

Chapter 9

Summary

The deep sea, the largest single ecosystem on earth is an extreme environment, and the least explored habitat. This is mainly due to the remoteness of the deep sea and the difficulties encountered in its exploration that have resulted in it being the least understood environments on the earth. The understanding of deep sea will unravel a lot of exciting information about the history of life existing on earth.

The deep sea was thought to be a very difficult environment for life forms to exist due to combination of different extremities in the form of low temperature, high hydrostatic pressure, low nutrient supply and absence of light. Due to these factors, deep sea was thought to be devoid of life till the mid 19th century. This assumption was first challenged in 1860 with the discovery of corals and sponges attached to a transatlantic cable that was hauled up from the seafloor for repair. Since then study of marine life forms has progressed a lot. It can be concluded safely that deep sea is one of most diverse habitat in terms of species richness on earth.

Marine sediments overlay two-thirds of the earth's surface and harbour diverse and abundant fauna. The organisms in these sediments can be divided into two categories as infauna (living within sediments) and epifaunal (living on surface of the sediment). Amongst these, bacteria are one of the most extensively studied microorganism with respect to their abundance, diversity, phylogeny, growth and enzyme profiles and different adaptations. Fungi in the marine environment have only been fully recognized since about 1960, and within the group, marine fungi have shown highest decadal indices (% increases in species number over a 10 year period) with respect of description of new species. In spite of this, fungi have remained largely neglected in the vast environment of deep sea, with some sporadic reports appearing

once in a while. With this study, it is tried to reduce this void by describing the occurrence and diversity of fungi from the Central Indian Basin.

For this, fungi were isolated from the sediments from the Central Indian Basin from an average depth of ~ 5000 m by different techniques viz. dilution plating, particle plating and pressure enrichment technique on board the vessel AA Sidorenko. The particle plating method yielded the lowest number of fungi while dilution plating and pressure enrichment resulted in almost similar recovery of fungi from the sediments. The highest number of species was often obtained at 0-2 cm depth of deep-sea sediment cores, while the numbers were much less below 25 cm depth (Table 2). *Aspergillus* species were the dominant fungi isolated, followed by non-sporulating and unidentified sporulating fungi. However, a one-way analysis of variance (ANOVA) comparing the number of species isolated from different subsections of each core down to 30 cm depth showed that differences between the subsections were not significant. None of the media used for isolation were selective for isolating specific type of fungi.

All the different culture techniques that we employed yielded filamentous fungi, belonging to the genera such as *Aspergillus*, *Penicillium*, *Cladosporium*, *Curvularia*, *Fusarium* and several non-sporulating forms which are known from terrestrial habitats. *Aspergillus terreus* was one of the most common fungi isolated. Colonies of the terrestrial fungi in our isolations from the sediments might have resulted either from dormant spores or actively growing mycelia. Among the various *Aspergilli* isolated, many showed abnormal morphology on solid media during the first couple of generations, which was not seen later.

When these deep sea isolates were compared with terrestrial species, they showed a different growth, physiological and genetic patterns indicating that these are completely different strains as can be deduced from their C- source utilization and ITS RFLP profile.

Along with filamentous fungi, some yeasts were also isolated from sediments and pore water (water trapped between the sediment particles). Two distinctly different groups were isolated from sediment and water as seen from the biochemical and molecular identifications. The orange yeasts isolated from the sediments belonged to *Rhodotorula mucilaginosa* while those from the pore water to *Rhodospiridium paludigenum*.

One of the main reasons for fungi remaining neglected is that they cannot be easily visualized in the sediments. In this study, an optical brightener, Calcofluor, was used in order to facilitate easy visualization of fungi in the sediments. But even with this, fungi could be visualized in only ~5 % of the sediment subsections. But when the same samples were treated with EDTA prior to staining with calcofluor, the number of sediment samples showing fungal presence doubled to ~ 13 %. This could have been because of disruption of the microaggregates by EDTA, the former entrap fungal hyphae within themselves.

Another method that of immunofluorescence staining, was used to visualize fungi in the sediment and to prove that the fungal cultures isolated were native of the deep sea sediments. Polyclonal antibodies were produced against one of the most frequently isolated fungus, *Aspergillus terreus*, in rabbit. Sediment samples of the core subsection from which this particular culture was isolated revealed fluorescing hyphae.

Being obtained from an extreme environment of high hydrostatic pressure and low temperature, these fungi should have been able to grow in these conditions simulated in laboratory. But, a large number of fungi (109 in all) did not show growth when directly incubated under elevated pressure of 500, 400 & 300 bar. Majority of these 109 isolates that did not show direct growth initially when incubated at elevated hydrostatic pressure showed growth at 50 bar pressure. Decreasing numbers of fungi showed viability and growth when they were gradually subjected to higher pressure. Only two strains of *Aspergillus terreus* and the yeast # A 344 grew at 300 bar and only the latter at 400 bar pressure. It may be hypothesized that although several fungi reach the seafloor, most of them remain dormant and only a few show active growth.

