Proteomic approach to study fungal growth under simulated deep-sea conditions

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by

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Under the guidance of

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Statement

As mentioned under the University ordinance (OA 19), I state that the present thesis entitled, "**Proteomic approach to study fungal growth under simulated deep-sea conditions**" is my original work and the same has not been submitted on any previous occasion. To the best of my knowledge, the present study is the first comprehensive work of its kind from the area mentioned.

The literature related to the problem investigated has been cited. Due acknowledgements have been made whenever facilities and suggestions have been availed of.

All the suggestions and corrections suggested by the examiners have been incorporated in the thesis.

Ms. Akhila Krishnaswamy

Certificate

This is to certify that the thesis entitled "**Proteomic approach to study fungal growth under simulated deep-sea conditions**" submitted by Ms. Akhila Krishnaswamy for the award of the degree of Doctor of Philosophy in Department of Microbiology is based on her original studies carried out by her under my supervision. The thesis or any part thereof has not been previously submitted for any degree or diploma in any University or Institution.

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Date:

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Acknowledgments and dedication

I duly take a moment to thank everyone who made this work possible. Heartfelt thanks, to……

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THIS WORK IS DEDICATED TO

KALPANA, KRISHNASWAMY, SHISHIR, APARNA, DURGA & SURENDRA

Abstract

While the existence, growth and possible role of fungi have been well established in deep-sea environment, the persisting question is to know how these microbes survive and adapt themselves to deep-sea conditions, which are otherwise challenging to terrestrial life. Proteomics techniques were selected for survival and adaptation study as proteins are direct indicators of cellular functions. Due to logistical constraints of carrying out this work in deep-sea, simulated deep-sea conditions were created in the laboratory by exerting various elevated pressures and providing low temperature (4^{o}C) for the organisms under study. Fungi being eukaryotic organisms, the observed results from this study can be extrapolated to understand how other eukaryotic organisms thrive in the deep sea. For this analysis, filamentous fungi (four isolates from deep-sea sediments and one terrestrial isolate obtained from a culture collection centre) was chosen. Fungi are known to have two distinct phases of life – the conidial phase and mycelial phase; consecutively both these phases were examined for growth in simulated deep-sea conditions. As microbes from the deep-sea environment are unknown for developing a considerable chunk of biomass, the first objective of this study was to optimise conditions for biomass production such that there was sufficient quantity of biomass for further analysis. Thirty-day incubation as triplicates in modified Czapek Dox Broth (sucrose replaced by 5% glucose) under various elevated pressures produced ~ 5–10 mg (dry weight) biomass for mycelia and less than 3 mg (dry weight) biomass of germinating conidia (in Malt Extract Broth). The second objective of the study was to compare the protein profile of these isolates. Protein studies from such miniscule biomass were challenging because traditional methods of protein extraction failed. Therefore, zirconium bead-beating technique for protein extraction using 3-buffer system, alcohol salting-out, and in-solution protocols were standardised. Protein digests were analysed by in-house LC-MS-QToF system that involved standardising protocol run time

for MS and solvents for LC. The third objective was the identification of proteins expressed in simulated deep-sea condition. Spectra that was generated by Protein Mass Fingerprinting was checked against a variety of databases by varying search parameters for generating peptide and protein summary. Organism-specific Swiss-Prot search being standardised in case of two isolates while two other isolates had Swiss-Prot genus-specific database maintained for protein summary generation. Study on the regulation of proteins is essential to understand its importance and so statistical analysis was performed with the obtained results by altering base-line values of interpretation. This analysis generated lists of commonly regulated proteins (can also be mentioned as proteins unique to a set of tested condition) whose placement in metabolic pathways was checked in the KEGG pathway database. Abundant expression of stress proteins and unknown proteins were expected in results. Instead, it was noticed that the regulation of essential and house-keeping proteins had a better role in survival and adaptations to simulated deep-sea conditions. Under simulated deep-sea conditions, there were more pathways expressed in germinating conidia than mycelia. Also, all organisms studied did not respond to the identically simulated deep-sea environment in the same manner, thus proving that each organism/isolate may have a unique/different response to similar stress. Results of pathway analysis with possible and speculated connectivity between the pathways has been discussed followed by mentioning the importance of this work in future perspective.

