a substantial portion of the organic nutrients present in the deep-sea sediments as well as suspended particulate matter [Banse, 1990; Smith *et al.*, 1992]. Raghukumar &

[Raghukumar \[1998\]](#), reported production of protease enzyme under simulated deep-sea conditions by two filamentous fungi isolated from deep-sea calcareous sediments.

Protease refers to a group of enzymes whose catalytic function is to hydrolyze (breakdown) peptide bonds of proteins. They are also called proteolytic enzymes or proteinases. They are ubiquitous in nature. Proteases differ in their ability to hydrolyze various peptide bonds. They are highly complex group of enzymes, which vary enormously in their physicochemical and catalytic properties. Each type of protease acts on a specific kind of peptide bond. They are produced intra- and extracellularly. Proteases execute a large variety of complex physiological functions. Their importance in conducting the essential metabolic and regulatory functions is evident from their occurrence in all forms of living organisms. Proteases play a critical role in many physiological and pathological processes such as protein catabolism, blood coagulation, cell growth and migration, tissue arrangement, morphogenesis in development, inflammation, tumor growth and metastasis, activation of zymogens, and transport of secretory proteins across membranes. In general, extracellular proteases catalyze the hydrolysis of large proteins to smaller molecules for subsequent absorption by the cell whereas intracellular proteases play a critical role in the regulation of metabolism [[Kalisz, 1988](#); [Rao \*et al.\*, 1998](#)].

Previously, proteases were classified by [Hartley \(1960\)](#) into four classes as,

1. Serine proteases,
2. Aspartic proteases,
3. Cysteine proteases and
4. Metalloproteases

According to the Nomenclature Committee of the International Union of Biochemistry, proteases are classified in subgroup 4 of group 3 (Hydrolases) [Dixon & Webb, 1979,]. This classification was further recommended by Nomenclature Committee of 1992 (International Union of Biochemistry, 1992). However, proteases do not comply easily with the general system of enzyme nomenclature due to their huge diversity of action and structure.

Currently, proteases are classified on the basis of three major criteria as,

1. Type of reaction catalyzed,
2. Chemical nature of the catalytic site and
3. Evolutionary relationship with reference to structure [Barret, 1994]

Even though a lot of work has been carried out to explore the possibility of harnessing extremophiles as potential protease producers, fungi from extreme conditions of deep sea have not yet been tapped for this purpose. Barophilic enzymes having characteristic substrate specificity would be very useful for industrial applications such as proteases and glucanases for detergents and DNA polymerases for PCR amplification. Michels & Clark [1997] purified and characterized a protease from *Methanococcus jannaschii*. This enzyme is the first protease to be isolated from an organism adapted to a high pressure and high temperature environment. The partially purified enzyme has a molecular mass of 29 kDa and narrow substrate specificity. Enzyme activity increased as the temperature increased up to 116°C and enzyme activity was measurable up to 130°C, one of the highest temperatures reported for the function of any enzyme. These results suggest that enzymes produced by these high-pressure adapted bacteria should be more functional under high-pressure conditions

than at atmospheric pressure [Horikoshi, 1998]. Thus, such enzymes may be very useful in high-pressure bioreactor systems.

Proteases are a unique class of enzymes, since they are of immense physiological as well as commercial importance. Since proteases are physiologically necessary, they occur ubiquitously in animals, plants and microbes. However, microbes are a gold mine of proteases and represent the preferred source of enzymes in view of their rapid growth, limited space required for cultivation, and ready accessibility to genetic manipulation. The proteases isolated from extremophilic organisms are likely to mimic some of the unnatural properties of the enzymes that are desirable for their commercial applications. Hence deep-sea fungi could be one of the candidate organisms for harnessing the proteases produced by them.

Apart from their ecological significance, proteases are one of the most valuable commercial enzymes. They are being used in food industry, dairy, detergents, pharmaceuticals, leather industry, research laboratories, bioremediation, effluent treatments, etc. More than 25% of the worldwide sale of enzymes is contributed by proteases alone, where mainly alkaline proteases are used.

## **7.2 Objectives**

The objectives for the present studies were –

1. To screen the deep-sea fungi isolated by different method for the production of alkaline and low temperature active protease.
2. To characterize protease produced by these cultures.

### **7.3 Methodology**

#### **7.3.1 Screening for protease producing fungi**

All the isolates obtained from the deep-sea sediments, deep-sea organisms as well as from the lagoon sediments were screened for the production of protease as per method described by Molitoris [2000]. The assay was a qualitative one indicating whether the culture is capable of producing protease. Briefly, mycelia of a growing culture were spot inoculated on a Czapek Dox (CD) agar plate supplemented with 1 % of skimmed milk. The plates were incubated till good growth was observed (4 to 5 days). The protease producers show a zone of clearance around them. If the zone was not clearly visible, the plates were flooded with coomassie blue solution for 10 minutes. The area around the protease producers is seen as a clear colourless zone as against blue coloured area where the milk protein is not degraded.

#### **7.3.2 Protease production**

The protease positive cultures were used for further quantification of the enzyme. For this, the cultures were first grown in MEB at 30°C. After 4 to 5 days, when sufficient growth was achieved, the biomass was homogenized mechanically using sterile glass beads and this was used as inoculum (the same procedure was followed for all the experiments involving protease production). This was inoculated in CD broth (Czapek Dox broth without agar) with 0.3% skimmed milk powder (Appendix A - IX). Protease production was compared at 5° and 30°C by growing the cultures at these temperatures. The cell free culture filtrates were obtained by filtering

through GF/F and 0.22  $\mu\text{m}$  nitrocellulose filter papers, successively. These filtrates were used to assay the protease enzyme activity.

The cultures were also tested for their ability to grow and produce protease at elevated hydrostatic pressures. Sterile plastic pouches containing 45 ml of Czapek Dox broth with 0.3% skimmed milk solution were inoculated with 5 ml of culture suspension (finely broken mycelia and spores in seawater). These were suspended in deep-sea culture vessels (Tsurumi & Seiki Co., Japan) filled with sterile distilled water. These vessels were pressurized at 50 bar and 100 bar (10 bar = 1 MPa) and incubated at 30°C and 5°C for 20 days. Three replicates were maintained for each treatment. After 20 days, the biomass was separated from culture broth by centrifugation and further filtered through sterile 0.22  $\mu\text{m}$  hydrophilic durapore membrane filters (Millipore, USA). Protease activity of the filtrate was estimated using azocasein as the substrate. The assay was carried out at the optimal conditions of the enzyme (pH = 9; 45°C and 1 bar pressure). The activity was also assayed under 50 and 100 bar pressure at pH 9 and 30°C.

The extracellular protease activity of the culture grown under atmospheric pressure (1 bar pressure) and 30°C and 50 bar/30°C was compared with activity under elevated hydrostatic pressure of 50, 100, 200 and 300 bar at pH 9 and 30°C. The  $K_m$  constants of the protease activity from cultures grown at 30°C and 1 bar pressure were measured at 1 bar/5°C and 50, 100, 200 and 300 bar/45°C using the substrate azocasein at pH 9.

### 7.3.3 Protease assay

The protease activity was assayed with 150  $\mu$ L of crude culture filtrate and 250  $\mu$ L of the substrate azocasein (Sigma Chemicals, USA) at 2 % concentration prepared in 2 buffers, 0.1 M boric acid-borax buffer pH 9 and 0.1 M phosphate buffer pH 7. Protease activity was measured by incubating the reaction mixture at 30°C and 5°C for 30 min. The reaction was stopped by addition of 1.2 mL of 10% trichloroacetic acid solution. The contents were centrifuged at 10000 rpm for 10 min. To the supernatant, 1.4 mL of 1N NaOH was added and the absorbance read immediately at 440 nm against appropriate blanks in a spectrophotometer (Shimadzu, Model 1210, Japan). One ACU (Azocasein Digestion Unit) is defined as the increase in absorbance by 0.001 per minute under the assay conditions [Hamamoto *et al.*, 1995].

Protease activity under elevated hydrostatic pressure was assayed in microcentrifuge tubes of 0.5 mL capacity [Raghukumar & Raghukumar, 1998]. The lids of the tubes were snapped off and sealed with parafilm after adding the reaction mixture, which contained the substrate azocasein, buffer at pH 9 and appropriately diluted enzyme. Care was taken to avoid trapping of air bubbles while sealing. The tubes were suspended in deep-sea culture vessel pressurized to the desired hydrostatic pressure and incubated under desired temperature for 30 min. At the end of the incubation period, the vessels were depressurized and without any delay the enzyme activity was arrested by adding TCA. Protease activity was measured as described above. Control was kept keeping in view the delay in pressurizing the vessels and that was considered as the starting point for the calculation of enzyme activity.

### **7.3.4 Characterization of protease produced by one of the deep sea fungus**

One of the cultures NIOCC # 20, identified as *Aspergillus ustus* (Bain.) Thom and Church (deposited at the Institute for Microbial Technology, Chandigarh, India under the accession number MTCC 5102) showed the highest protease activity at pH 9, both at 5° and 30°C temperatures among all the deep-sea fungal cultures and therefore was selected for further studies. The conditions for obtaining maximum protease production were partially optimized with reference to the time of harvesting and different inducers.

#### **7.3.4.1 Temporal production of biomass and protease**

The culture, NIOCC # 20, was inoculated in Czapek Dox broth with 0.3 % skimmed milk. Every day, culture filtrate was collected by filtering first through glass fibre (GF/F) filters (Whatman, USA) and then by durapore 0.22µm (GVWP04700, Millipore, USA). The biomass was dried to constant weight and recorded. The culture filtrate was assayed for protease activity at pH 9 and 45°C, the optimum pH and temperature for activity.

#### **7.3.4.2 Substrate inducers**

The culture was inoculated in Czapek Dox broth supplemented with different inducer substrates like molasses, corn steep liquor, skimmed milk, tween 80 and also in plain CD broth and MEB. The culture filtrate was collected after 7 days of incubation at 30°C and 1 bar as described above and assayed for protease activity at pH 9 and 45°C.

#### **7.3.4.3 Harvesting of enzyme**

It was observed that the culture is producing a lot of exopolysaccharides, which might be binding the extracellular protease and thereby resulting in the loss of the amount of protease being reported. To circumvent this problem, just before harvesting the culture filtrate, EDTA was added to it, mixed properly and then the contents filtered as described previously.

#### **7.3.4.4 Purification and characterization of purified enzyme**

The culture was inoculated in CD broth with 0.3 % skimmed milk and incubated at 30°C. After 7 days of incubation, the culture filtrate was collected as described previously and used for further studies. The cell free culture filtrate was concentrated in a vacuum concentrator (Biotron, Korea) to 1/10<sup>th</sup> volume. The concentrate was passed through an anion exchange column 'Resource Q' (Amersham Biosciences, Upssala, Sweden). It was eluted using a gradient of NaCl (0 – 0.25 M) prepared in 10 mM phosphate buffer at pH 7. The flow rate was adjusted to 0.1 mL min<sup>-1</sup> and eluted fractions of 1 mL each were collected. Fractions showing protease activities were pooled, concentrated and further subjected to size exclusion column chromatography using Superdex 200 column (Amersham Biosciences, Upssala, Sweden). The enzyme was eluted with 0.2 M NaCl prepared in 100 mM acetate buffer pH 4.5. The flow rate was adjusted to 0.5 mL min<sup>-1</sup> and the eluted fractions of 2 mL each were collected. Fractions showing protease activity were pooled and used for characterization of the enzyme.

At each step of purification, the enzyme activity and protein content [Lowry *et al.*, 1951] (Appendix A-V) were assayed to calculate the efficiency of the purification process.

The homogeneity of the fractionated enzyme was confirmed by running a native PAGE with 10% acrylamide at 60 V. The gel was blotted on 1.5% agarose containing 1% casein for 1 h. The agarose blot was stained with 0.15% amido black (Sigma Chemicals, USA) and 0.3% coomassie blue in methanol: acetic acid: water at a ratio of 4:1:5 [Oh *et al.*, 1999]. The molecular weight of the purified enzyme was determined using SDS-PAGE [Laemmli, 1970] (Appendix A-VI). The electrophoresis was carried out on 12% resolving gel at a constant voltage of 60V. The protein was detected using silver staining method [Heukeshovan & Dernick, 1985] (Appendix A-VII). The pI of the purified enzyme was estimated by running an isoelectric focusing tube gel. Broad range ampholytes of pH 3-10 were used. The pH gradient was formed by running the gel at 250 V for 30 min following which the sample was loaded onto the gel. The isoelectric focusing was carried out at 500 V for 4 hours.

#### **7.3.4.5 Optimum pH for enzyme activity**

The optimum pH for the activity of the purified enzyme activity was determined using eight different buffers (100 mM): sodium acetate (pH 5), citrate phosphate (pH 5-7) phosphate (pH 6-8), tris HCl (pH 8 & 9), borax-boric acid (pH 8 & 9), glycine-NaOH (pH 9 & 10) borax-NaOH (pH 10) and carbonate-bicarbonate

(pH 10 & 11) at 45°C. The substrate was prepared in the above buffers and the assay was carried out at 45°C as described previously.

#### **7.3.4.6 Optimum temperature for enzyme activity**

The optimum temperature for the protease activity was determined by assaying at 2, 10, 15, 30, 45, 50, 60, 70, 80 and 90°C at pH 9, the optimum pH for the enzyme activity.

#### **7.3.4.7 $K_m$ constant and $V_{max}$ for the protease**

$K_m$  constant and  $V_{max}$  for the enzyme were determined from Lineweaver Burke plot at pH 9 and 45°C using the substrate azocasein. The enzyme assay was carried out with varying substrate concentrations of 1 to 10 mg mL<sup>-1</sup>.

#### **7.3.4.8 Thermostability of purified enzyme**

Thermostability of purified enzyme at different temperatures was assayed by incubating the enzyme samples at 40, 50, 60, and 70°C for 10 minutes and then the residual activity was measured at its optimum pH and temperature i.e. pH 9 and 45°C. Thermostability of purified enzyme at its optimum temperature of activity (45°C) was estimated by incubating the enzyme at 45°C and the residual activity was assayed at an interval of 10 minutes. The assay was carried out at pH 9 and 45°C as described previously.