In a comparative study, the terrestrial cultures obtained from the culture collection of IMTECH, Chandigarh also showed growth at 200 bar pressure, but the biomass produced by these was less than the similar deep sea isolate of *Aspergillus terreus* (# A 4634).

All the sporulating cultures tested for germination of spores, failed to show germination at low temperature of 5°C at both 1 bar and elevated hydrostatic pressures. All the fungi that we tested, irrespective of whether they were isolated from deep-sea sediments, shallow coral reef lagoon waters or terrestrial sources, grew and produced substantial biomass when mycelial inocula were used. Contrary to hyphae, spores may be poor candidates for propagation of fungi in the deep-sea sediments. None of the four fungi that were tested, including 3 terrestrial and 1 deep-sea isolate, germinated at 5°C at 1 or 200 bar. The terrestrial isolates germinated very poorly at 200 bar 30°C, while the marine isolate showed substantial germination. Thus, pressure appears to have different effects on mycelium and spores of fungi.

At 30°C and elevated hydrostatic pressures, where germination was recorded, it was seen that the germination rate is inversely proportional to the hydrostatic pressure. The viability of the spores is also inversely proportional to the hydrostatic pressure. But here also, the deep - sea isolates showed a lot of variability amongst themselves. The viability decreased gradually from 92.3 to 12.8 % at 100 and 500 bar respectively in case of # A 4634, while in case of other deep-sea isolates, the viability was not much affected by the incubation pressure, but was on the lower side.

Not only pressure, low temperature also adversely affected the viability of the spores. The spores of the two deep sea isolates tested showed better viability than the similar terrestrial isolate and another deep sea isolate.

Due to the “Feast & Famine” conditions of the nutrients prevailing in the deep sea, effect of nutrient concentrations was also tested, but it did not show any substantial effect on the germination rates. There was a random change in the germination rates irrespective of the nutrient concentrations. This could be because of requirement of less stringent nutrient conditions for the viability and germination of spores.

Use of a known stress protectant, DMSO as reported in yeasts, did not seem to affect the growth of the deep sea fungi in this case. The biomass production or spore germination of these fungi in presence of DMSO did not increase indicating that DMSO is not playing any role here.

Apart from this external stress protectant, the deep sea yeasts were tested for trehalose (another stress protectant) production under different temperatures. Although, reported previously to be produced under the stress of low temperature, in the yeast cultures tested, maximum trehalose production was seen at higher

temperatures of 15 & 30°C, and not at 5°C. This can be further proof of these yeasts being deep sea inhabitants, hence low temperature not really proving to be a stress.

When the deep sea cultures, both the filamentous fungi and the unicellular yeast were analyzed for intracellular proteins by the means of electrophoresis, no conclusive results were obtained which could point to a specific stress protein being induced or repressed due to the elevated pressure. Although a large number of proteins were induced or repressed, these could not be assigned to any of the described stress protein families.

Along with the intracellular proteins, these deep sea fungi were also analyzed for one of the extracellular enzymes produced by them, the protease. Proteases were selected for study as these occupy a very important position in the nutrient cycling in the deep sea environment mainly because proteins and peptides constitute a substantial portion of the organic nutrients present there. This enzyme was produced by majority of the fungi isolated, in varying amounts. But, not all the cultures were producing protease active at alkaline pH and low temperature. Similar to the growth and spore germination, protease production and activity was also affected by low temperature. The number of fungi showing protease activity at 30°C was much higher than those showing at 5°C. Although, growth was fairly unaffected by elevated pressure and low temperature (inoculum in the form of mycelia), protease production was reduced 10 to 100-fold under similar conditions.

One of the deep sea cultures, *Aspergillus ustus* (# NIOCC 20) was found to produce maximum amount of protease in the primary screening. It showed a substantial biomass at elevated hydrostatic pressure (100 bar). But, when the fungus was grown under elevated hydrostatic pressure, whether at 30 or 5°C, little or

negligible amount of protease production was seen. In contrast, the enzyme produced by fungus at 30°C / 1 bar was more tolerant to elevated pressure, with almost same activity recorded up to 300 bar of pressure. However, with increasing pressure, the K_m value for the enzyme also increased indicating a low substrate affinity.

The enzyme produced at 30°C and 1 bar pressure was further characterized. The fungus produced a maximum of 1639 ACU mL⁻¹ of protease by day 7. The enzyme, with molecular mass of 32 kDa showed several interesting properties. It had a broad pH range of 6–10, with an optimum at pH 9. The optimum temperature for protease activity was 45°C and approximately 10% of the activity was retained at 2°C. The enzyme was totally inhibited in the presence of 2 mM PMSF suggesting it to be a serine protease. It was active in the presence of several commercial detergents and in the presence of 0.5M NaCl, equivalent to 29 parts per thousand salinity. In the presence of stabilizing agents such as glycerol, CaCl₂, its thermostability at 60°C was enhanced. Heavy metal ions Cu, Hg, Fe, Ni and Zn did not inhibit the enzyme activity considerably.