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Details of chemicals with catalogue number

Chemicals	Catalogue number
β-mercapto-ethanol	Merck; 8057400250
0.22µm filter paper	Millipore; GSWP 04700
0.45µm filter paper	Millipore; HAWP 04700
100bp DNA ladder	Axygen; M-DNA-100bp
1kB ladder	Genei; StepUp™ 1 kb DNA Ladder Axygen; M-DNA-HR
10X buffer for DNAse	Roche; 04 716 728 001
10X Taq buffer with 15mM $MgCl_2$	Genetix biotech asia; B16
Acetone (AR)	SD Fine chemicals; 20003
Acetonitrile	Sigma-aldrich; 34851
Acrlyamide	Sigma; 01698
Agarose	Sigma-aldrich; 05077
Agarose, low melting	Sigma-aldrich; A9539
Ammonium acetate	Fluka; 73594
Ammonium bicarbonate	Fluka; 40867
Ammonium format	Fluka; 55674
Ammonium sulphate	Himedia; RM 1273
Ammonium per sulphate (APS)	Genei; 105355
Biorad 2D starter kit	Biorad; 1632105
Biospin microspin chromatography columns	Biorad; 732-6221
Bisacrylamide	Sigma; M7972
Bromophenol blue	Himedia; RM 117
Calcoflour	Sigma; F-6259

Chemicals	Catalogue number
CDB*	(*Sucrose replaced with glucose)
Dipotassium phosphate	Fisher scientific; P290-212
Ferrous sulphate	SD Fine chemicals; 38400
Glucose	Difco; DF-0155-17-4
Magnesium sulphate	SRL; 85611
Potassium chloride	Merck; 104936
Sodium nitrate	Qualigens; Q2787E
CHAPS	Sigma; C9426
Coomassie blue stain (G250)	Himedia; RM 1219
DEPC	Sigma; D5758
DNAse (10000 U)	Roche; 04 716 728 001
dNTP (2.5Mm)	Chromus; BIO30
Di thio thereitol (DTT)	Sigma; D9779
EDTA	Merck; 324503
Ethidium bromide (EtBr)	Himedia; MB071
Ethanol	Tedia; ER 0514-196
Formaldehyde	Qualigens; NL- 353 5607
Formamide	Calbiochem; 344206
Formic acid	Fluka; 94318
Fungal DNA extraction kit	Zymo research; D6005
Gel loading dye (DNA)	Thermo scientific; R0611
Glacial Acetic acid	Qualigens; 11007
Glycine	Sigma-aldrich; G8898
Glycerol	Sigma; G5150-1L
Hydrochloric acid	Qualigens; 29507
IEF filter papers (Electrophwick)	Pharmacia Biotech; 80-1129-52
In gel blue	G Biosciences; 786-681
In gel silver	G Biosciences; 786-241

Chemicals	Catalogue number
Iodoacetamide (IAA)	Sigma; I 1149
IPG Biolyte solution (3-10, ampholyte)	Biorad; 163-1113
IPG buffer	GE healthcare; 17-6000-88
IPG Strips (3-10)	Serva; IPG 12182
Lysate tube - Lysing Matrix A	MP Biomedicals
Magnesium chloride	Fluka; 63068
Malt extract agar (MEA)	Himedia; M-137
Malt extract broth (MEB)	Himedia; M255
Methanol (AR)	Fisher scientific; 32407
Methanol (MS Grade)	Fluka; 34966
Mineral oil	BioRad; 163-2129
MS Vials	Agilent; 5188-2788
MS Vials lid	Agilent; 5182-0541
Off gel kit	Agilent; 5067-0202
Orthophosphoric acid	Qualigens; 29217
PAGE PERFECT	G-Biosciences; 786-123
PCR clean-up kit	Axygen; AP-PCR-250
Potato dextrose broth (PDB)	Himedia; M403
PERFECT FOCUS	G-Biosciences; 786-124
pH papers	Fisher scientific; 38141
Phenol	SD Fine Chemicals; MB 54917
Potassium ferricyanide (III)	Sigma; 455946
Prot chip	Agilent; Prot ID chip 150 II 300A C18 150mm column
Protease inhibitor cocktail (PIC)	Serva; 39104
Protein molecular weight marker (PMW) – Medium	Genei ; 105979