#### **7.3.4.9 pH stability of the purified enzyme**

The stability of purified enzyme at different pHs was carried out by incubating 25  $\mu$ L of enzyme with 75  $\mu$ L of different buffers as given above for one hour and then the residual protease activity was measured at 45°C and pH 9. Different additives were used to increase the thermostability of the enzyme at 60°C.

#### **7.3.4.10 Effect of inhibitors and stabilizers**

The effect of different protease inhibitors like PMSF, EDTA, chlorides of heavy metals like Ni, Zn, Cu, Hg, Fe, reducing agents like DTT, glutathione,  $\beta$ -mercaptoethanol, sodium thioglycolate, on the enzyme activity was determined by assaying in presence of these substances. The effect of varying ionic strength on the protease activity was also determined by assaying the enzyme in presence of varying sodium chloride concentration up to 1M. Keeping in mind one of the possibilities of the enzyme being a potential candidate for use in detergents, the activity was determined in presence of various commercially available detergents and bleaching agents at pH 9 and 45°C.

#### **7.3.5 Protease production by terrestrial *Aspergillus ustus* (MTCC 2200)**

A comparison was carried out with respect to protease production between the deep-sea isolate # NIOCC 20 (*A. ustus*) and the terrestrial isolate MTCC 2200 (*A. ustus*). The protease of the two cultures was compared with respect to the temporal production, different salinities, pH and temperature for activity. The biomass produced by the two in CD broth was also compared.

## 7.4 Results

### 7.4.1 Screening of deep sea fungi for protease production

A total of 221 and 22 fungi from deep-sea and shallow depth sediments respectively were obtained by employing different techniques of isolations.

**Table 7.1 Growth and alkaline (pH 9) protease production by deep-sea and shallow water isolates of fungi**

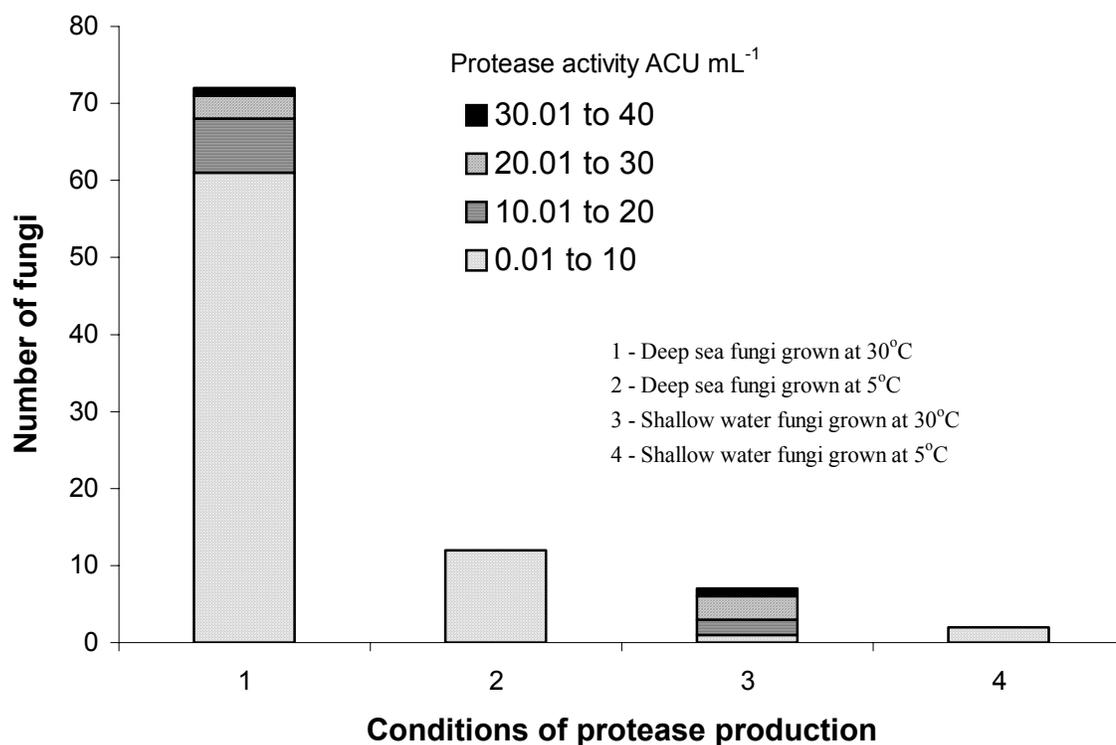
Source of Fungi	Total Number isolated	Culture condition	Number and (%) isolates showing growth	Cultures with protease activity at 30°C*	Cultures with protease activity at 5°C*
Deep sea	221	1 bar and 30°C	221 (100 %)	105 (48 %)	73 (33 %)
		1 bar and 5°C	113 (51 %)	15 (13 %)	12 (11 %)
Shallow water	22	1 bar and 30°C	22 (100 %)	10 (45 %)	3 (14 %)
		1 bar and 5°C	7 (32%)	2 (29 %)	2 (29 %)

\* percentage of cultures with protease activity was calculated from the total number of cultures showing growth (numbers in the 4<sup>th</sup> column)

Among these, 33 % of the deep-sea and 14 % of shallow water fungal isolates when grown at 30°C and 1 bar pressure-produced protease, which was active at alkaline pH and low temperature of 5°C. Up to 51% of the deep-sea isolates and only

32 % of the shallow water isolates showed ability to grow at low temperature of 5°C. Among the cultures showing growth at low temperature, 11 % of the deep-sea isolates and 29 % of the shallow water isolates produced protease that was active at alkaline pH (9.0) and 5°C (Table 7.1). The number of isolates showing protease activity at 30°C was much higher than those showing activity at 5°C. A large number of isolates showed alkaline protease activity within a range of 1 – 10 ACU mL<sup>-1</sup> (Fig. 7.1).

**Fig. 7.1** Number of fungi isolated from deep-sea and shallow-water sediments showing protease activity in the range of 1 to 40 ACU mL<sup>-1</sup> when grown at 5° and 30°C. The NIOCC #20 belonged to the group showing 30-40 ACU mL<sup>-1</sup>



Measurement of protease activity using azocasein was found to be very sensitive because as low as 1 ACU could be detected

#### 7.4.2 Protease production by NIOCC 20 under different conditions

A culture of *Aspergillus ustus*, (NIOCC # 20) isolated from the deep-sea sediments, during the cruise AAS 34, from core # BC 3, subsection 15-20 cm, from a depth of 5294 m by particle plating method showed maximum protease production initially in the range of 30-40 ACU mL<sup>-1</sup> when assayed at 30°C and 1 bar pressure. It showed growth at elevated hydrostatic pressure and 5°C and was selected for further characterization of the enzyme. The culture showed growth under elevated hydrostatic pressures at 30 and 5°C (Table 7.2).

**Table 7.2 Characteristics of the deep-sea isolate NIOCC # 20 when grown for 20 days in CD broth with 0.3 % skimmed milk powder under various pressure and temperature conditions**

Growth temperature	1 bar	50 bar	100 bar	Parameters
30°C	208.2	69.4	44.1	Biomass produced (mg dry weight.)
5°C	121.4	93.7	85.8	
30°C	36.02	3.67	0.78	Protease activity (ACU mL <sup>-1</sup> ) assayed at 30°C and 1 bar
5°C	4.45	0.45	0.22	
30°C	100	10.2	2.17	% activity against the highest value
5°C	12.35	1.25	0.61	
30°C	0.173	0.15	0.014	Protease activity /mg dry biomass
5°C	0.037	0.013	0.007	

Protease production by this fungus was assayed both at 30 and 5°C. The protease produced at 1 bar pressure at both 30°C and 5°C was active up to 300 bar. The activity was fairly stable but the protease production seemed to be repressed considerably at 5°C (Table 7.3).

**Table 7.3 Activity of the protease of the deep-sea fungus NIOCC #20 assayed under elevated hydrostatic pressures and 30°C**

Pressure (bar)	Protease activity (ACU mL <sup>-1</sup> )	
	A	B
1	427.33 ± 4.51	ND
50	356 ± 2.12	3.67 ± 0
100	363.33 ± 4.72	3.33 ± 0
200	417.33 ± 10.24	4.33 ± 0.24
300	428.33 ± 4.95	4.67 ± 0

A – Protease produced when grown at 1 bar pressure /30°C

B – Protease produced when grown at 50 bar pressure / 30°C

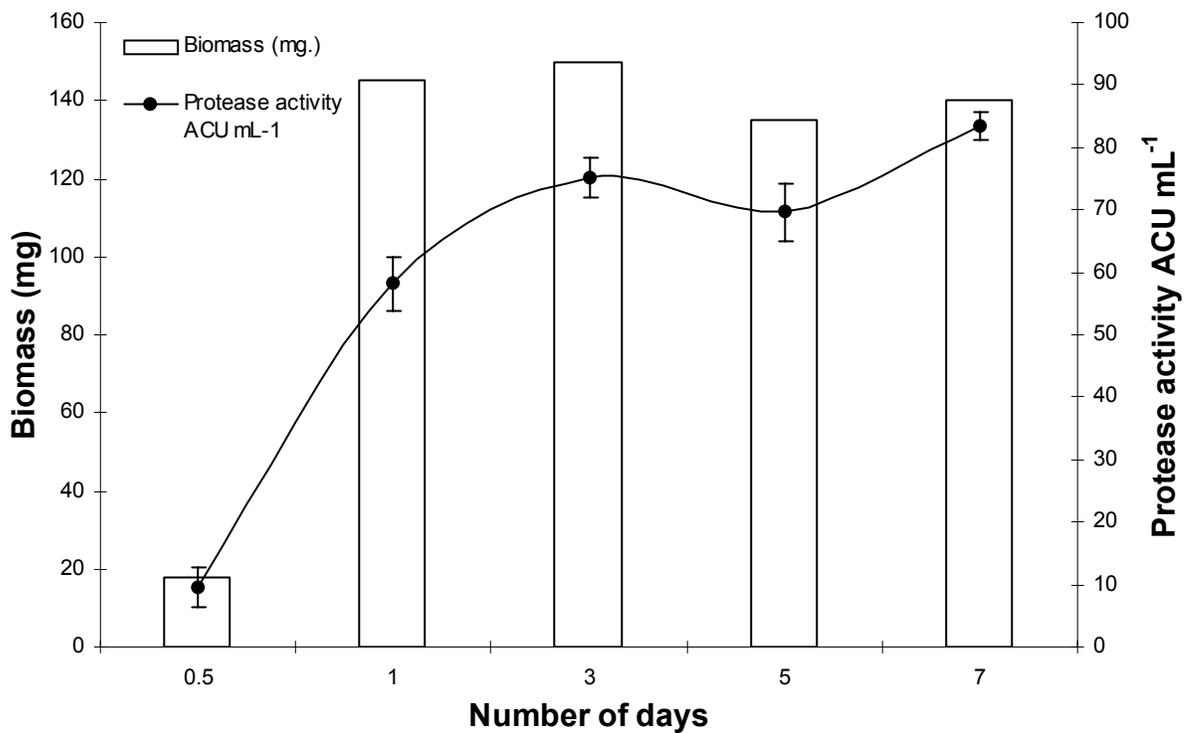
ND – not detectable

The enzyme produced at elevated hydrostatic pressure and low temperature revealed negligible or non-detectable levels of protease in all the assays at 45°C. Therefore further comparisons of protease production under different growth conditions of elevated hydrostatic pressures were made by assaying at 30°C.

### 7.4.3 Optimization of culture conditions for protease production by NIOCC 20

The culture was showing highest amount of protease production after 7 days of incubation at 30°C (Fig. 7.2). The biomass production was very high by day 1 with the

**Figure 7.2 Temporal production of biomass and protease by NIOCC #20 grown in CD broth with 0.3 % skimmed milk powder**

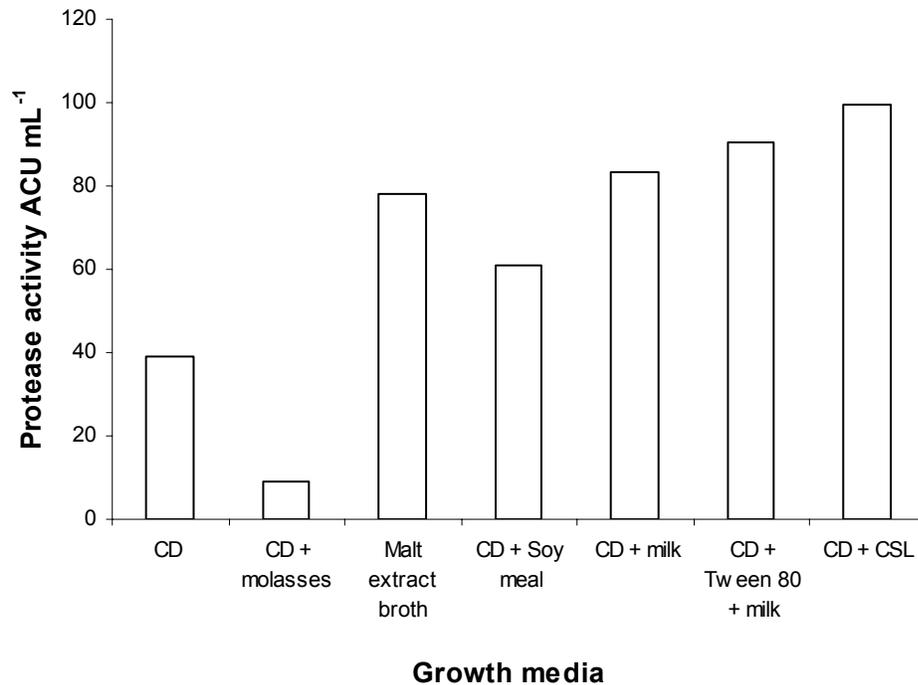


maximum being observed by day 2. The protease production attained maxima by day 7, which was more or less similar from day 3.

