

Chapter 5  
Summary

## 5.1 Summary

Deep-sea covers ~ 65% area of the total earth surface (Svendrup et al., 1942) and is usually characterized by the extreme conditions of temperature, hydrostatic pressure and nutrient conditions. There are several reports on diversity of bacteria from deep-sea (Li et al, 1999a; Kato and Qureshi, 1999; Schauer et al, 2010) but fungi have not received much attention from such extreme environments. Fungi in marine ecosystem occupy an important position as they are ubiquitous and involved in the decomposition and mineralization of organic matter. Deep-sea fungi were first reported in shells collected from deep-sea waters of 4610 m depth (Hohnk, 1961 & 1969). Recently few studies have reported diversity of fungi from deep-sea environments using culture-dependent as well as culture-independent approaches (Lopez-Garcia et al, 2003; Malosso et al, 2006; Wang et al, 2008; Lai et al, 2007; Bass et al, 2007; Burgaud et al, 2010; Liu et al, 2010). Various filamentous fungi were isolated from calcareous sediments and were also observed to show germination of spores at elevated hydrostatic pressure (Raghukumar and Raghukumar, 1998). Diverse fungal forms have been reported from the deep-sea sediments of the Indian Ocean at a depth of ~ 5000 m (Damare et al, 2006). The cultures were identified by classical taxonomic methods in these studies. However, the non-sporulating forms could not be identified on the basis of morphological features by this method.

Therefore, the objectives of the present study were to isolate and identify fungi from deep-sea sediments of the Central Indian Basin (CIB) using culture-dependent as well as culture-independent approaches by molecular methods. Further, the effect of simulated deep-sea conditions was analyzed on the growth, spore germination and protein profiles of a few selected cultures in order to understand their adaptation strategies under such extreme conditions.

Sediment samples were collected from 20 and 2 stations of Central Indian Basin during two cruises i.e. #ABP26 and # ABP38 respectively at an average

depth of ~ 5000 m. A total of 28 and 19 fungi were isolated during cruises #ABP26 and # ABP38 respectively using four different isolation techniques and four culture media. Fungal isolates belonged mostly to Ascomycota and Basidiomycota with majority being the filamentous fungi. Pressure Incubation yielded maximum number of fungi (Tables 2.3 and 2.4). Fungi could be isolated from deeper depths i.e. 35-40 cm of sediment core also (Table 2.6). Best medium for isolation was found to be MEA during cruise #ABP26 whereas both CDA and PDA yielded maximum number of fungi during the cruise # ABP38 (Tables 2.5 and 2.6). The fungi were identified by classical taxonomic method as well as by amplification and sequencing of their 18S and ITS regions of SSU rDNA. During the cruise #ABP26, fungi belonging to the phylum Ascomycota were clustered into 7 subgroups, namely *Aspergillus* sp., *Sagenomella* sp. (Fig 2.4), *Exophiala* sp. (Fig 2.5), *Capronia* sp., *Cladosporium* sp., *Acremonium* sp. and *Tritirachium* sp. based on 18S sequences (Fig. 2.6). Based on ITS sequences they grouped into 6 clusters showing maximum similarity to *Aspergillus* sp., uncultured member of Hypocreaceae family, *Exophiala* sp., an uncultured soil fungus clone DQ682584.1, *Hypocreales* and *Trichothecium* sp. (Fig. 2.7). Species of filamentous fungi *Sagenomella*, *Exophiala*, *Capronia*, and *Tilletiopsis* are being reported for the first time from the deep-sea sediments. Majority of the yeast belonged to genera *Sporobolomyces* sp., *Sporidiobolus*, *Rhodospiridium*, *Rhodotorula* and *Cryptococcus* sp. of the phylum Basidiomycota. The cultures were deposited in a recognized microbial culture repository, Microbial Type Culture Collection (MTCC) at Chandigarh, India, under the accession numbers, MTCC9332-MTCC9334 and MTCC9336-MTCC9341.