While the deep sea *Aspergillus ustus* produced substantial amounts of protease enzyme as described, the terrestrial isolate of *Aspergillus ustus* obtained from IMTECH, Chandigarh (MTCC 2200) secreted negligible amounts of protease.

A conclusive proof for the existence of fungi in the deep sea sediments has been provided by us, but the question does not end here. The major question that still remains to be answered is that “are they merely present there or growing actively to play a role in the deep sea environment?” This can be answered by determining their ability to grow in the native conditions being provided to them in the laboratory. The deep sea isolate # A 4634 showed growth in the sediment extract, without any

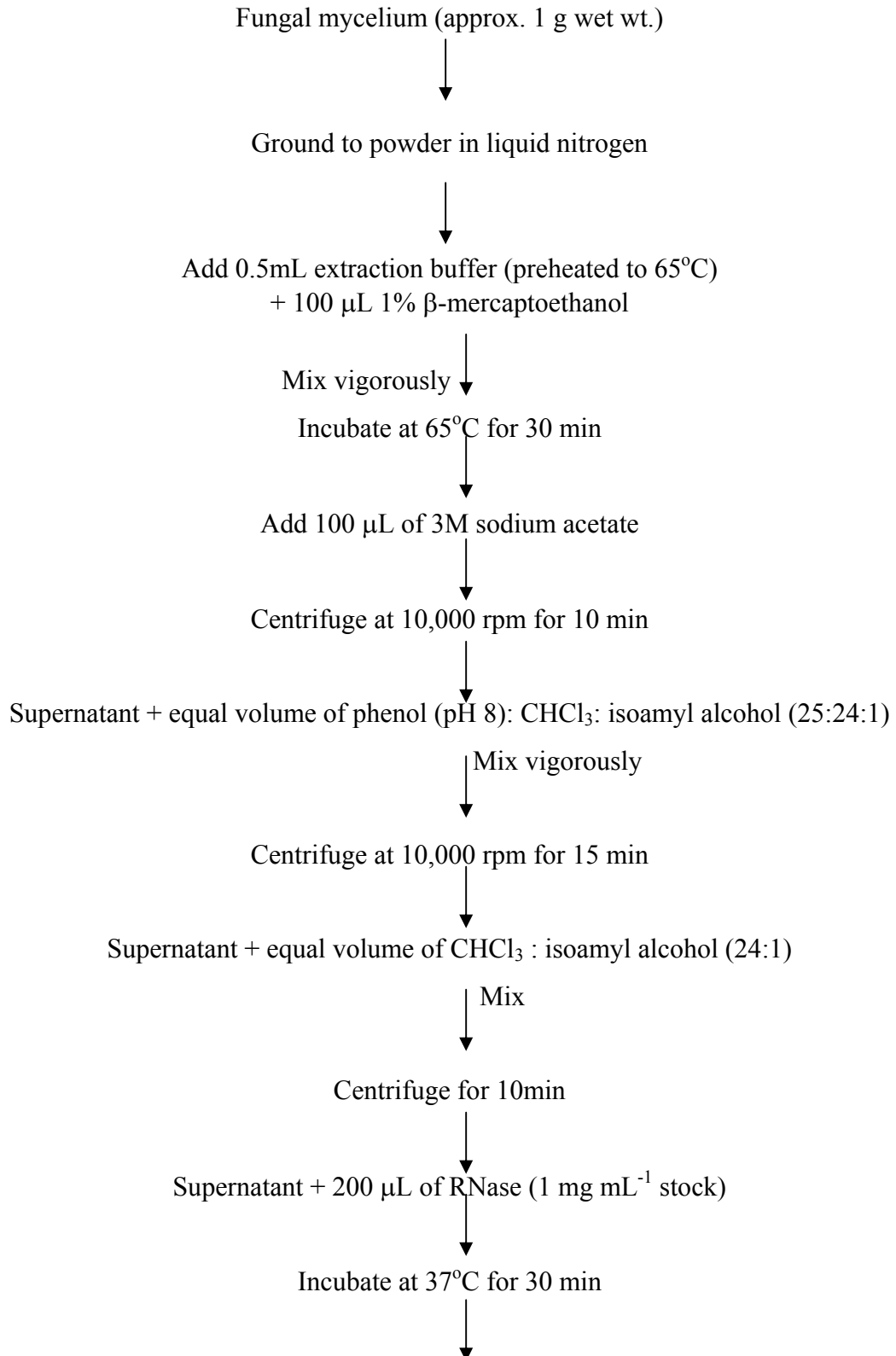
additional nutrients suggesting that, they can not only survive but also grow normally in these conditions. The biomass produced by the culture in the sediment extract was similar to the biomass produced in laboratory culture medium. Not only the biomass production, the growth morphology was better in the sediment extract under elevated hydrostatic pressure than in malt extract broth, indicating sediment to be better environment for these deep sea cultures.