There was increase in the amount of protease production in the presence of inducers (Fig.7.3) Protease production was also observed constitutively in CD broth with only glucose as an organic source. The maximum protease production was observed in the presence of corn steep liquor (CSL), with the production decreasing in

CD + tween 80 + skimmed milk, CD + skimmed milk, malt extract, CD + soy meal and plain CD. The protease production was repressed in the presence of molasses.

**Figure 7.3 Protease production by NIOCC #20 when grown at 30°C in different media**



Addition of EDTA to the culture before harvesting was found to increase the protease yield by 1.5 to 2 times. When the enzyme was harvested after addition of EDTA, the yield of the enzyme was around 2400 ACU mL<sup>-1</sup> as against that of 1200 ACU mL<sup>-1</sup> without addition of EDTA.

Amongst the different methods of concentrations used, vacuum concentration was found to be the most efficient as compared to dialysis and ultra filtration (Table 7.4).

**Table 7.4 Efficiency of different concentration techniques**

Concentration method	Total volume (mL)		Protease activity (ACU mL <sup>-1</sup> )	
	Before concentration	After concentration	Before concentration	After concentration
PEG concentration using dialysis	100	10	1639	1890
Ultrafiltration using 3 kDa cut off	100	20	1639	1485
Vacuum concentration	100	15	1639	2365

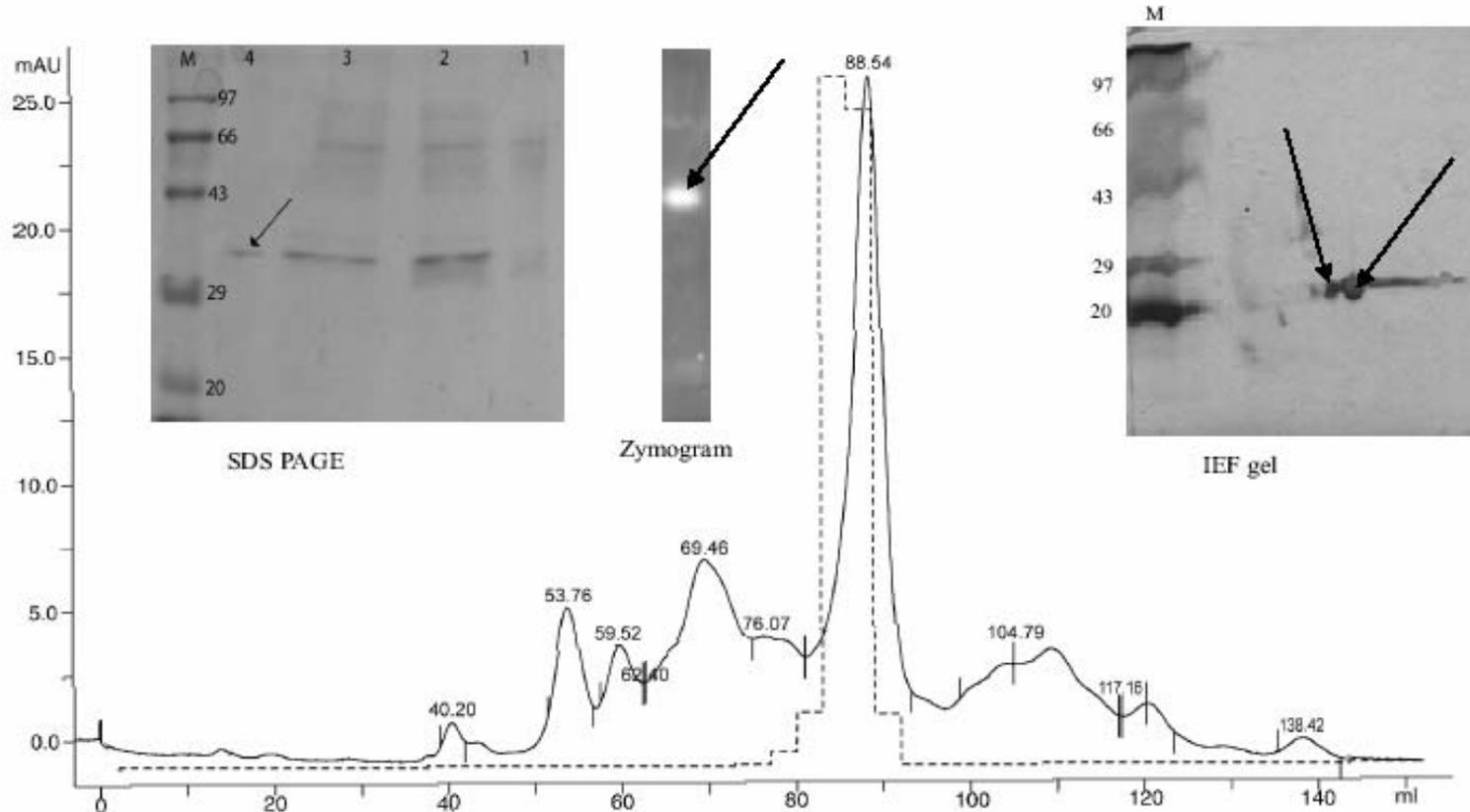
#### 7.4.4 Purification and characterization of protease produced by NIOCC 20

The concentrate when passed through Resource Q column, protease enzyme was eluted as an unbound fraction. There was no protease activity recorded in any of the bound fractions. These fractions when pooled, concentrated and subjected to size exclusion chromatography, resolved in different proteins. The protease enzyme was

**Table 7.5 Purification of protease of the deep-sea fungus NIOCC #20 grown at pH 9.0 and 30°C**

	Total enzyme units ACU	Total Protein mg	Sp. Activity (ACU mg <sup>-1</sup> protein)	Purification (fold)	% Recovery
<b>Crude filtrate</b>	981,000	131.08	7,484	1	100
<b>Vacuum concentrated</b>	205,800	93.06	2,212	0.3	21
<b>Resource Q</b>	166,786	82.61	2,019	0.27	17
<b>Superdex-200</b>	206,800	6.82	30,323	4.05	21

**Figure 7.4 FPLC profile with Zymogram, SDS and pI profile as insets**



FPLC profile after passing through Superdex 200, showing active enzyme fraction (dotted line, solid line indicates protein)

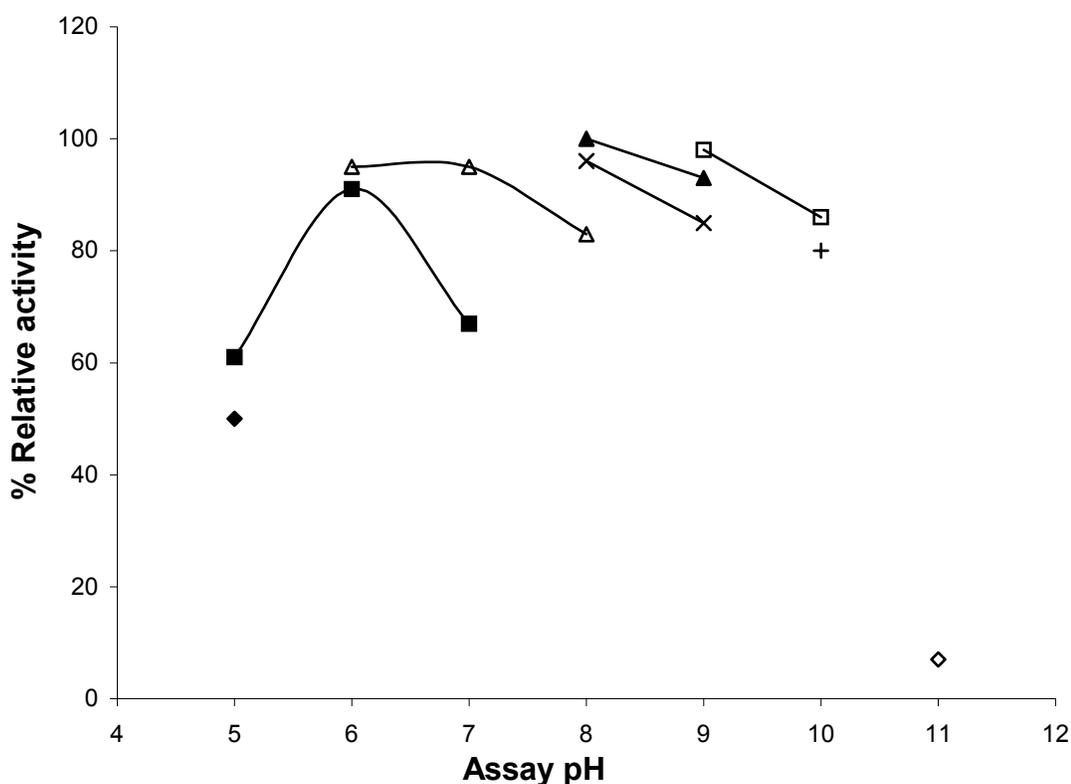
Zymogram of the active fraction on native page showed activity staining for protease. The clear zone in casein as a result of protease activity appears as a band (arrow);

The SDS-PAGE of the protease; Lane 1= crude culture filtrate, Lane 2=vacuum-concentrated culture filtrate, Lane 3= Resource Q-pooled fraction, Lane 4= pooled active fraction obtained by gel filtration using Superdex 200 showing single band of 32 kDa (arrow);

IEF gel showing isoforms with pI values of 6.6 and 6.9 (arrows).

obtained in pure form distributed over 10 mL (Fig. 7.4). This part was concentrated under vacuum and when checked for homogeneity showed a single activity band on a zymogram, with casein as substrate. The protease enzyme showed a single band of 33 kDa on a SDS PAGE, indicating that it is monomer (Fig. 7.4). On the isoelectric gel, the enzyme was found to give two bands corresponding to pI values of 6.6 and 6.9 (Fig. 7.4). The enzyme was purified 4-fold with the specific activity of the pure enzyme being more than 30000 ACU mL<sup>-1</sup> and a total yield of 21 % (Table 7.5).

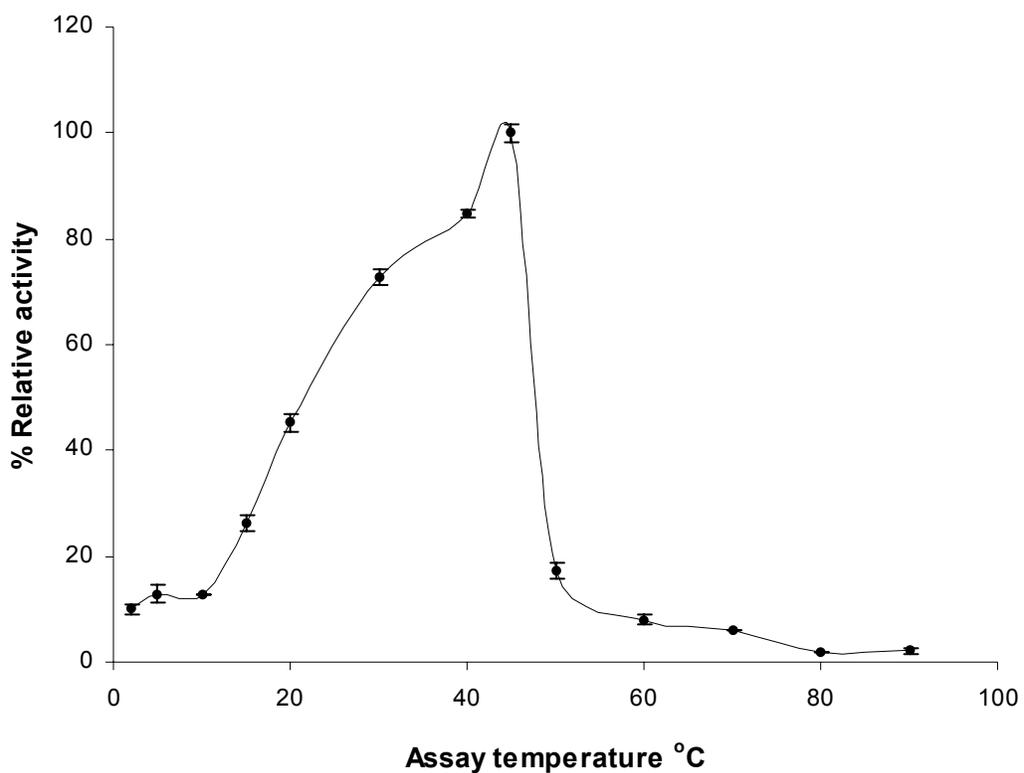
**Figure 7.5** Relative activity of protease of NIOCC #20 at different pHs and 45°C



◆ acetate buffer; ■ citrate phosphate buffer; △ phosphate buffer; × Tris-HCl buffer; ▲ boric-borax buffer; □ Glycine-NaOH buffer; + borax-NaOH; ◇ carbonate-bicarbonate buffer.

The enzyme exhibited pH optima of 9 with >80% of activity between pH 6-10 at 45°C (Fig. 7.5). The maximum enzyme activity was recorded at 45°C and about 45% of the maximum activity was detected at 20°C and about 10 % at 2°C (Fig.7.6). The Q<sub>10</sub> values at 2 and 15°C were above 1 while those at 30, 45 and 60°C were less than 1 (Table 7.6).