During cruise #ABP38, a total of 19 fungi were isolated belonging to 12 distinct genera. Majority of the forms belonged to Ascomycota on the basis of 18S sequences and included members from *Aspergillus* sp., *Eurotium* sp., *Cladosporium* sp., *Pleospora* sp., *Chaetomium* sp., *Ascotricha* sp., *Penicillium* sp., *Sagenomella* sp. among filamentous fungi (Fig. 2.10). Two isolates matched

with a black yeast i.e. *Hortaea* sp. Among basidiomycota, two fungal genera belonging to *Cerrena* and *Trametes* sp. were identified (Table 2.8). The isolation of *Cerrena* and *Tremetes* sp. is being reported for the first time from the deep-sea sediments of the Central Indian Basin. Some of the fungi such as *Aspergillus*, *Cladosporium*, *Penicillium* and *Sagenomella* sp. were isolated during both the cruises, #ABP26 and #ABP38. Diversity and species richness of the fungi was higher during the cruise #ABP26 than #ABP38 (Tables 2.9 and 2.10).

Diversity of fungi was also evaluated from deep-sea sediments of CIB collected during two cruises #ABP26 and #ABP38 using culture-independent approach. Sediment DNA samples from the three stations (A, B and C) were amplified using fungal-specific ITS1F/ITS4, “primer pair a” as well as universal ITS1/ITS4, “primer pair b”, and universal 18S rDNA NS1/NS2 primer set, “primer pair c”. For the station SVBC-33 of the cruise #ABP26, sediment DNA sample was amplified using fungal-specific primer pair, ITS1F/ITS4 as well as universal 18S rDNA primers, NS1/NS2, Euk18S-42F/Euk18S-1492RE and Euk18S-555F/Euk18S-1269R (Tables 3.1 and 3.3). Sediment DNA samples isolated during the cruise #ABP38 were amplified using fungal specific ITS1F/ITS4 and universal 18S rDNA NS1/NS2 primer set (Tables 3.2 and 3.3). A total of 768 clones were sequenced from the 16 environmental libraries for the 3 stations (A, B and C) of the cruise #ABP26. Of the resultant sequences, 257 sequences were found to be fungal, resulting in a total of 39 OTUs and 32 distinct taxa, after clustering on a basis of 98% sequence identity criterion. For the station SVBC-33, a total of 48 and 144 clones from ITS and 18S primer pairs respectively were sequenced from the environmental libraries. This resulted in 27 fungal OTUs and 20 distinct fungal species. During the cruise #ABP38, a total of 192 clones resulted in 46 OTUs found to be affiliating with distinct fungal species. All the OTUs obtained during these three studies belonged to Ascomycota and Basidiomycota.

Among Ascomycota the major classes amplified were Sordariomycetes, Dothideomycetes, Eurotiomycetes and Saccharomycetes. Basidiomycete phylotypes included members from Tremellomycetes, Agaricomycetes, Agaricostilbomycetes, Cystobasidiomycetes, Microbotryomycetes, Wallemiomycetes, Exobasidiomycetes and Ustilaginomycetes. During cruise #ABP38, two OTUs affiliating with eukaryotic sequences, other than fungi were also recovered (Table 3.6). Some of the OTUs during all the three studies matched at a % similarity of  $\leq 97$  to their closest relative suggesting them to be novel phylotypes (Tables 3.4, 3.5 and 3.6). This is the first report of occurrence of a halophilic and osmophilic fungus *Wallemia sebi* from deep-sea sediments. Phylotype belonging to *Malassezia* sp., was amplified from samples of both the cruises. Some of the classes such as Eurotiomycetes, Sordariomycetes, Dothideomycetes, Agaricomycetes, Saccharomycetes and Wallemiomycetes recovered during all the three studies (Tables 3.1 and 3.2) suggesting them to be abundant in deep-sea sediments of CIB. Fungal OTUs affiliating with *Aspergillus*, *Penicillium* and *Cerrena* species obtained by culture-dependent as well as culture-independent approach (Table 3.8). Higher diversity of fungi was observed by using multiple primer approach with the station SVBC-33 in spite of screening less number of clones (Table 3.5). Some of the OTUs recovered affiliated with sequences of fungi which have been reported previously from deep-sea environment (Tables 3.4, 3.5 and 3.6).