Apart from the protease production as discussed above, these fungi could also be playing a role in nutrient cycling by production of other enzymes like alkaline phosphatase. Alkaline phosphatase activity is widely used as indicator of microbial activity in the sediments. When individual contribution of fungi and bacteria towards APA was determined at elevated hydrostatic pressure, the share of fungal APA was more as compared to the bacterial APA, indicating that deep sea fungi could be contributing more towards APA than the deep sea bacteria used.

In conclusion, this study proves an active role of fungi in the deep sea sediments of the Central Indian Basin. It would be worth while to conduct similar studies in other oceans (deep-sea sediments) for comparison.

Appendix

A - I. DNA Extraction Method [van Burik *et al.*, 1998]



Equal volume of phenol (pH 8) : CHCl₃ : isoamyl alcohol (25:24:1)

↓ Mix vigorously

Centrifuge at 10,000 rpm for 15 min

↓

Supernatant + equal volume of CHCl₃ : isoamyl alcohol (24:1)

↓ Mix

Centrifuge for 10 min

↓

Supernatant + 2.5 volumes of chilled isopropanol

↓

Centrifuge at 10,000 rpm for 10 min at 4°C

↓

Wash with 70% cold ethanol

↓

Dissolve in minimum volume of sterile d/w

↓

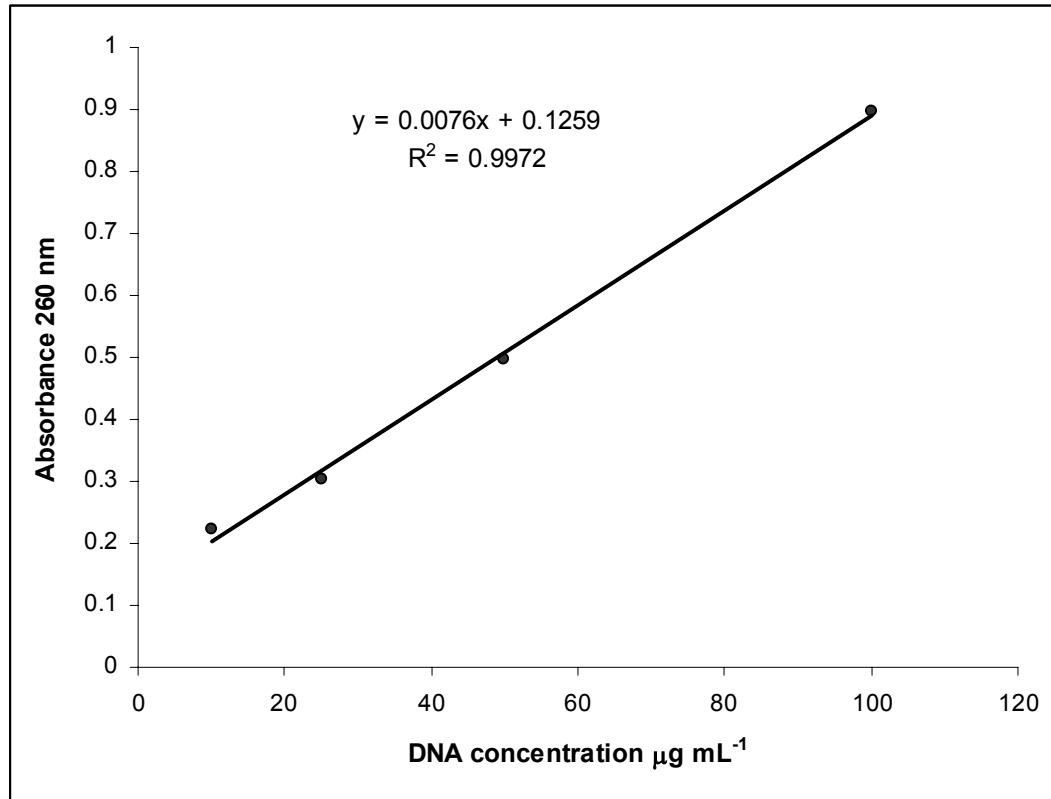
Store in -20°C

Extraction buffer:	Tris HCl	100 mM
	Na ₂ EDTA/Na ₄ EDTA	20 mM
	CTAB	2 % (w/v)
	NaCl	1.4 M
	Poly vinyl pyrrolidine	1 % (w/v)