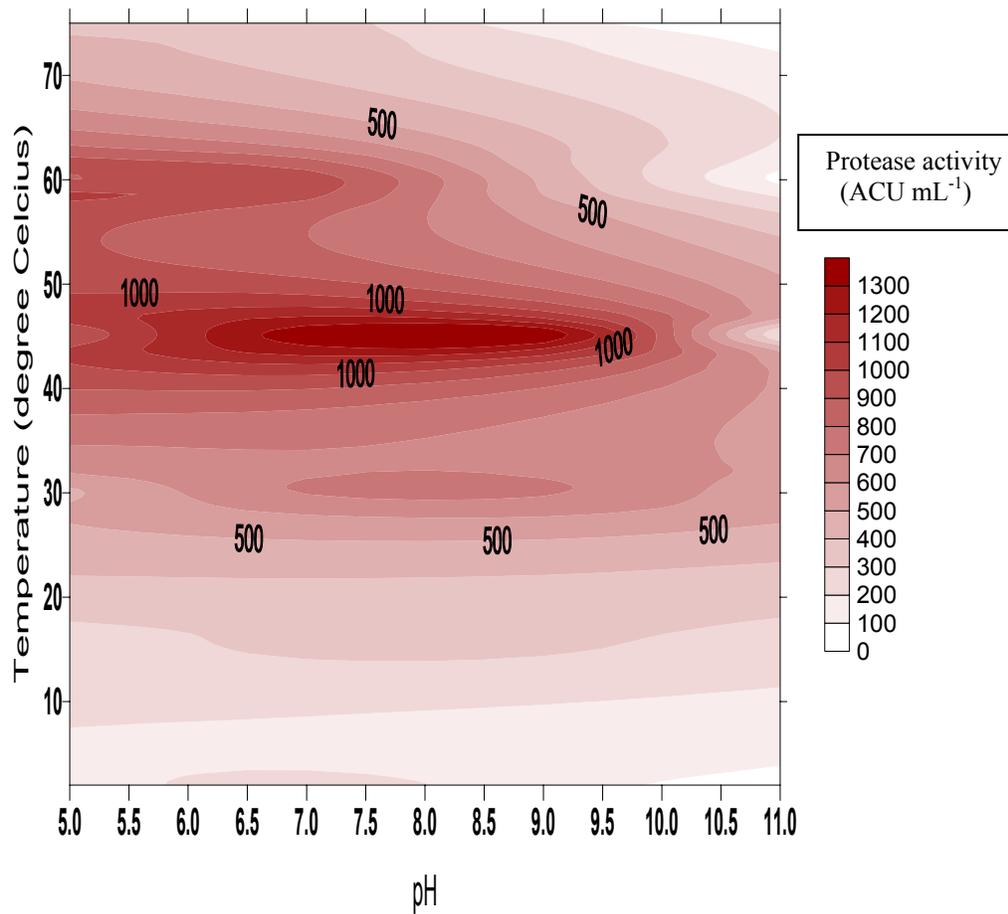
**Figure 7.6** Relative activity of protease at different temperatures measured at pH 9



**Table 7.6**  $Q_{10}$  values for the purified protease enzyme produced by NIOCC # 20

$T_1$	$T_2$	$Q_{10}$ value	$T_1$	$T_2$	$Q_{10}$ value
2	15	1.53	30	45	1.74
2	30	1.78	30	60	0.89
2	45	1.77	30	75	0.73
2	60	1.22	30	45	1.74
2	75	1	30	60	0.89
15	30	1.82	30	75	0.73
15	45	1.78	45	60	0.46
15	60	1.13	45	75	0.47
15	75	0.92	60	75	0.49

**Figure 7.7** Activity of purified protease at different pH and temperature



When the enzyme activity was assayed at all the pH values at all the temperatures mentioned above, it showed a fairly constant activity in the pH range 6 to 8 and a temperature range of 30 to 50°C (Fig 7.7).

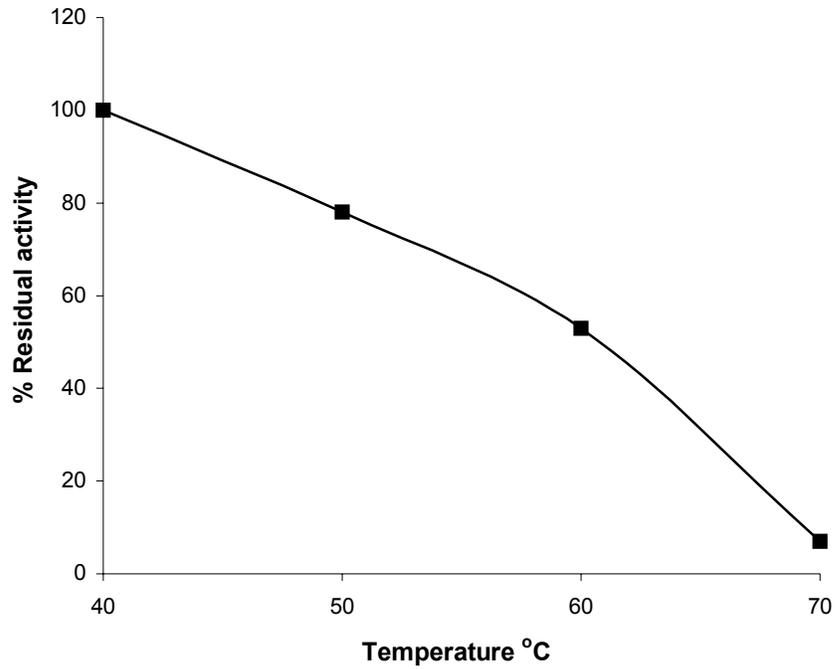
The protease of the culture grown at 1 bar pressure showed a  $K_m$  constant of 2 mg mL<sup>-1</sup> when measured at pH 9 and 45°C and this increased marginally at elevated hydrostatic pressures of 50 and 100 bar (Table 7.7). There was a marked increase in  $K_m$  constant at 5°C/1 bar, 45°C/ 200 and 300 bar pressure. The  $V_{max}$  values were not much affected by the elevated hydrostatic pressures except a substantial drop at 50 bar pressure from that at 1 bar pressure (Table7.7).

**Table 7.7**  $K_m$  constant and  $V_{max}$  values of the purified protease of the deep-sea fungus NIOCC # 20 measured under different conditions

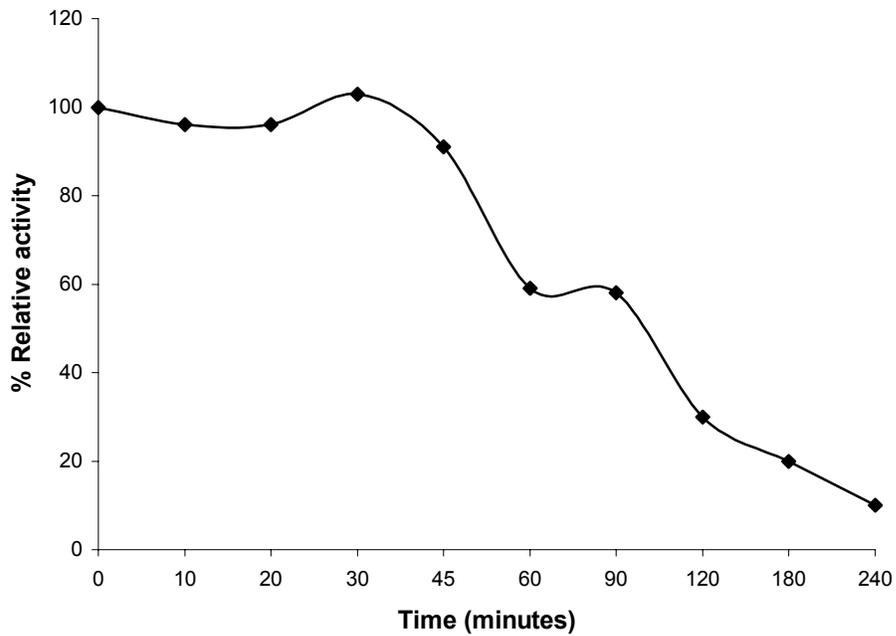
Enzyme activity assayed at	$K_m$ constant (mg mL <sup>-1</sup> azocasein)	$V_{max}$ (ACU mL <sup>-1</sup> )
1 bar and 45°C	2.0	26.3
50 bar and 45°C	2.2	19.2
100 bar and 45°C	2.4	24.4
200 bar and 45°C	3.3	25.0
300 bar and 45°C	5.0	25.0

Thermostability studies showed that the enzyme retained 80 and 55% of its activity at 50 and 60°C respectively for 10 min at pH 9 (Fig. 7.8). At its optimum temperature of activity (45°C), the enzyme retained 100 % of its activity for 30 min after which it steadily decreased (Fig. 7.9). The enzyme was most stable at pH 4.5 in the absence of the substrate (Fig. 7.10), with the stability decreasing with alkalinity.

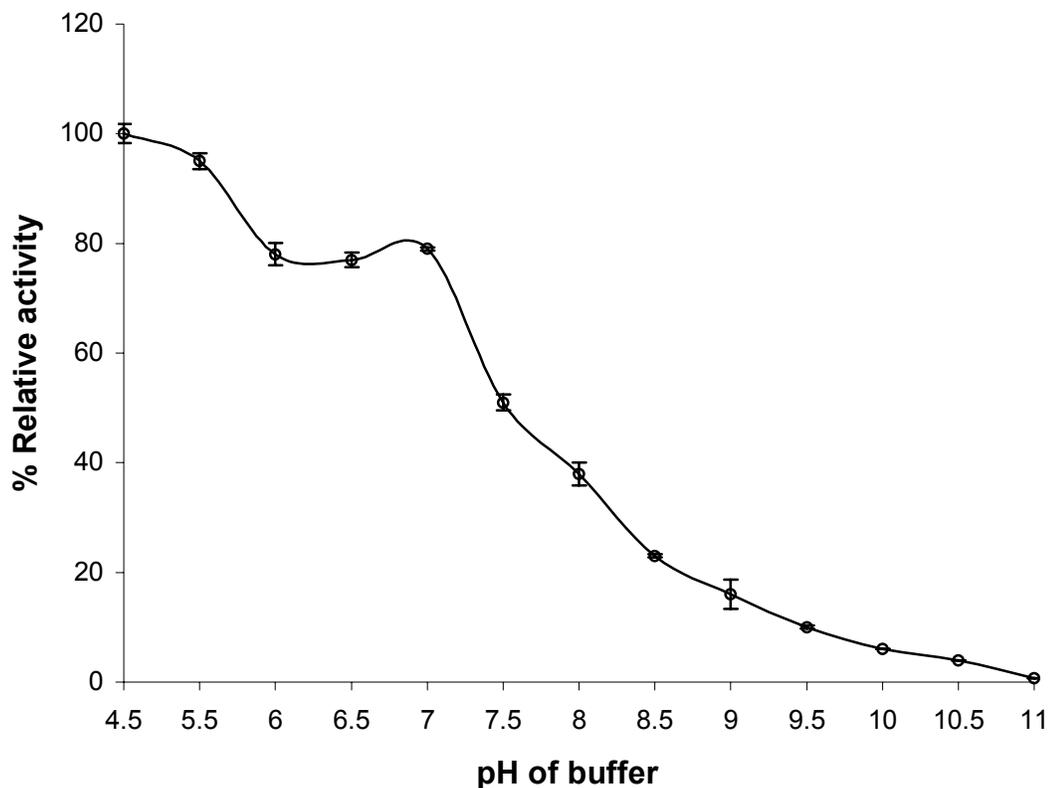
**Figure 7.8** Residual activity of protease measured after incubating the enzyme sample at pH 9 for 10 min at different temperatures



**Fig. 7.9** Stability of purified protease of NIOCC # 20 at its optimum temperature of activity (45°C)



**Fig. 7.10** Stability of protease at different pHs checked by incubating the enzyme at specified pHs without the substrate for 60 min. Activity was measured at its optimum temperature (45°C) and expressed as percent residual activity.



The protease activity was totally inhibited by 2 mM PMSF suggesting it to be a serine protease. EDTA at 5 and 100 mM concentration hardly inhibited the protease activity (Table 7.8) indicating that it was not a metalloprotease. The protease of NIOCC #20 is thiol-independent because reducing agents as DTT and mercaptoethanol did not affect the activity considerably (Table 7.8) At 1 mM concentration of Hg only 45 % inhibition of the enzyme was observed. Addition Fe, Ni and Zn at 1 mM hardly inhibited the enzyme activity. On the other hand, Cu at 1 mM concentration inhibited the protease activity totally (Table 7.8). About 70% of the activity was retained in the presence of sodium chloride of 0.5 M concentration, which equals to 29 ppt salinity of seawater (Table 7.8).

**Table 7.8. Effect of various inhibitors, heavy metals, varying ionic strengths (sodium chloride) and reducing agents on protease activity of the deep-sea fungus NIOCC #20.**

<b>Inhibitors</b>	<b>Residual activity (%)</b>
<b>Protease Inhibitors (mM)</b>	
None (control)	100
PMSF (2)	0
EDTA (5)	99
EDTA (100)	93
<b>Heavy metals (mM)</b>	
NiCl <sub>2</sub> (1)	72
ZnCl <sub>2</sub> (1)	81
HgCl <sub>2</sub> (1)	55
CuCl <sub>2</sub> (1)	0
FeCl <sub>3</sub> (1)	74
<b>Sodium chloride (M)</b>	
0	100
0.1	88.6
0.25	80
0.50	70
0.75	61.4
1.0	40
<b>Reducing Agents (%)</b>	
None	100
DTT Dithiothereitol (0.1)	91
DTT (0.5)	73
β-mercaptoethanol (0.1)	77
β-mercaptoethanol (0.5)	75
Sodium thioglycolate (0.1)	45
Sodium thioglycolate (0.5)	27
Sodium thioglycolate (1.0)	27
Glutathione (0.1)	0

Chemical surfactants like Triton X-100 and Tween 80 (both at 1.0%) and commercial detergents at 2 g L<sup>-1</sup> concentrations did not reduce the protease activity, although SDS (0.1%) totally inhibited its activity (Table 7.9). About 50% of the enzyme activity was lost after treatment at 60°C for 10 min. However, thermostability of the enzyme at 60°C increased on addition of glycerol, CaCl<sub>2</sub>, PEG 6000, sucrose, mannitol, sorbitol and starch during enzyme assay (Table 7.10).