Effect of simulated deep-sea conditions on growth, spore germination and protein profiles of a few selected cultures was analyzed. All of the deep-sea fungi, mycelial as well as the yeasts isolated during the cruise #ABP26, showed growth under 20 MPa pressure and 5°C temperature suggesting their adaptation to grow under such extreme conditions (Figs. 4.1 and 4.2). Also these isolates grew better in media prepared with seawater than with distilled water. Two Basidiomycete fungi belonging to *Cerrena* and *Trametes* sp. were compared for their growth under simulated deep-sea conditions and production of oxido-reductase enzyme.

Deep-sea isolates grew better at elevated pressure and low temperature and did not show production of oxido-reductase enzyme in contrast with mangrove counterparts suggesting their different physiological characteristics (Fig. 4.6). Spores of four sporulating deep-sea fungi showed germination of spores under 20-50 MPa pressure at 30°C and no dependence on salinity (Fig. 4.9). Among yeast isolates, psychro-tolerant yeast NIOCC#PY13 showed considerable growth at 5°C. It also showed higher viability at elevated 50MPa/5°C than 30°C (Fig. 4.11) suggesting its adaptation to extreme conditions of deep-sea. This yeast grew better with higher nutrient conditions when grown at 5°C whereas the growth was not found to be affected by nutrients at 15 or 28°C. The protein profiles observed were distinct under different pressure and temperature stresses suggesting the role of different stress proteins in survival of the yeast #PY13 under extreme conditions of deep-sea (Fig. 4.13). These proteins further need to be identified using advanced level techniques such as 2D-Mass Spectrometry in order to understand their contribution towards survival of cells under deep-sea conditions.

## 5.2 Conclusions

Major inferences that could be drawn from this study are:

- ❖ Diversity of culturable as well as uncultured fungi was found to be comparatively low in deep-sea sediments of the Central Indian Basin.
- ❖ Fungal isolates could be identified till genus or species level using molecular approaches for identification.
- ❖ Fungal communities detected in deep-sea sediments mostly belonged to phyla Ascomycota and Basidiomycota using both culture-dependent and culture-independent approaches. No other groups could be recovered.
- ❖ Culturable filamentous fungi are mostly from ascomycetes whereas, yeast belonged to basidiomycetes. Some of the species such as *Tilletiopsis*, *Sagenomella Exophiala* and *Cerrena* spp. are being reported for the first time from the deep-sea.

- ❖ Fungal OTUs belonging to marine sequences in existing database were detected in all the studies.
- ❖ The recovery of fungi such as *Aspergillus*, *Penicillium* and *Cerrena* species with both the culture-dependent as well as culture-independent approaches suggest them as the major component of fungal community in the deep-sea.
- ❖ Occurrence of similar types of phyla/subclasses in the environmental libraries from different stations of the CIB suggests their abundant nature in such extreme environments.
- ❖ Increase in biomass and spore germination of the cultures isolated from deep-sea sediments suggests their adaptation under such extreme conditions.
- ❖ Increased viability of one of the psychro-tolerant yeast at elevated hydrostatic pressure and low temperature conditions suggests its survival at such extreme conditions.
- ❖ The protein patterns observed for the above yeast at different pressure and temperature conditions were distinctly different.