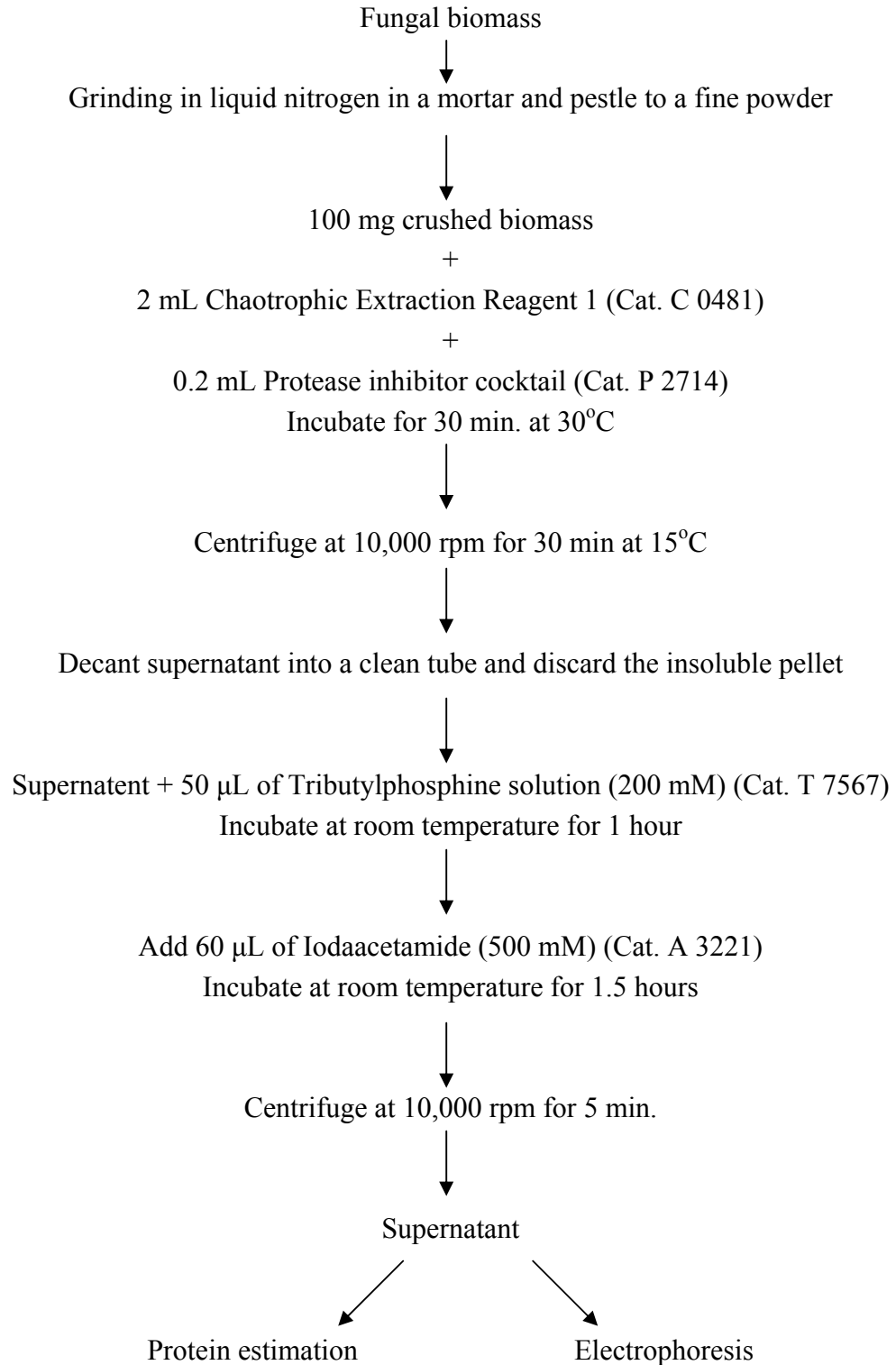
T.E buffer	Tris-HCl (pH 8)	10 mM
	EDTA	1 mM

A - II. DNA Standard

DNA (HiMedia, Cat. RM 511) was dissolved in sterile distilled water to get the desired concentration and the absorbance was read at 260 nm.



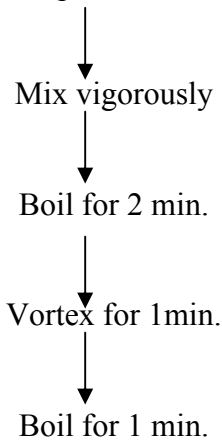
**A – III. Protocol for the intracellular protein extraction using
Sigma total protein extraction kit [Cat. PROT-TOT]**



A - IV. Urea buffer protein extraction method

[[Nir Osherov & Gregory May](#), Webpage – Department of Cell Biology, Baylor college of Medicine, Houston, Texas, 77030 USA; *Optimization of protein extraction from Aspergillus nidulans for gel electrophoresis*]