**Table 7.9 Effect of detergents and bleaching agents on protease activity of the deep-sea fungus NIOCC #20**

Compound (Concentration in %)	Residual specific activity (%)
None	100
Tween 80 (1.0)	142
Triton X-100 (1.0)	139
SDS (0.1)	0
Ala (Commercial fabric bleach) (2.0)	20
Sodium hypochlorite (1.0)	2
H <sub>2</sub> O <sub>2</sub> (2.0)*	40
Commercial Detergent Wheel (2 g L <sup>-1</sup> )	91
Mr. White	85

#### **7.4.5 Comparison of protease production between deep sea (NIOCC 20) and terrestrial isolate (MTCC 2200) of *Aspergillus ustus***

Both the terrestrial (MTCC 2200) and the deep-sea culture (NIOCC 20) produced almost similar biomass in Czapek Dox broth (Fig. 7.11). By third day, the

cultures reached stationary phase, after which the biomass was almost constant. But the protease production showed a lot of difference, with the deep-sea isolate producing

**Table 7.10 Effect of additives on thermostability of the crude enzyme of the deep-sea fungus NIOCC #20 with reference to 10 minutes of heat treatment at 60°C without any additive.**

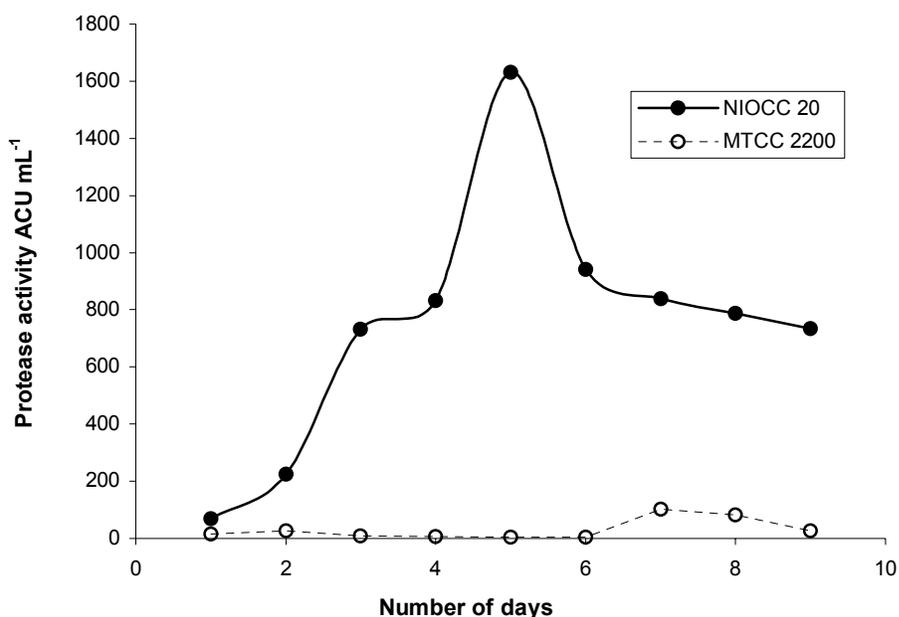
Additive present during the heat treatment	% Residual activity
Control (untreated)	100
No additives	51
Glycerol (1%)	101.5
CaCl <sub>2</sub> (1 mM)	108.5
CaCl <sub>2</sub> (5 mM)	110.6
CaCl <sub>2</sub> (10 mM)	102.4
PEG 6000 (1%)	111.1
Sucrose (1%)	84.8
Mannitol (1%)	79.2
Sorbitol (1%)	85.6
Starch (1%)	98.8

a maximum of 1600 ACU mL<sup>-1</sup>, while the terrestrial culture showed only 102 ACU mL<sup>-1</sup> (Fig. 7.12). The pH for the activity of the protease produced by both the

terrestrial and the deep-sea isolate were found to be similar with the activity increasing with the increasing pH, more in the alkaline range (Table 7.11).

NIOCC 20 showed about 50-fold increased protease production in the presence of sea water at different salinities as compared to MTCC 2200. In case of NIOCC 20, the protease production increased gradually up to 20 ppt, above which salinity did not affect it. Whereas in case of MTCC 2200, no uniform effect was observed (Table 7.12).

**Fig. 7.11 Temporal production of protease by NIOCC 20 and MTCC 2200**



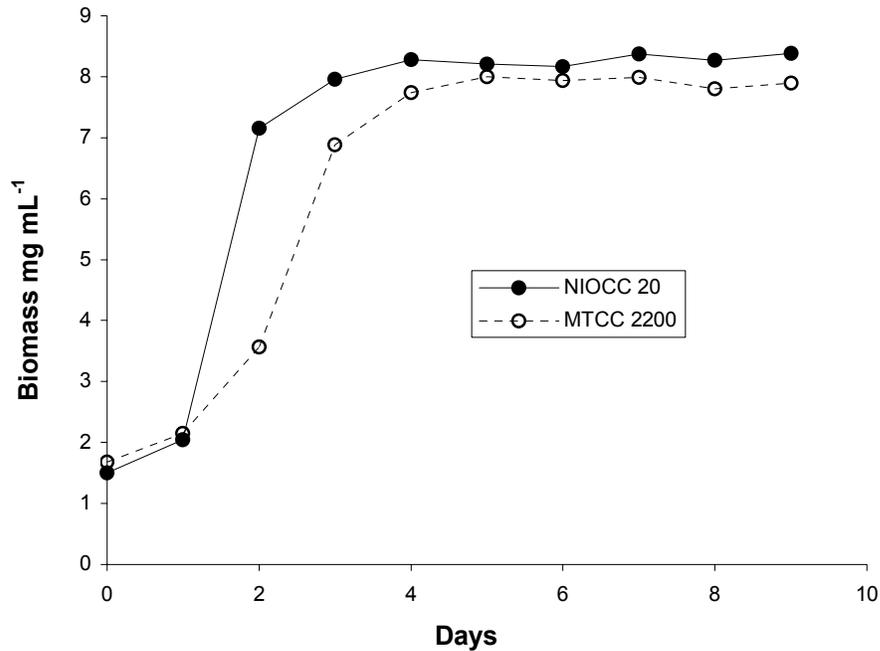
**Table 7.11 Comparative activity of protease produced by the terrestrial isolate MTCC 2200 and the deep-sea isolate NIOCC 20 at different pH values**

Assay pH	Protease activity ACU mL <sup>-1</sup>	
	NIOCC 20	MTCC 2200
5	522	5.3
7	817	26
9.5	861	55.1

**Table 7.12** Protease produced by the deep-sea isolate # NIOCC 20 and terrestrial isolate MTCC 2200 in Czapek Dox broth prepared in seawater with varying salinities after 8 days of incubation

Salinity (parts per thousand) ppt	Protease activity	
	NIOCC 20	MTCC 2200
0	729	16
5	828	20
10	926	28
15	1119	35
20	1140	17
25	1149	24
30	1151	28
35	1154	25

**Fig. 7.12** Biomass produced by NIOCC 20 and MTCC 2200



## 7.5 Discussion

Protease enzyme was produced by majority of the fungal isolates tested suggesting that it is one of the major extracellular enzymes being produced by the fungi playing a major role in their nutritional requirements. But again, not all the fungal cultures were showing protease production, which was active in the alkaline pH range and at a low temperature of 5°C, suggesting that the protease being produced by one culture is enough to degrade the proteinaceous matter present into easily assimilable products and supply the required nutrients for growth to all the non-protease producers also. Further, although all the isolates were isolated from a cold habitat of deep sea, only 51 % of these showed protease production, which was active at low temperature. The number of fungi showing protease activity at 30°C was much higher than those showing activity at 5°C, with large number of isolates showing activity in the range of 1 - 10 ACU mL<sup>-1</sup>. This is keeping in view with the theory put forth in previous chapter that these peizotolerant isolates are terrestrial forms that have managed to reach the ocean bottom and have got adapted to the conditions there.

The measurement of protease activity using azocasein was found to be very sensitive because as low as 1 ACU could be detected and also the assay was replicable with low standard deviation values.

*Aspergillus ustus* isolated from deep-sea calcareous substrates has been reported to produce protease [Raghukumar & Raghukumar, 1998], which was active under elevated hydrostatic pressure and low temperature. The culture, *Aspergillus ustus*, NIOCC # 20, isolated in the present study, showed highest amount of protease production active at alkaline pH and low temperature. It showed substantial growth at

5°C, under 50 and 100 bar pressure (corresponding to 1000 m). Under the above two conditions, it attained a biomass equivalent to 58 and 41 % respectively of that produced at 30°C/1 bar pressure (Table 7.2). However, when the fungus was grown at 5°C under 1, 50 or 100 bar and the enzyme activity assayed at 5 or 30°C under different hydrostatic pressures, little or negligible amount of cold-tolerant protease was detected. In contrast to the above, the enzyme produced by the fungus at 30°C and 1 bar was much more versatile and retained about 10 % of activity at 2°C at 1 bar compared to its activity at optimum temperature. The enzyme produced at 30°C under 1 or 50 bar also retained its activity when assayed under elevated hydrostatic pressures up to 300 bar (Table 7.3). However, the increasing  $K_m$  constants of protease at elevated hydrostatic pressures of 200 and 300 bar (Table 7.7) indicates low enzyme-substrate affinity under these extreme conditions. The total enzyme activity *per se* is not affected by elevated pressures (Table 7.3), which is also indicated by the almost constant  $V_{max}$  values (Table 7.7).

Although growth was fairly unaffected by elevated pressure and low temperature, protease production under similar conditions was reduced 10 to 100 folds. This appears to be a general trend with all the deep-sea isolates that were isolated (Table 7.1). Thus, a higher percentage of deep-sea isolates (51 %) were capable of growing at 5°C, when compared to those isolated from shallow waters (32 %). Besides, a high number of deep-sea isolates produced low-temperature active protease when grown at 1 bar and 30°C, but very few of these did so when grown at 1 bar and 5°C (Table 7.1). Compared to the deep-sea isolates, fewer isolates from shallow water produced cold-

tolerant protease when grown at 1 bar pressure and 30°C and their growth was also poor at 5°C.

A culture of *Aspergillus ustus* obtained from the culture collection IMTECH, showed negligible amounts of protease production, the activity of which was very low at both the pH and temperatures checked indicating that NIOCC # 20 is a totally different strain isolated from the deep sea sediments. NIOCC # 20 appears to be a strain adapted to deep-sea conditions, since it grew better at 5°C / 100 bar than at 30°C / 100 bar, although its production of protease under the former conditions was low (Table 7.3). This is in contrast to the report on a deep-sea bacterial strain DB6705, which grew better at higher rather than low temperatures when cultured at 500 bar hydrostatic pressure [Kato *et al.*, 1996]. Growth and metabolic activities under extreme conditions are known to be low [Wirsen & Jannasch, 1975]. Our results with NIOCC #20 also show similar trends.

During the standardization of the medium components, it was found that the presence of corn steep liquor induced maximum protease production followed closely by skimmed milk (Fig. 7.3). But for all the standardization and characterization of the enzyme, skimmed milk was used as the inducer due to its easy availability and more defined nature. Apart from this CSL, being dark coloured might also interfere in the enzyme assay.

The enzyme yield was found to be better when EDTA was added to the culture broth just before harvesting of the enzyme. This could have happened due to release of the enzyme that was trapped in the exopolysaccharides produced by the culture that came in solution form due to chelating of the EPS by EDTA [Comte *et al.*, 2006]. The

protease produced by NIOCC # 20 showed several important features. Although the optimum temperature for activity of the enzyme is 45°C, it is described as a cold tolerant protease. Enzymes from cold-adapted organisms have been classified into 3 groups [Ohgiya *et al.*, 1999]. Group I enzymes are heat-sensitive, but the other enzymatic characteristics are similar to mesophilic enzymes. Group II enzymes are heat-sensitive and relatively more active than mesophilic enzymes at a low temperature. Group III enzymes have same thermostability as mesophilic enzymes but are more active than mesophilic enzymes at a low temperature. Accordingly, the protease of this isolate falls into the group II. The protease of mesophilic *Penicillium* sp. did not show any activity below 20°C [Germano *et al.*, 2003]. The alkaline protease from another mesophilic species of *Penicillium* did not show any activity below 35°C [Agrawal *et al.*, 2004]. On the other hand, NIOCC # 20 showed about 45, 26 and 12% of the maximum activity at 20, 15 and 5°C. It loses about 50% of its activity at 60°C (Fig. 7.8). Moreover, at its optimum temperature of activity (45°C), it is stable only for 30 min after which it rapidly loses activity (Fig. 7.9).

The enzyme is stable for more than 24 h at 5°C and pH 4.5. Its half-life under these conditions is about 196 hours. These results suggest its placement in the group II. The effect of temperature on the enzyme activity is determined by the temperature coefficient  $Q_{10}$  [<http://www.csupomona.edu/~seskandari/Q10.html>]. It is the factor by which the rate increases when the temperature is raised by ten degrees. If the rate of the reaction is completely temperature-independent, the resulting  $Q_{10}$  will be 1.0. If the reaction rate increases with increasing temperature,  $Q_{10}$  will be greater than 1. Thus, the more temperature-dependent a process is, the higher will be its  $Q_{10}$  value. The

calculated  $Q_{10}$  value of 1 between 2° to 10°C in the present work (Table 7.6) indicates temperature-independent enzyme activity at lower temperatures. On the other hand, the increased  $Q_{10}$  values (above 1) at 15° to 45°C indicate direct relation to increasing temperatures. Above 45°C, the  $Q_{10}$  value is less than one suggesting negative effect of temperature on the enzyme activity.

The protease of NIOCC #20 is a serine protease because it was totally inhibited by PMSF, which is a known inhibitor of serine protease. EDTA did not inhibit it and therefore it is not a metalloprotease. It was not substantially inhibited by the heavy metals Ni and Zn, which are the inhibitors of cysteine protease. Its activity did not increase in the presence of thiol compounds, indicating that it was thiol-independent serine protease. Protease produced by this isolate showed several special features. It was active in the presence of several commercial detergents when assayed under its optimum conditions. The enzyme was active in the presence of 0.5 M NaCl equivalent to 29 ppt of seawater salinity. Its stability at 60°C increased in the presence of various stabilizing agents. Such features in proteases are often much sought after for use as additives in detergents for low temperature wash [Ohgiya *et al.*, 1999]. Most alkaline proteases have been reported to be significantly stabilized in the presence of additives such as those used in this study [Beg & Gupta, 2003].