Chemicals	Catalogue number
Qubit protein assay kit	Invitrogen; Q33211
Soyabean casein digest (SBCD)	Himedia; M011
Sodium dodecyl sulphate (SDS)	Sigma; L3771
SDS sample loading buffer (Tris-glycine buffer)	Serva; 42527
Silver nitrate	SRL; 194545
Sodium carbonate	SRL; 1949157
Sodium hydroxide	Merck; 106482
Sodium pyruvate	Fluka; 15990
Sodium thiosulphate	Sarabhai-Merck; 106512
Sucrose	SD Fine chemicals; 20274
Sulphuric acid	Qualigens; Q29997
Taq Polymerase 3U/µL	Genei; 105926
TEMED	Merck; 1107320100
Thiamine	Himedia; CMS182
Thiourea	Sigma; 88810
Trichloroacetic acid (AR)	SD Fine chemicals; 20286
Tris	Merck; 1.08382.0500
Trypsin	Sigma-aldrich; T6567
Tryptophan	SD Fine chemicals; 47153
Tween-80	Himedia; RM 159
Urea	Sigma; U-5378
YBB buffer	MP biomedicals; 6550-400
Zirconium beads	Zymo Research; D6006

Details of instruments with model number

Name of the instrument	Company and model number
Autoclave	Equitron (fully automatic #7407PAD); Equitron (manual operation #7451PAD)
Bioanalyzer	Agilent (2100)
Casada reverse osmosis system	Pall (CROB 240 565, CBDG 240 809)
Centrifuge	Remi (C-24 BL); Eppendorf (5430R)
DNA sequencer	Genetic analyzer (Applied biosystems, 3130x1, USA)
Dry bath	Axygen (D1200)
Electrophoresis units	Biorad (1704467, 1653308); Mini protean electrophoresis cell (1658002EDU)
Freeze dryer	Lab conco: Freezer (120356054D), Pump (129407923)
Glassware washer	Smeg (GW3060S)
Gel documentation system	Syngene (GBOX CHEMI XRQ 6)
Homogenizer	FastPrep®-24 (MP biomedicals, USA)
Icemaker	Icematic (F-80)
IEF Cell	BioRad (1653308)
Incubator	Innova (42 R/1)
LAF (MS sample prep)	Biosystem
LC-MS-QToF	Agilent (G6538UHD Accurate Mass QTOF LC/MS, Agilent Technologies, USA)
Microscope	Olympus (BX53F)
Nano-drop	Spectrophotometer (ND-1000)
Off gel fractionator	Agilent (G3100AA)
PCR Master cycler	Eppendorf (Mastercycler pro)
pH meter	Fisher scientific (3636AB15P)
Pressure cultivation chambers	Autoclave engineers, Japan; Tsurumi and Co, Yokohama, Japan

Pressure pumps	Pall (081 000 5703, 081 000 57 30)
Ultrasonic bath	Grant Bio (XB-2)
Vacuum concentrator	Eppendorf (5305 plus)
Weighing machine	Denver (TB215DDE); Mettler-Toledo [CUB H (0.75/1.5)]

List of softwares employed

Alpha imager software (GEL DOC)
Clustal W sequence alignment
Mass hunter software version 5.0 (Agilent technologies, USA)
Mega 5.05
MPP version 13.0 build 210916
PQuest 8.0 2D analysis software
Spectrum mill MS proteomics workbench" rev B.04.01.141

List of abbreviations

APS: Ammonium per sulphate
CDB: Czapek dox broth
CIB: Central indian basin
DEPC: Di ethyl pyrocarbonate
DTT: Di thio thereitol
EtBr: Ethidium bromide
IAA: Iodoacetamide
IEF: Iso electric focussing
IPG: Immobilized pH gradient
LAF: Laminar air flow
LC-MS-QToF: Liquid chromatography- mass spectrometry- quadrapole time of flight
MEA: Malt extract agar

MEB: Malt extract broth
MS: Mass spectrometry
PCA: Principle component analysis
PCR: Polymerase chain reaction
PDB: Potato dextrose broth
PIC: Protease inhibitor cocktail
PMF: Peptide mass finger printing
PMW: Protein molecular weight marker
PTM: Post translational modification
SBCD: Soyabean casein digest
SDS: Sodium dodecyl sulphate
TE: Tris-EDTA

As on 11th Jan 2019, total number of annotated proteins in UniProtKB

Organism	Total proteins (reviewed + unreviewed)
A. terreus (NIH2624)	10417 (380 + 10037)
A. flavus (NRRL 3357)	13500 (226 + 13274)
Aspergillus sp.	792543 (4752 +787791)
Penicillium sp.	327461 (512 + 338902)