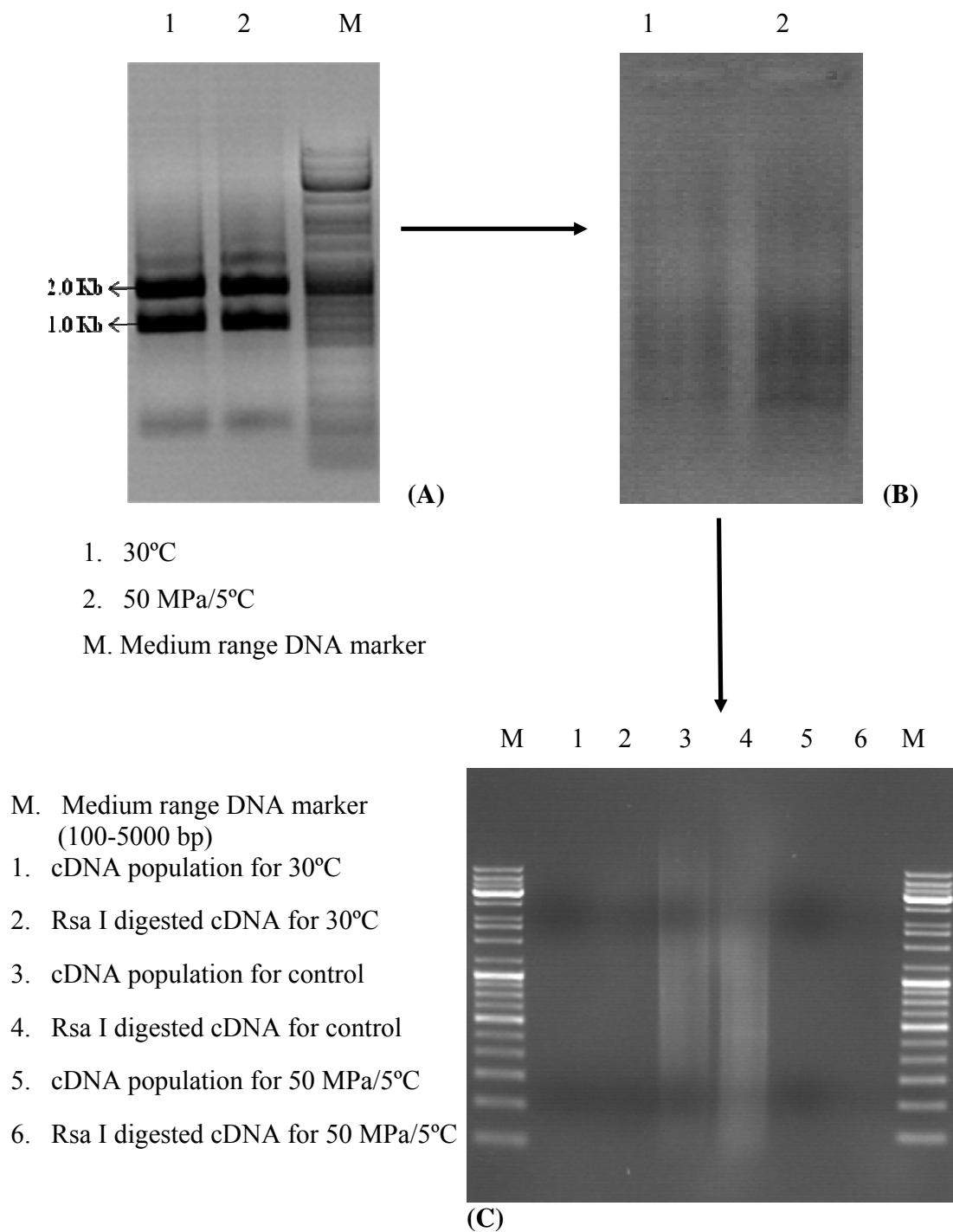
The protein profiles observed under different elevated pressure and temperature shocks were distinct and need to be identified by using advanced techniques in order to understand the cellular mechanism of yeast under stress. The following analysis is being carried out as a continuation of the present study.

**Suppression Subtractive hybridization (SSH) for identification of differentially expressed genes:**

Suppression Subtractive hybridization (SSH) is a powerful technique that may be used to compare two populations of mRNA and obtain clones of genes that are expressed in one population but not in the other. Although there are several different methods, the basic theory behind subtraction is simple. First, both mRNA populations are converted into cDNA, containing specific i.e. differentially expressed transcripts as **tester**, and the reference cDNA as **driver**. Tester and driver cDNAs are hybridized, and the hybrid sequences are then removed. Consequently, the remaining un-hybridized cDNAs represent genes that are expressed in the tester yet absent from the driver mRNA. Sets of genes or proteins associated with salinity adaptation have been effectively identified in non-model organisms by the SSH, proteomics and cDNA microarray experiments that are based on normalized cDNA libraries from non-model species or on cross-species hybridization (Gracey et al, 2001; Podrabsky and Somero, 2004; Buckley et al, 2006). SSH used in conjunction with high throughput differential screening has been reported for rapid and easy identification of differentially expressed genes in yeast and mycelia forms of *Ophiostoma piceae* (Dogra and Breuil, 2004). Marques et al. (2004) reported identification of genes preferentially expressed in the pathogenic yeast phase of *Paracoccidioides brasiliensis*, using suppression subtraction hybridization and differential macroarray analysis.