Protease of *Aspergillus ustus* (NIOCC #20) with an optimum pH of 9 and showing about 45% and 25 % of its activity at 20°C and 15°C respectively is an alkaline protease. Its performance in terms of activity can be further improved by optimization of growth and other parameters. Terrestrial isolate of *Aspergillus ustus* (isolate # MTCC 2200) obtained from IMTECH culture collection showed extremely

low protease production (23 ACU mL<sup>-1</sup>) under similar assay conditions. This indicates that deep-sea fungi are useful candidates in the search for alkaline proteases and that the enzyme can have multiple uses right from detergents to food to biotechnological field.

# Chapter 8

## Role of Fungi in Deep-sea Sediments

## 8.1 Introduction

Roughly 62 % of the globe's surface, the ocean floor, is covered by seawater. This huge volume and area, however, has to be viewed as a desert of life where life is strictly limited by the source of organic carbon and biochemically useful energy [Jannasch, 1994]. The sea surface overlaying the deep-sea provides only about 21 % of the total global photosynthetic useful energy. About 95 % of this amount is recycled in the upper 100 - 300 m of surface water, while the remaining 5 % sink down in the water column in particulate form by sedimentation. Small portions of this material are large chunks of organic matter such as fish carcasses, decapods and large shrimp. Their relatively high sinking rates of 50 – 500 m hour<sup>-1</sup> [Vinogradov & Tseitlin, 1983] prevent a substantial en route decomposition. Another portion of organic matter is represented by faecal pellets of jellyfish and zooplankton with sinking rates of 2700 and 50 - 200 m/day, respectively [Bruland & Silver, 1981; Komer *et al.*, 1981; Madin, 1982]. The potentially much lower sinking rates of phytoplankton debris often aggregate to “marine snow” particles [Aldredge & Gotschalk, 1988] and much of it becomes part of the food for zooplankton, largely containing compounds undigestible for zooplankton. The faecal pellets overgrown with bacteria are eaten repeatedly on their way to the deep sea. These faecal pellets act as a matrix for bacterial growth, a system that can be termed as an extraorganismic enteric symbiosis [Jannasch, 1994].

Most of this material is decomposed during this descent providing food for the pelagic and deep populations of animal and microorganisms in the water column. The fraction of local plankton production reaching the sediments is mainly a function of water depth [Suess, 1980; Jørgensen, 1983]. The transport from the surface to the

deep-sea can be affected by various factors [Michaels *et al.*, 1990]. Where it is rapid and not diverted by lateral transport, seasonal signals of surface water productivity can be observed at the deep-sea floor [Asper *et al.*, 1992]. Generally, less than 1 % of the photosynthetically produced organic matter reaches the deep-sea sediment at a depth of about 4000 - 5000 m and even less in deeper parts of the oceans.

The major function of microorganisms in the deep sea is commonly seen in the conversion of dissolved organic matter into the particulate food for zooplankton often termed as the “microbial loop”. The total concentration of organic carbon is of less importance for its potential microbial decomposition than the metabolic availability of its constituents. The concentration of those compounds that are susceptible to microbial attack will decrease faster with depth than the concentration of total organic carbon, and the relative quantity of recalcitrant compounds will increase.

Because of their omnipresence and highly diverse metabolic capabilities, microorganisms are playing the major role as biocatalysts for the control of inorganic and organic carbon in the biosphere. Their ability to metabolize organic substrates at extremely low levels and to survive long periods of starvation, as well as adapt to life at low temperature and high pressure, helps them to use almost any biochemically exploitable form of organic carbon available at even low concentration [Jannasch, 1994].

The global carbon cycle is greatly influenced by the activities of both bacteria and fungi in terrestrial soils, which govern the rates of mineralization, as well as carbon sequestration [Bronick & Lal, 2005]. Individually, though, fungi and bacteria play different roles in soil biogeochemistry, bacteria have greater rates of

rem mineralization but lower C assimilation efficiencies than fungi, the latter thus being more significant in terms of carbon sequestration [Adu & Oades, 1978]. Therefore, it is important to consider both groups for a holistic understanding of microbial dynamics and their role in the carbon budget [Bailey *et al.*, 2002]. Particulate organic carbon deposition in deep-sea sediments is one of the mechanisms for removal of atmospheric carbon dioxide in the context of the global C cycle and marine sediments are the largest source of organic carbon on earth [Seiter *et al.*, 2004; Ittekkot, 1996].

As in terrestrial soils, microorganisms are major players in transformation of organic matter in deep-sea sediments. In contrast to land, however, most studies on deep-sea sediments have focused exclusively on bacteria and have demonstrated their intense metabolic activities therein [Danovaro *et al.*, 1995; Turley & Dixon, 2002]. The fungi and their role in the deep-sea sediments have remained neglected mainly due to the fact that they are not easily seen / observed. This is because most of the times, fungi remain embedded in the sediments and hence go unnoticed. The fungi are known to stabilize the soil by the way of aggregation in the terrestrial ecosystems [Lynch & Bragg, 1985]. Soil aggregation is defined as the naturally occurring cluster or group of soil particles in which the forces holding the particles together are much stronger than the forces between the adjacent aggregates [Martin, 1955]. A number of studies have attempted to elucidate the role of soil microflora in the formation and stabilization of soil aggregates [Harris *et al.*, 1964; Molohe *et al.*, 1987]. Although fungi are generally believed to be more effective at aggregate stabilization than other soil microflora, several studies have concluded that their primary contribution to aggregation is through hyphal entanglement of soil particles [Molohe & Page, 1986].

Reducing sugars and amino acids, formed, as by-products of microbial metabolism in terrestrial sediments are known to undergo nonenzymatic polymerization to form a series of compounds like 3-carbon aldehydes, ketones and reductones and hydroxymethyl furfurals. These highly reactive compounds readily polymerize in the presence of amino compounds to form brown-coloured products, constituting humus [Tisdall & Oades, 1982]. The humic material combines with soil particles to form microaggregates. Fungal hyphae further act as binding agents themselves to form macroaggregates by trapping fine particles into the microaggregates [Kandeler *et al.*, 1999]. Fungi or bacteria can thus remain protected in certain particle size classes [Suberkropp & Meyers, 1996]. Cations such as  $\text{Si}^{4+}$ ,  $\text{Fe}^{3+}$ ,  $\text{Al}^{3+}$  and  $\text{Ca}^{2+}$  form bridges between particles in terrestrial microaggregates [Bronick & Lal, 2005].

Several enzymes are known to be involved in the cycling of nutrients and can be used as potential indicators of nutrient cycling processes. In aquatic ecosystems like that of deep sea where P-limitation exists, alkaline phosphatase activity (APA) plays an important role in the regeneration of inorganic phosphate through its catalysis of the breakdown of organic esters to inorganic P [Chrost, 1991]. Alkaline phosphatase activity is often repressed by highly dissolved reactive P (DRP) concentrations by feed back inhibition. The general name 'phosphatases' has been used to describe a broad group of enzymes that catalyze the hydrolysis of both esters and anhydrides of phosphoric acid. Amongst these, the acid phosphatase has studied more as compared to the alkaline phosphatase.

Sediment microorganisms, which depend upon the activities of their extracellular enzymes to supply them with nutrients, are confronted with several

problems. In the first instance, the sediment environment is inhospitable for extracellular enzymes meaning, non-biological denaturation, adsorption and inactivation, and degradation by proteolytic microorganisms all conspire to take their toll on enzymes once they have left the protection of the cell [Burns, 1978]. However, it is obvious that if an enzyme is to be of value to the cell that secreted it, that enzyme must evade any destructive forces as long as it takes to locate and react with an exogenous substrate. There is also a strong possibility that at any particular moment, there is no suitable substrate in the vicinity of the enzyme-producing microorganism or that any substrate, which is present, is either too low a concentration to elicit a response or in form which is unavailable for enzymatic attack. So an enzyme has to survive long enough to locate and bind its substrate and even then, the physical conditions for catalysis may be inappropriate. Even if it is assumed that the enzyme overcomes all these obstacles then the next stage in the process is for the product of the catalysis to be relocated in the vicinity of the cell, which has produced the extracellular enzyme in the first place. Only when this stage is reached can adsorption of the product of extracellular catalysis take place and such responses occur as enzyme induction, chemotaxis and ultimately growth.

This is where fungi play a very important role, possibly in deep-sea sediments also. Fungi are known to produce a lot of exopolysaccharides [Selbmann *et al.*, 2003; Tang & Zhong, 2002], which in turn help the aggregation of these. This is particularly helpful feature in natural environment where it also helps other microorganisms like bacteria to stay together as consortia. This prevents the extracellular enzymes from diffusing away from the cells secreting it. Thus, these humic-enzyme complexes have

an important role to play in overall nutrient dynamics of the sediments [Burns, 1977, 1978].

## **8.2 Objectives**

The main aim was to study the role of fungi in the deep-sea sediments with the following objectives:

1. To estimate the contribution of fungi to the carbon in the deep-sea sediments
2. To illustrate the microaggregation caused by fungi under laboratory conditions
3. To estimate the contribution of fungi to alkaline phosphatase in the deep-sea sediments.

## **8.3 Methodology**

### **8.3.1 Fungal biomass (biovolume) in the deep-sea sediments**

The fungal biomass in the sediments was estimated by measuring the total biovolume under a microscope. For this, about 0.5 g of sediment sample was suspended in 3 mL sterile seawater in a sterile vial and fixed with 1 mL formalin solution (final concentration of ~ 10 %). To 600  $\mu$ L of this fixed sediment sample, 100  $\mu$ L of 0.1 % solution of filter sterilized Calcofluor, an optical brightener, was added and mixed thoroughly. The excess stain was washed by centrifuging twice with sterile seawater. Microscopic mount of the sediment was then examined under ultraviolet light filter of an epifluorescence microscope to check for brightly fluorescing fungal hyphae. The same staining procedure was followed for the formalin fixed sediments mixed with 300  $\mu$ L of sterile 10 % EDTA solution.

The biovolume of all the fluorescing fungal hyphae was calculated using an ocular micrometer and converted to  $\mu\text{g}$  of C  $\text{g}^{-1}$  of dry sediment [Van veen & Paul, 1979]. The approximate length and breadth (width) of the fungal hyphae detected under the microscope was measured. Using this, the volume of the filament was calculated assuming it to be a cylinder. The volume of the hyphae was then converted to the equivalent biomass in terms of C using the formula,  $1 \mu\text{m}^3$  of fungal biomass is equivalent to  $1 \text{ pg C}$  [Van veen & Paul, 1979]. This was then converted to  $\mu\text{g}$  of C  $\text{g}^{-1}$  of dry sediment to get the contribution of fungal biomass in terms of C in the deep-sea sediments in the Central Indian Basin (CIB).

### **8.3.2 Microaggregation by fungal cultures under simulated laboratory conditions**

The ability of cultures for microaggregation was detected by growing them in sediment extract as sole nutrient source. The sediment extract was prepared as described in Chapter 5.

The deep-sea isolate # A 4634 (*Aspergillus terreus*) was used for this experiment. Finely ground quartz ( $< 100 \mu\text{m}$ ) was used as an inducer for aggregation [Dietrich & Lampky, 1981]. The culture was grown in MEB for 2 to 3 days and harvested just before sporulation. The mycelial inoculum was homogenized using glass beads and used as inoculum for the further experiment. In a sterile plastic pouch, to 4 mL of the sediment extract, one mL of the inoculum was added. Two sets of experimental pouches were made, one with quartz and one without quartz. Both the sets were incubated under following conditions:

1. 200 bar / 30°C
2. 200 bar / 5°C
3. 1 bar / 30°C
4. 1 bar / 5°C

The bags were incubated at the above-mentioned conditions for 20 days. At the end of the incubation period, the contents of the pouches were fixed with formalin. The biomass was observed under the microscope for the presence of microaggregates. The biomass obtained was also stained to check for the humic material, polysaccharides and proteinaceous matter using benzidine [Sieberth & Julseth, 1968], alcian blue and coomassie blue staining [Long & Azam, 1996] respectively (Appendix A - X).

### **8.3.3 Fungal and bacterial contribution to alkaline phosphatase activity**

Various types of enzymes are responsible for the degradation of organic materials present in the sediments. Every organism contributes differently, both in terms of quality and quantity, to accomplish this. One of the approaches to study this is to add growth inhibitor for specific type of organism and study the effect. To study the bacterial and fungal contributions, antibacterial antibiotic and fungicides have been used previously [Flegler *et al.*, 1974]. In the present study, antibacterial antibiotics (Penicillin and Streptomycin) and fungicide (Bavistin) were used to study the individual contribution of alkaline phosphatase activity in the sediment by fungi and bacteria with respect to. A deep-sea fungal isolate *Aspergillus terreus* along with bacterial isolates from the deep sea were used as control. Ampicillin (0.1 g in 100 mL of medium used) and penicillin (4000 U in 100 mL) were used as antibacterial

antibiotics and Bavistin™ (0.5% final concentration) as antifungal antibiotics. The concentrations of the fungicide and antibacterial antibiotic used in the study were standardized by growing different bacteria and fungi in varying concentrations of both the inhibitors. The second lowest concentration not showing cross inhibition (i.e. fungicide not inhibiting bacteria and vice versa) were used in the study. To simulate the sporadic enrichment of the deep-sea sediments with phytodetritus and dead animals, the sediment was spiked with detritus and artemia (cysts and larvae) as additional nutrient sources. The detritus was collected from shallow waters from Dona Paula Bay, using 200 µm plankton net. The detritus was a mixture of phyto-and-zooplankton.