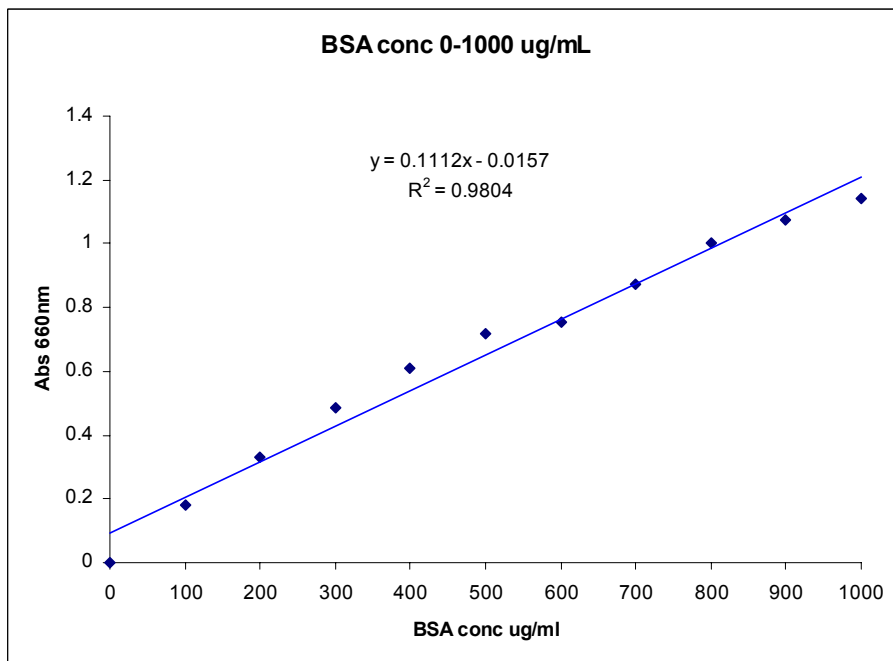
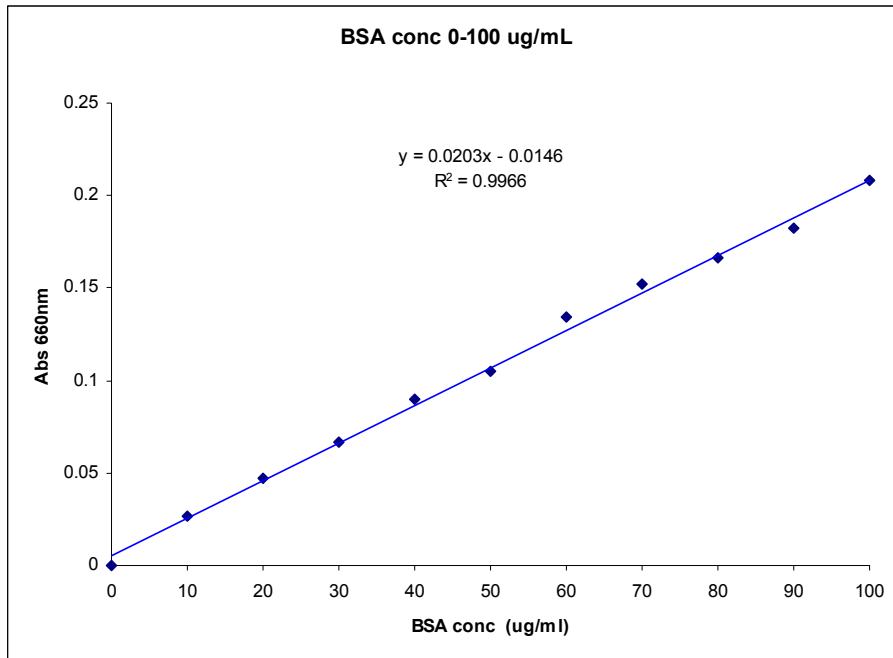
Lyophilized fungal biomass powder in 0.2 mL urea sample buffer



Urea buffer – 1 % SDS
9 M Urea
25 mM Tris-HCl pH 6.8
1 mM EDTA
0.7 M mercaptoethanol

A – V. Protein standard [Lowry *et al.*, 1951]

Bovine Albumin (BSA) (SRL chemicals, Cat. 0140299) dissolved in d/w was used as standard for protein. The dilutions required were done using d/w and the absorbance was read at 660 nm on a Shimadzu spectrophotometer (Model UV 1201V)



A – VI. SDS PAGE ----- Reagents and Gel Compositions [Laemmli, 1970]

Acrylamide / Bis : Acrylamide - 29.2 %
 N²N²-bis-methylene-acrylamide - 0.8 % in d/w

10% (w/v) SDS in d/w

Resolving buffer : 1.5 M Tris-HCl pH 8.8 (adjust pH with 6 N HCl)

Stacking buffer : 0.5 M Tris-HCl pH 6.8 (adjust pH with 6 N HCl)

% gel	D/w mL	Acrylamide/bis mL	Gel buffer mL	10% SDS mL
4	6.1	1.3	2.5	0.1
5	5.7	1.7	2.5	0.1
6	5.4	2.0	2.5	0.1
7	5.1	2.3	2.5	0.1
8	4.7	2.7	2.5	0.1
9	4.4	3.0	2.5	0.1
10	4.1	3.3	2.5	0.1
11	3.7	3.7	2.5	0.1
12	3.4	4.0	2.5	0.1
13	3.1	4.3	2.5	0.1
14	2.7	4.7	2.5	0.1
15	2.1	5.0	2.5	0.1
16	2.1	5.3	2.5	0.1
17	1.7	5.7	2.5	0.1