Elevated pressure and low temperature are also known to induce expression of various genes in yeast enabling it to survive under such extreme conditions (Iwahashi et al, 2003; Domitrovic et al, 2006). In my study, I tried to isolate total RNA from yeast cells after giving shocks of elevated hydrostatic pressure and low temperature according to manufacturer protocol (Takara Clontech, U.S.A.) (Fig. 5.1A). It was followed by purification of total mRNA (Fig. 5.1B).





**Fig 5.1 (A) Total RNA, (B) mRNA, (C) cDNA populations**

Total cDNA populations were constructed with these mRNA for both the shock conditions along with control according to the steps mentioned in the kit (Fig. 5.1C).

The concentration of cDNA obtained was not enough in comparison with control to proceed for the next hybridization step. The possible reason for the above may be not enough biomass of yeast to get required concentration of cDNA. By standardizing the concentration of cDNA, subtractive hybridization may be performed for the yeast subjected to different pressure and temperature shocks. This may provide insight in to identification of differentially expressed genes under various pressures and temperature stresses and therefore better understanding of cellular mechanism of yeast adopted for survival.

### **5.3 Future directions**

- ❖ Assessment of biological activity of fungi in the deep-sea sediments by the application of functional genomics.
- ❖ Improvise isolation techniques from deep-sea sediments to get yet uncultured fungi in culture.
- ❖ To study the ecological role of fungi in deep-sea sediments.
- ❖ To develop probes for identification of differentially expressed genes under elevated hydrostatic pressure and low temperature stresses.
- ❖ Analysis of fatty acid profiles of fungi subjected to different pressure and temperature shocks.
- ❖ To study the osmo-regulatory mechanism of fungi subjected to elevated hydrostatic pressure and low temperature stress using microscopic and biochemical methods.
- ❖ Screening of biologically active metabolites from these fungi.

Appendix

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## 6.1 Media

### a) Malt Extract Agar (1/5 strength)

Malt Extract Agar Base (MEA) powder	1 g
Agar powder	1.5 g
Seawater	100 ml

### b) Czapek Dox Agar (1/5 strength)

Czapek Dox Agar (CDA) powder	1 g
Agar powder	1.5 g
Seawater	100 ml

### c) Corn Meal Agar (1/5 strength)

Corn Meal Agar (CMA) powder	0.35 g
Agar powder	1.5 g
Seawater	100 ml

### d) Potato Dextrose Agar (1/5 strength)

Potato Dextrose Agar (PDA) powder	0.75 g
Agar powder	1.5 g
Seawater	100 ml

### e) Sabaraud Dextrose Agar (1/5 strength)

Sabaraud Dextrose Agar (SDA) powder	1.25 g
Agar powder	1.5 g
Seawater	100 ml

### f) Malt Extract Broth

Malt Extract Broth base (MEB) powder	2.0 g
Seawater	100 ml

**g) Yeast Extract, Peptone, Dextrose Broth**

Yeast Extract powder	0.5 g
Peptone powder	1.0 g
Dextrose	1.0 g
Seawater	100 ml

**h) Boyd and Kohlmeier (B&K) agar medium**

Yeast Extract powder	0.1 g
Peptone powder	0.2 g
Dextrose	1.0 g
Agar powder	1.5 g
Seawater/Distilled water	100 ml

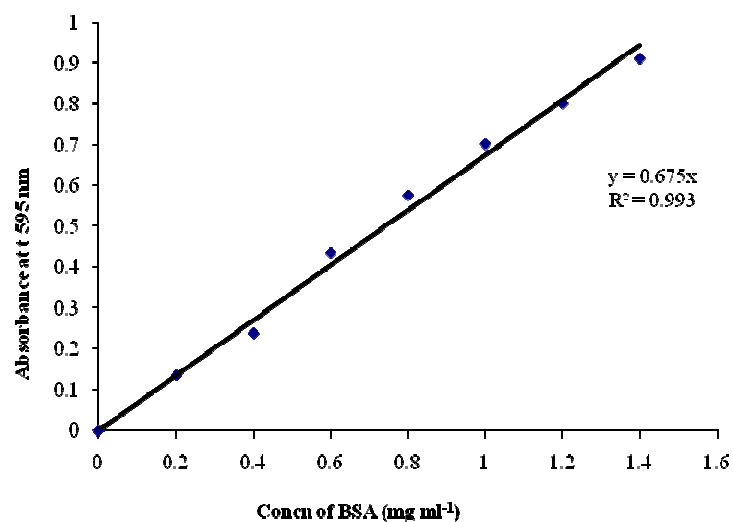
**6.2 Protein extraction and estimation methods****a) Merck Yeast buster protein extraction reagent (Cat. 71186) , protocol**