Following combinations were used to study the exact contribution of each one to alkaline phosphatase activity:

1. Plain sediment
2. Sediment + fungicide (to suppress fungal growth)
3. Sediment + antibiotics (to suppress bacterial growth)
4. Sediment + detritus
5. Sediment + detritus + fungicide
6. Sediment + detritus + antibiotics
7. Sediment + artemia
8. Sediment + artemia + fungicide
9. Sediment + artemia + antibiotic
10. Sediment + detritus + antibiotic + fungus
11. Sediment + detritus + fungicide + bacteria

All these inoculations were done in quadruplicates in sterile plastic pouches, and incubated at following conditions for 20 days ---

1. 200 bar and 5°C
2. 500 bar and 5°C

At the end of the incubation period, the pouches were cut open and the contents centrifuged to obtain cell free supernatant, which was used for estimating the total alkaline phosphatase activity. The activity was estimated using 1mM MUF-phosphate as substrate prepared in methyl cellosolve [Hoppe, 1983; Ammerman & Glover, 2000]. The contents of the plastic pouches were transferred to 100 mL sterile conical flasks. To this, 10 mL of sterile 0.1 M tris HCl buffer pH 8.3 and the substrate, 100 µL of MUF phosphate were added. Toluene (25 µL) was added to avoid any bacterial activity and the flask was incubated in dark for 24 h at 5°C. When the enzyme hydrolyzes the substrate, equal amounts of orthophosphate and fluorescent non-phosphate moiety (methylumbelliferon) are produced. At the end of the incubation period, the suspension was centrifuged to obtain a clear supernatant. The methylumbelliferon (MUF) produced after the assay was detected as increase in fluorescence by using a Shimadzu RF-5301 spectrofluorometer, with excitation at 350 nm and emission at 450 nm. The fluorescence emitted is directly proportional to the alkaline phosphatase activity. A standard curve with MUF (Sigma) was used to quantify the amount of MUF produced by alkaline phosphatase activity (Appendix A - XI).

## 8.4 Results

### 8.4.1 Fungal biomass in the sediments of CIB

Actively growing fungi are found as filamentous hyphae in their natural habitats, in contrast to dormant spores. In order to detect such hyphae in the sediments, the sediments were stained with the fluorescent brightener, Calcofluor (Sigma, USA). Only 5.1 % (Table 8.1) of the deep-sea sediment samples examined

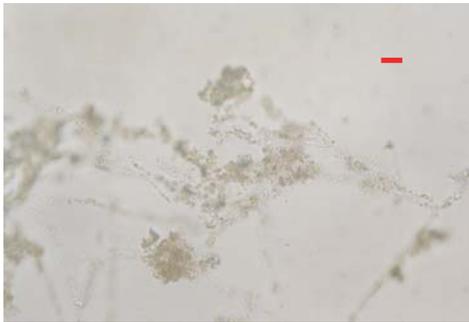
**Table 8.1 Fungi in deep sea sediments detected in presence and absence of EDTA**

Sub-surface depth (cm.)	Number of cores showing presence of fungi					
	Cruise # AAS 61			Cruise # ABP4		
	Total cores	Fungi observed in cores treated with EDTA	Fungi observed in cores without EDTA treatment	Total cores	Fungi observed in cores treated with EDTA	Fungi observed in cores without EDTA treatment
0 – 2	36	22 (61)	4 (11)	5	4 (80)	2 (40)
2 – 4	36	7 (19)	3 (8)	5	0	0
4 – 6	36	12 (33)	4 (11)	5	0	1 (20)
6 – 8	36	5 (14)	3 (8)	5	1 (20)	0
8 – 10	36	2 (6)	3 (8)	5	2 (40)	1 (20)
10 – 15	36	0	2 (6)	5	3 (60)	0
15 – 20	36	2 (6)	2 (6)	5	1 (20)	1 (20)
20 – 25	35	3 (9)	3 (9)	5	0	0
25 – 30	32	0	0	4	0	0
30 – 35	23	2 (9)	1 (4)	4	0	0
35 – 40	11	0	0	4	0	0
40 – 45	3	0	0	1	0	0

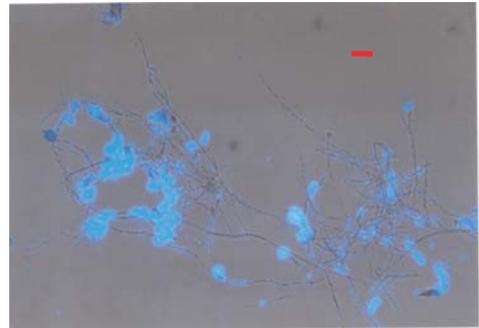
\* figures in bracket indicate % cores showing presence of fungi

revealed fungal hyphae using this staining. Fungal biomass, estimated based on the total biovolume of fungal hyphae detected by epifluorescence microscopy ranged from 2.3 to 6.3  $\mu\text{g C g}^{-1}$  sediment (Table 8.2). The same sediments samples treated with EDTA prior to staining with Calcofluor, revealed fungi in 12.8 % of sediment samples, an increase of two-fold over control treatments without EDTA. Fungal biomass carbon of EDTA-treated, Calcofluor stained sediment samples ranged from 2.3 to 5.1  $\mu\text{g g}^{-1}$  dry sediment, which was similar to the samples without EDTA treatment. The treatment resulted in disrupting the soil to reveal numerous microaggregates, all of which contained fungal hyphae. Numerous microaggregates were connected by fungal hyphae to form a complex. Surfaces of such hyphae were encrusted with particulate matter. These encrustations stained positive for humic material (Fig. 8.1 a, b). Results obtained by this method could still be conservative estimates, since biomass estimated by hyphal length may often underestimate the true values, as compared to phospholipid estimations [Balsler *et al.*, 2005].

The deep-sea fungal isolate # A4634 (*Aspergillus terreus*) showed good growth in the sediment extract. The three terrestrial cultures obtained from IMTECH were also able to grow in the sediment extract, but their growth was better in MEB, whereas in the case of deep sea isolate # A 4634, the biomass produced was almost the same in sediment extract and MEB when incubated under elevated pressure of 200 bar and 5°C (Table 8.3).



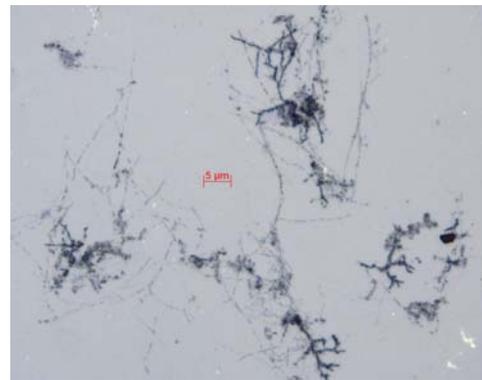
(a) Benzidine stained humic material in the vicinity of hyphae as seen under bright field microscope. Bar equals 10  $\mu\text{m}$ .



(b) The same material as seen in (a) under epifluorescence microscope. Bar equals 10  $\mu\text{m}$ .



(c) Coomassie blue stained proteinaceous material in the vicinity of hyphae grown in sediment extract. Bar equals 5  $\mu\text{m}$ .



(d) Alcian blue stained polymeric substances in the vicinity of hyphae grown in sediment extract. Bar equals 5  $\mu\text{m}$ .

Fig. 8.1 The aggregates produced by *Aspergillus terreus* (# A4634) in the sediment extract stained positive for humic substances (a & b), proteins (c) and exopolymeric substances (d).

**Table 8.2 Comparison of fungal biovolume and carbon contribution to sediments of different habitats**

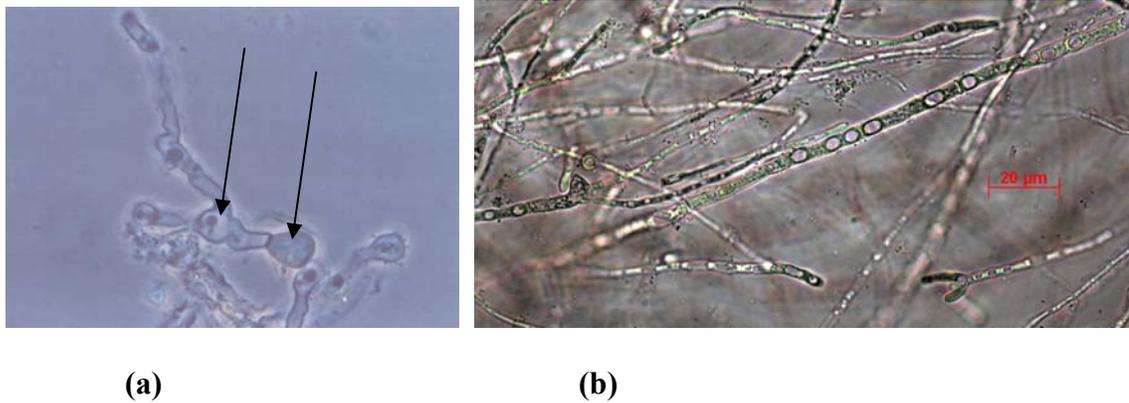
Source	Location	CFU	Biomass (fungal carbon in $\mu\text{g g}^{-1}$ dry sediment)	Reference
Sea water	Arctic	1000-3000 L <sup>-1</sup>	---	Gunde-Cimermann <i>et al.</i> , 2003
Forest Soil	Brazil	50-75x10 <sup>4</sup> g <sup>-1</sup>	---	Viera & Nahas, 2005
Grassland soil	Denmark	---	36	Klein & Paschke, 2000
Grassland soil	Not mentioned	---	453	Chiu <i>et al.</i> , 2006
Forest soil	Not mentioned	---	3375	
Forest soil	Denmark		250-3500	Ekelund <i>et al.</i> , 2001
Deep-sea sediments (~5000 m depth)	Central Indian Basin	18-2130 g <sup>-1</sup>	2.3-6.3 (bacterial carbon= 10-40)	Present work

**Table 8.3 Growth of deep sea and terrestrial isolates in sediment extract**

Culture	Biomass produced (mg) in			
	Sediment		MEB	
	200 bar / 5°C	1 bar / 30°C	200 bar / 5°C	1 bar / 30°C
A 4634	24.9	5.2	29.1	31.4
MTCC 279	8.2	28.4	17.8	28.0
MTCC 479	17.4	20.6	29.2	40.6
MTCC 635	7.4	6.2	7.8	8.3
A 344	3.8	10.2	7.4	14.2

It showed better growth in sediment extract at 200 bar and 5°C as compared to growth in MEB at 1 bar and 30°C. For rest of the cultures (except # A4634), the growth was better at 1 bar and 30°C than at 200 bar and 5°C in MEB.

**Fig. 8.2 Growth of the deep-sea isolate *Aspergillus terreus* in malt extract broth(a) and sediment extract(b) grown under hydrostatic pressure of 200 bar**



Apart from the differences in the biomass produced, the deep-sea isolate also showed morphological differences when grown in MEB and sediment extract. The isolate when grown in MEB under elevated hydrostatic pressure, showed abnormal swellings of hyphae (Fig. 8.2 a, arrows), which were not seen when the culture was grown under elevated hydrostatic pressure in the sediment extract (Fig. 8.2 b).

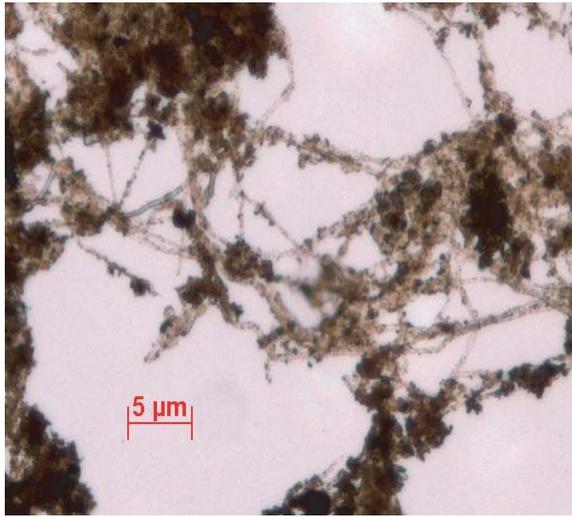
In the previous chapter, it is seen that most of the fungi showed growth under elevated pressure very easily when the inoculum was in the form of growing mycelia, and that the spores failed to germinate at low temperature, at elevated hydrostatic pressure as well as at 1 bar pressure. Therefore, the ability of spores to germinate in

the sediment extract was determined at low temperature. Although, morphology of the fungal hyphae was normal in the sediment extract (Fig. 8.2 b), the

**Table 8.4 Germination of spores of deep-sea *Aspergillus terreus* # A4634 in malt extract broth (MEB) and sediment extract**

Incubation conditions	Spore germination in soil and MEB growth medium			
	Growth medium	Number of spores showing germination	Number of spores not showing germination	% germination (with SD)
200 bar / 30°C	MEB	145	115	56 ± 12
	Sediment	208	102	67 ± 8
200 bar / 5°C	MEB	8	212	4 ± 3
	Sediment	12	187	6 ± 4
1 bar / 30°C	MEB	190	33	85 ± 21
	Sediment	388	98	80 ± 12
1 bar / 5°C	MEB	4	245	2 ± 2
	Sediment	6	173	3 ± 2

spores showed negligible germination at 5°C at both 1 and 200 bar (Table 8.4). The spores showed 67 % germination in sediment extract at 200 bar/30°C, which was slightly better than the germination in MEB. The germination was not much different when the nutrient supply was limited (1: 100 diluted medium) as discussed in the previous chapter.