10% Ammonium persulphate : freshly prepared

For 10 mL gel volume: for resolving --- 50 µL 10% APS & 5 µL TEMED
 for stacking --- 50 µL 10% APS & 10µL TEMED

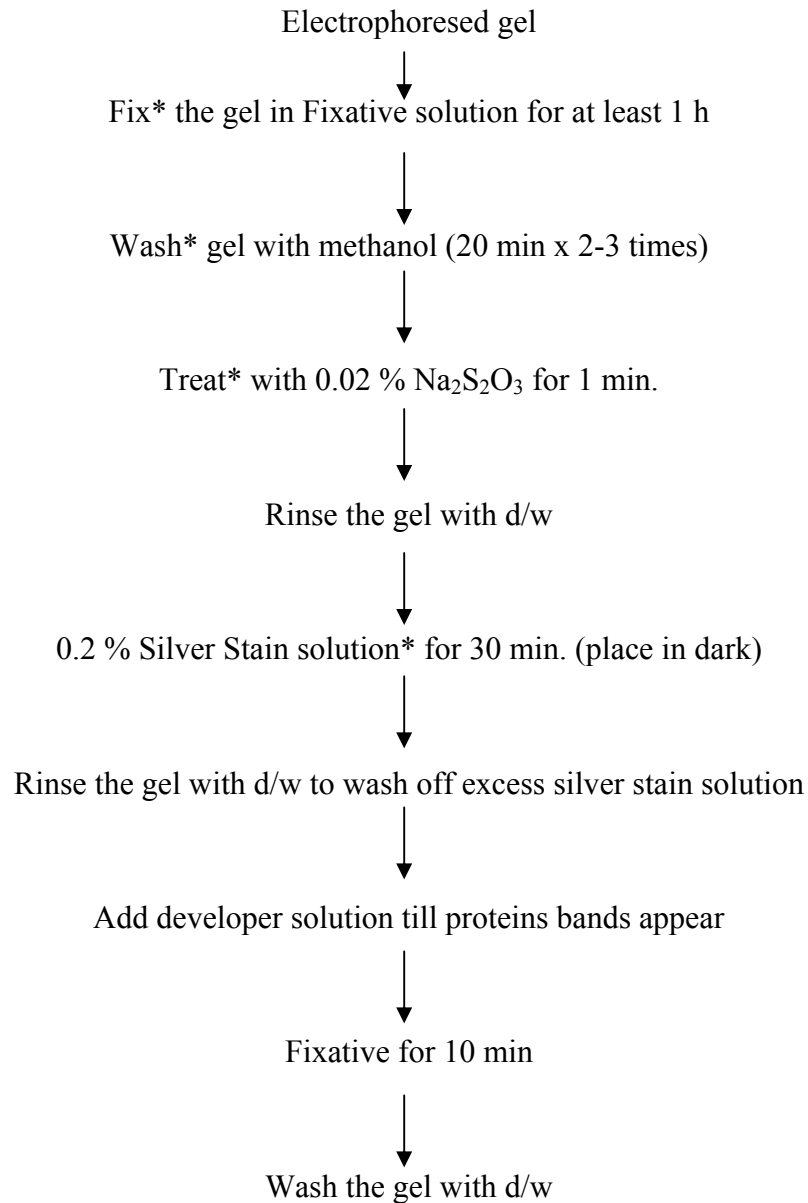
Sample buffer (SDS reducing buffer)

10X electrode running buffer, pH 8.3

D/w	3.55 mL	Tris base	30.3 g/L
0.5M Tris-HCl, pH 6.8	1.25 mL	Glycine	144 g/L
Glycerol	2.5 mL	SDS	10 g/L
10% SDS	2.0 mL		
0.5% (w/v) bromophenol blue	0.2 mL		
Total volume	9.5 mL		

Use 1X buffer for the electrophoresis run

A – VII. Silver staining protocol [Heukeshovan & Dernick, 1985]



* The gel was placed on gel rocker during these steps.

A – VIII. Reagents for Western blotting

Transfer buffer: Tris - 3.03 g
Glycine - 14.4 g
Dist. Water - 800 mL
Methanol - 200 mL

TBST buffer : Tris - 2.42 g
NaCl - 8 g
Tween 20 - 500 µL
Dist. Water - 200 mL
Adjust pH to 7.2 to 7.5 and make up volume to
1000 mL using dist. Water

A – IX. Czapek Dox (CD) medium composition (with modification)

Glucose*	-	10 g
Sodium nitrate	-	3 g
Dipotassium hydrogen phosphate	-	1 g
Magnesium sulphate	-	0.5 g
Potassium chloride	-	0.5 g
Ferric sulphate	-	0.02 g
Sea water	-	1000 mL

Wherever required, milk solution (prepared using skimmed milk prepared in d/w) was added to this basal medium, after autoclaving separately

* in original composition, sucrose is used instead of glucose.