One ml of overnight grown yeast cells were harvested by centrifugation and resuspended in 200  $\mu$ l of yeast buster extraction buffer containing 2  $\mu$ l of THP solution (provided with the kit). Cells were incubated at room temperature for 20 min with gentle shaking and harvested by centrifugation. Supernatant was collected and protein estimation was carried out.

**b) Protein Estimation (Bradford's Method) ([Bradford, 1976](#); [Stoscheck, 1990](#))**

Bovine serum albumin (BSA), 2 g was dissolved in 1 ml distilled water and used as stock for the preparation of protein standards for protein estimation by Bradford's method. Different dilutions were prepared with distilled water.

To 30  $\mu$ l of each protein dilution, 900  $\mu$ l of Bradford's reagent was added and incubated at room temperature for 30 min. The absorbance was recorded spectrophotometrically at 595 nm and a standard graph was plotted.



**Calculations:** Protein (mg ml<sup>-1</sup>) = (A<sub>595</sub>/0.675)

Where A<sub>595</sub> = Absorbance at 595 nm.

Protein was expressed as mg ml<sup>-1</sup>.

### c) SDS-PAGE Composition and Reagents (Sambrook and Russell, 2001)

Components	12% Resolving gel (ml)	5% stacking gel (ml)
Distilled water	3.4	5.7
Monomer solution	4.0	1.7
Gel buffer	2.5	2.5
SDS (10%)	0.1	0.1
APS (10%), freshly prepared	0.05	0.05
TEMED	0.02	0.01

#### Resolving gel buffer:

Tris-HCl	1.5 M
pH	8.8 (adjusted with 6N HCl)

**Stacking gel buffer:**

Tris-HCl	0.5 M
pH	6.8 (adjusted with 6N HCl)

**Monomer solution: (Acrylamide-bisacrylamide solution, 30%):**

Acrylamide	29.2 g
N'N'-bis-methylene-acrylamide	0.8 g
Distilled water	100 ml

Solution was filter sterilized through 0.22  $\mu$ m filter and stored at 4°C.

**SDS-PAGE sample loading buffer (6x):**

Tris-HCl	300 mM (pH 6.8)
$\beta$ -mercaptoethanol	7.5%
SDS	10%
Glycerol	60%
Bromophenol blue	0.6%

**SDS-PAGE running buffer (pH 8.3, 10x)**

Tris base	30.2 g
Glycine	144 g
SDS (10%)	100 ml

Final volume made to 1000 ml with distilled water.

**d) Silver staining protocol (Chevallet et al, 2006):**

After electrophoresis, the gel was fixed in 30% ethanol, 10% acetic acid for at least 30 min. The gel was rinsed twice in 20% ethanol, and then twice in water, for 10 min each wash. Sensitization was done by soaking the gel for one min in 0.02 %  $\text{Na}_2\text{S}_2\text{O}_3$  solution. The gel was rinsed twice with water for 1 min each wash. Gel was impregnated with 12 mM  $\text{AgNO}_3$  for minimum 20 min. After staining the  $\text{AgNO}_3$  was rinsed off quickly with de-ionized water. The gel was shifted with gloved hands to a box containing developer solution and shaken gently till protein bands appeared. The reaction was stopped by placing the gel in



stop solution. The gel was then placed in water for complete rehydration and the image was then documented using the gel documentation system.

**Developer (prepared freshly):**

Na <sub>2</sub> CO <sub>3</sub>	3 g
Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> solution (0.02%)	1 ml
Formalin	25 µl
Distilled Water	49 ml

**Stop Solution:**

Tris-HCl	4 g
Acetic acid	2 ml
Distilled water	100 ml

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