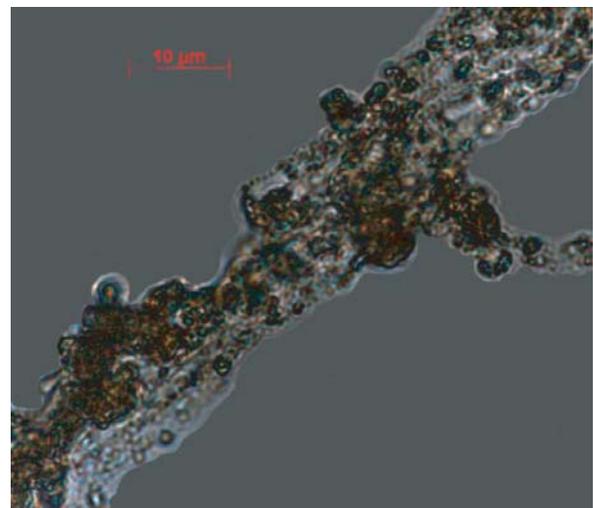
(a)



(b)



(c)

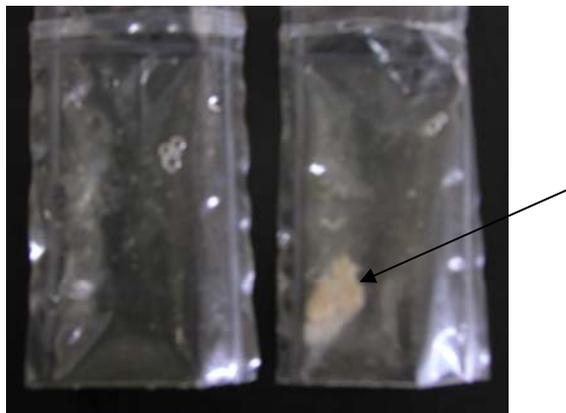


(d)

Fig. 8.3 (a – d) Stepwise microaggregate formation by *Aspergillus terreus* (# A 4634) during growth in sediment extract

**8.4.2 Microaggregate formation:** The culture showed microaggregate formation both in the sediment extract and MEB. There was no significant difference in microaggregation in presence or absence of the quartz. The microaggregation was seen in a step wise manner (Fig. 8.3 a - d). The fungi first showed granular precipitation around the hyphae, which acted as starter for all the further steps leading to aggregation. The end product of this process was formation of quite thick bundle-like aggregations of the fungal hyphae. The aggregates formed in the sediment extract appeared like loose, fluffy material to naked eye (Fig. 8.4). The aggregates stained positively for humic substances with benzidine (Fig. 8.1 a, b), for proteinaceous material with coomassie blue (Fig. 8.1 c) and exopolymeric substances with alcian blue (Fig. 8.1 d).

**Fig 8.4 Formation of aggregate (arrow) in presence of quartz by the deep sea fungus *Aspergillus terreus* during growth in sediment extract**



\* The plastic pouches in which the cultures were incubated at elevated hydrostatic pressure.

### 8.4.3 Alkaline Phosphatase Activity (APA)

Plain deep-sea sediment showed APA responsible for hydrolysis of 5.57 and 4.64  $\mu\text{g L}^{-1} \text{h}^{-1}$  substrate at 200 bar and 500 bar and 5°C respectively. Addition of antibiotic affected the activity marginally, whereas addition of fungicide affected to a greater extent (Table 8.5).

**Table 8.5 Alkaline phosphatase activity (APA) in the sediment in the presence and absence of additional nutrient sources**

Experimental condition		Release of MUF ( $\mu\text{g L}^{-1} \text{h}^{-1}$ )	
		200 bar / 5°C	500 bar / 5°C
1	Plain sediment	5.57 $\pm$ 0.3	4.64 $\pm$ 0.9
2	Sediment + antibacterial antibiotic	4.52 $\pm$ 1.2	4.94 $\pm$ 0.1
3	Sediment + Fungicide	1.47 $\pm$ 0.9	0.19 $\pm$ 0.1
4	Sediment + detritus	101.21 $\pm$ 25.4	98.57 $\pm$ 21.1
5	Sediment + detritus + antibacterial antibiotic	90.01 $\pm$ 8.4	96.96 $\pm$ 15.9
6	Sediment + detritus + Fungicide	73.53 $\pm$ 34.9	70.13 $\pm$ 34.9
7	Sediment + artemia	4.99 $\pm$ 1.6	2.63 $\pm$ 0.4
8	Sediment + artemia + antibacterial antibiotic	3.88 $\pm$ 0.6	1.99 $\pm$ 0.7
9	Sediment + artemia + Fungicide	3.38 $\pm$ 1.5	2.64 $\pm$ 1.2
10	Sediment + detritus + Deep-sea fungus + antibacterial antibiotic	108.02 $\pm$ 0	93.85 $\pm$ 9.3
11	Sediment + detritus + Deep-sea bacteria + Fungicide	84.85 $\pm$ 16.6	67.41 $\pm$ 0.6

When the nutrient source was added in the form of detritus to this sediment, the APA went up drastically at both the pressures of incubation. When fungicide was added to the mixture of sediment and detritus, the APA decreased by 25 %, whereas

addition of antibacterial antibiotic decreased the APA by 10 % as compared to plain sediment with detritus and without any inhibitor. But when the nutrient source was added in the form of artemia larvae and cysts, there was no detectable increase in the APA, rather a drop in the activity was seen. When the deep sea fungal isolate # A4634 was added along with antibiotic to sediment and detritus mixture, the APA increased almost 20 times as compared to plain sediments at both 200 and 500 bar pressure, whereas addition of deep sea bacteria along fungicide, increased the APA only 15 times at both 200 and 500 bar pressure.

## 8.5 Discussion

The fungal biomass constitutes only a portion of the potentially mineralizable organic matter associated with aggregates in terrestrial environment, which is mineralized only upon aggregate disruption [Gupta & Germida, 1988]. In this study also, the fungal biomass contribution is 2.3 - 6.3  $\mu\text{g C g}^{-1}$  dry sediment, which is much less than the bacterial carbon reported in the sediments of CIB, ranging from 3 to 40  $\mu\text{g C g}^{-1}$  dry sediment [Raghukumar *et al.*, 2001].

The deep-sea isolate # A4634 was able to grow better in the sediment extract as compared to MEB at 200 bar and 5°C. It appears that these conditions are closer to the existing conditions in the sea than the rich culture media. In contrast, the terrestrial cultures failed to emulate this growth in the sediments and were more suited to grow in MEB.

The sediment extract, when extracted without heating or boiling supported better fungal growth than the extract prepared by boiling. For culturing actinomycetes

routinely in laboratory, sediment extract is prepared by boiling sediment for one hour and filtering just before use. It is possible certain essential heat labile substances are present in the sediment, which help fungi to grow better. Addition of EDTA during sediment extract preparation was found to be helpful. EDTA has been shown to be an effective mild extractant of soil organic matter, extracting as much as 30 % organic matter from soils [<http://www.ar.wroc.pl/~weber/ekstrak2.htm>]. Although alkali extraction (NaOH) has been shown to be best extractant, able to extract as much as 80 % of soil organic matter, it has many undesirable features, due to which EDTA extraction was favoured. Other extractants like acetylacetone, cupferron, hydroxyquinolone, and formic acid [<http://www.ar.wroc.pl/~weber/ekstrak2.htm>] are also used but these were not used here, as these could be deleterious to fungal growth. The sediments of CIB contain LOM (labile organic matter) up to 3.845 mg g<sup>-1</sup> dry sediment [[Raghukumar et al., 2001](#)], out of which over 60 % (2.4 mg g<sup>-1</sup> dry sediment) is contributed by carbohydrates. EDTA is shown to be effective in solubilising the polysaccharides [[Liu et al., 2002](#)] so that it is available for further activity, for uptake by other organisms or analytical purpose.

The microaggregates stained positively for humic substances indicating that the fungal activities are responsible for the formation of humus possibly in deep-sea sediments also as described previously for terrestrial environments [[Stevenson, 1982](#)]. Reducing sugars and amino acids, formed, as by-products of microbial metabolism in terrestrial sediments are known to undergo nonenzymatic polymerization to form a series of compounds like 3-carbon aldehydes, ketones and reductones and hydroxymethyl furfurals. These highly reactive compounds readily polymerize in the

presence of amino compounds to form brown-coloured products, constituting humus [Tisdall & Oades, 1982]. The humic material combines with soil particles to form microaggregates. Fungal hyphae further act as binding agents themselves to form macroaggregates by trapping fine particles into the microaggregates [Kandeler *et al.*, 1999]. Fungi or bacteria can thus remain protected in certain particle size classes [Suberkropp & Weyers, 1996]. Microaggregates in the present study also stained positive for polysaccharides and proteins. Cations such as  $\text{Si}^{4+}$ ,  $\text{Fe}^{3+}$ ,  $\text{Al}^{3+}$  and  $\text{Ca}^{2+}$  form bridges between particles in terrestrial microaggregates [Bronick & Lal, 2005]. The fungi, thus present, concealed inside these microaggregates, contribute to the nutrient cycling in the deep-sea habitat, by acting upon them from inside the aggregates. Fungi can be important for both the formation as well as stabilization of soil aggregates [Lynch & Bragg, 1985]. Although fungi are generally believed to be more effective at aggregate stabilization than other soil microflora, several studies have concluded that their primary contribution to aggregation is through hyphal entanglement of soil particles [Molope & Page, 1986]. But the polysaccharide-mediated binding by fungi has also been shown equally effective [Aspiras *et al.*, 1971; Burns & Davies, 1986]. This aggregation protects the soil organic matter within water-stable aggregates, which is an important, biotically regulated mechanism for accumulation and maintenance of organic matter in soil [Elliot, 1986; Gupta & Germida, 1988; Beare *et al.*, 1994]. The results support the previous reports [Beare *et al.*, 1994] that fungi contribute significantly to the stabilization of soil aggregates, but the fungal biomass constitutes only a portion of the potentially mineralizable organic matter associated with aggregates, which is mineralized only upon aggregate

disruption [Gupta & Germida, 1988]. This could also be the reason for increased sighting of fungi after treating the sediments with EDTA as reported here (Table 8.1).

The enzyme phosphatase in sediments has received considerable attention over the last years. Previous research indicates that it plays a crucial role in the cycling of P [Ayyakkannu & Chandramohan, 1971; Degobbis *et al.*, 1984; Kobori & Taga, 1979; Silva & Bhosale, 1990]. Sabil *et al.* [1994] found, in the sediment phase of the Venice Lagoon within an area of shallow water, the Palude della Rosa, that the immobilized enzymes, including phosphatase, showed marked heat stability and an increased resistance to environmental changes. They could be useful as diagnostic factors of the ecosystem, since their presence is related to the waste products and their life span is prolonged by their insolubilization. Thus, bioassessment can reveal long term effects on sediments after the cause of the impact has passed and is itself undetectable, making the temporal scale of the enzymatic response appropriate for pollution. Most of the time, high phosphatase activity corresponds to low total P and low inorganic P, but high organic P in the sediments [Silva & Bhosale, 1990]. Alkaline phosphatase activity (APA) is one of the parameters for describing the microbial activity in the sediments. Here it was tried to differentiate the contribution of fungi and bacteria towards the APA. The plain sediment without any additional nutrients showed very low APA. When the detritus was added, the APA increased 20-fold, indicating acute increase in microbial activity, typical of a 'feast and famine' deep-sea habitat, where the nutrient supply is mostly in the form of pulse, meaning sometimes available in plenty and sometimes not available for days together. APA was higher in the presence of antibacterial antibiotic, indicating a major share of fungi towards the APA. This

indicates that fungi might play an important role in degradation of detritic material as reported in the terrestrial and mangrove environments [Newell, 1996; Ananda & Sridhar, 2004]. In contrast, addition of autoclaved artemia larvae and cysts had no effect on the APA. Protease enzyme might play a direct role in degradation of such animal detritus. The deep-sea fungal isolate #A4634, in the presence of antibacterial antibiotics showed a very high APA at both the pressures of incubation as compared to APA shown by deep-sea bacteria in the presence of fungicide, indicating that deep-sea fungus used could be contributing more towards APA than the deep-sea bacteria used.

In conclusion, these studies suggest that fungi are present in abundance in deep-sea sediments and may play following major roles.

- 1) By virtue of a biomass of about 25 % of that of the bacterial standing crop [Raghukumar *et al.*, 2001] as shown here, they form an important component of the microbial consortium and contribute substantially to carbon mineralization and sedimentary carbon on a global scale. The polymers of fungal cell wall melanin and chitin are not easily degradable whereas, phospholipids forming the main component of bacterial cell wall are readily degraded. Thus, in some instances, fungal C in terrestrial leaf litter is known to be about 26 times greater than that of bacterial sequestration [Okada *et al.*, 2005]. Fungal mediated C storage is expected to be more persistent in contrast to the bacterially sequestered one [Bailey *et al.*, 2002]. Understanding the relative contribution of C in the deep-sea sediments by fungi and bacteria may allow prediction of longevity of stored C in deep-sea sediments. Such a sequestration might be enhanced by the formation of aggregates composed of humic material, as demonstrated in the experiments that were carried out.

2) Fungi in deep-sea sediments might also be important in the food web involving meiofauna. The labile portion of the sediment organic matter consists mainly of simple sugars, fatty acids and proteins, which are rapidly mineralized [Fry, 1987]. Alongi [1990], analyzing the deep-sea sediment of the Solomon and Coral Seas, did not find any relationship between bacterial parameters and organic carbon content; he hypothesized that bacterial activity, even in deep sediments, may be correlated with standing amounts of specific labile compounds, such as lipids, amino acids and proteins.

3) Nematodes found in abundance in sediments from CIB are an important component of deep-sea sediment fauna. Nematodes constituted more than 25 % of the benthic macrofauna recorded in the sediments of CIB [Ingole *et al.*, 2001]. They are avid feeders of fungi in terrestrial soil [Okada & Kadota, 2003; Okada *et al.*, 2005]. The nematodes fed on fungi have been shown to exhibit a higher population growth rate. Deep-sea fungi may provide a direct food source to nematodes, contributing in a significant manner in the deep-sea food chain also. Fungal feeding habit of nematode is also a classification tool. Based on the presence of a particular type of fungus-feeding nematode, it may be also possible to predict the presence or absence of the host fungus [Okada & Kadota, 2003].