A – X. Staining reagents used for visualization of humic substances, proteins and exopolysaccharides (TEP) in sediment extracts

1. Benzidine reagent for humic substances [[Sieburth & Jensen, 1968](#)]

Equal volumes of 10 % aq. sodium nitrite and 5 g of benzidine in 25 mL conc. HCl diluted to 1 : 1

2. Coomassie brilliant blue stain for protein particles [[Long & Azam, 1996](#)]

Stock solution - 1 % (w/v) in sterile d/w

Working solution - Dilute the stock 25-fold in 0.2 µm filtered seawater to 0.04% concentration and pH 7.4

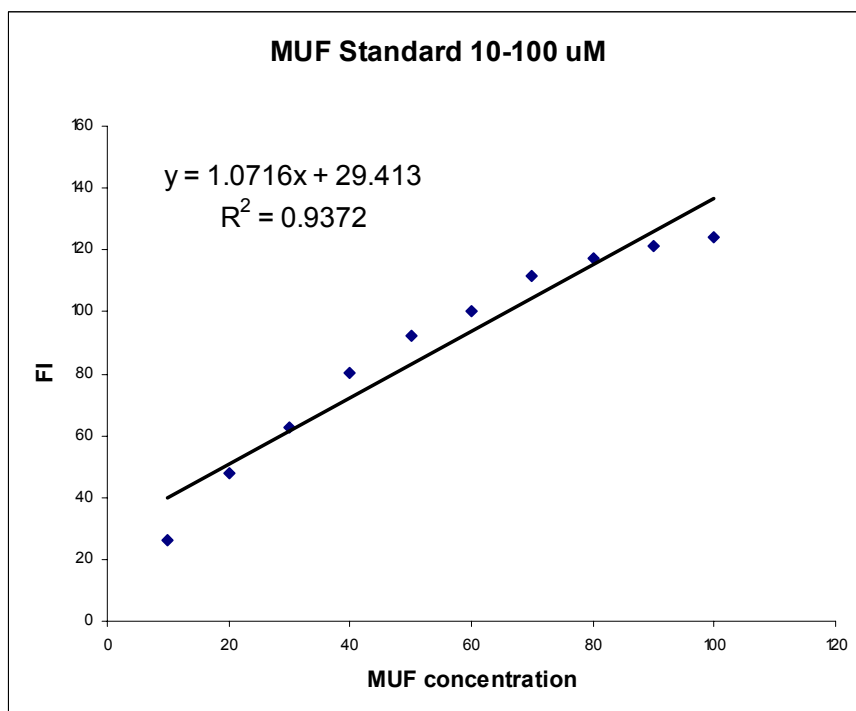
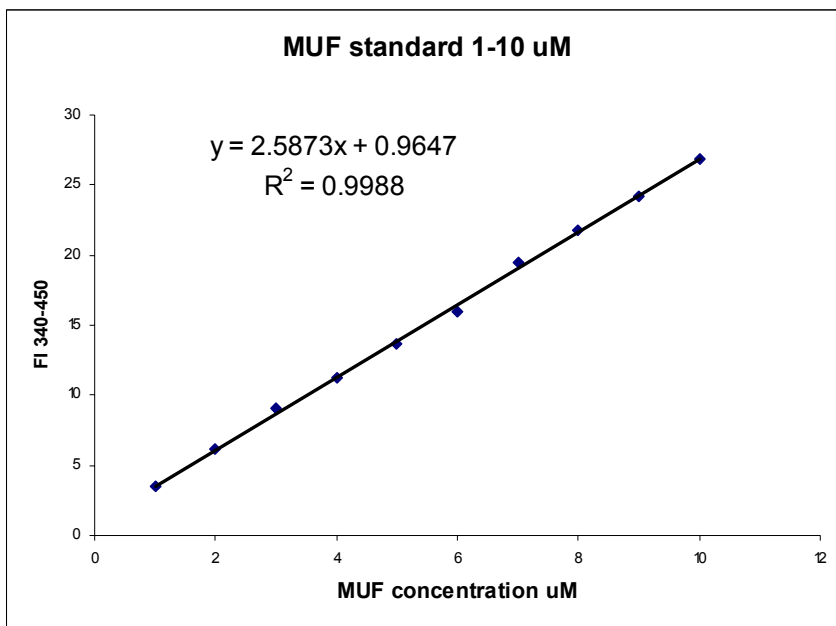
3. Alcian blue stain for TEP [[Long & Azam, 1996](#)]

0.02 % alcian blue in 0.06 % acetic acid (pH 3.3)

A – XI. Methylumbelliferone (MUF) standard [Hoppe Hans - Georg, 1983]

4 - Methylumbelliferone (Sigma Chemicals, USA, Cat. M 1381) was dissolved in methycellosolve to get the desired concentrations. The fluorescence was measured at 340 nm excitation and 450 nm emission on a Shimadzu spectrofluorimeter (Model RF1501).

The standards were done for 1-10 mM and 10 – 100 mM MUF concentration range.



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