Chapter ONE

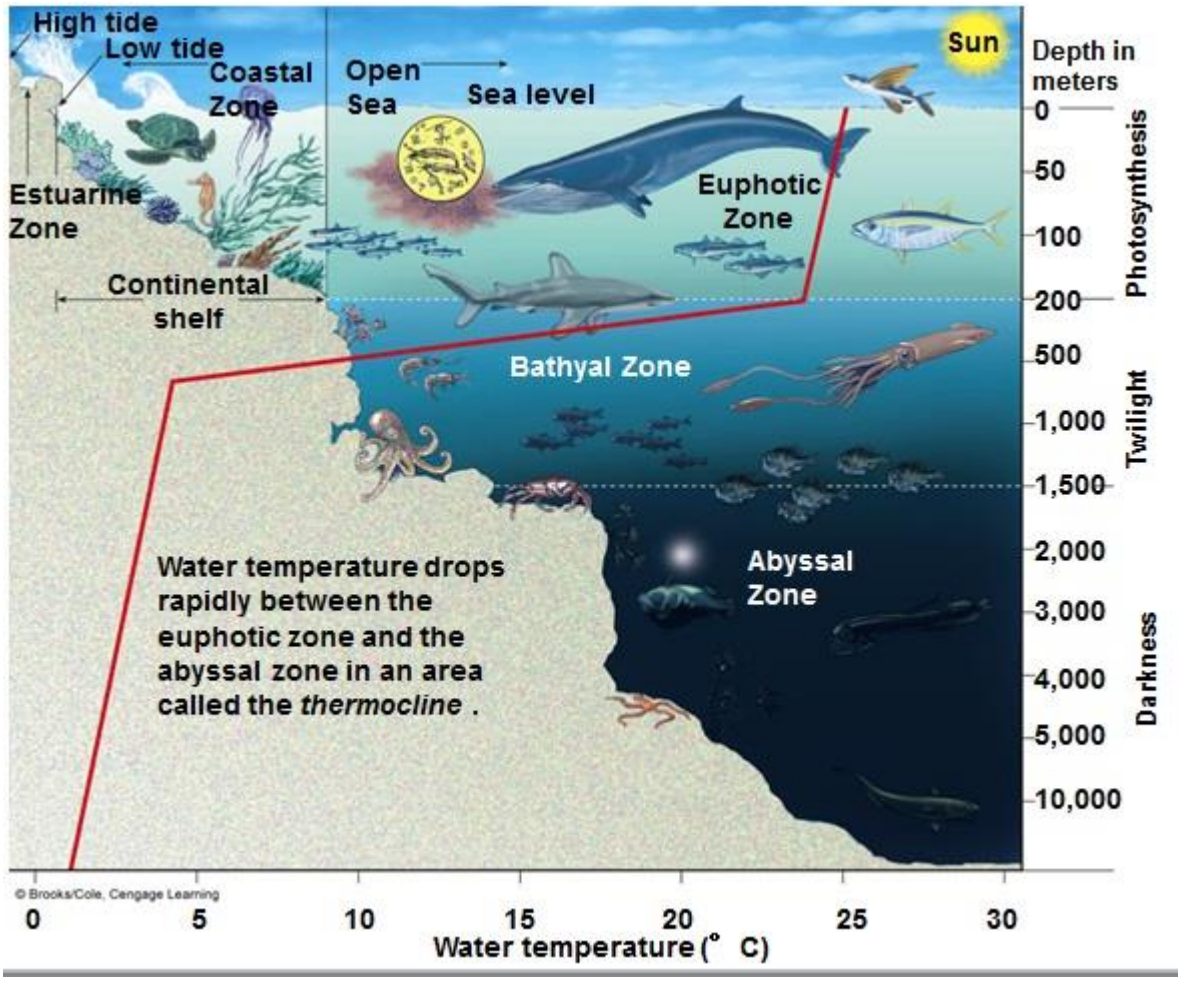
INTRODUCTION

Chapter 1 – Introduction

1.1 Literature and data available

The ocean can be classified into different zones based on the intensity of sunlight penetration regarding depth. The euphotic zone extends from coastal zone to the continental shelf (roughly 200 m). The bathyal or twilight zone starts from 200 m and extends till 1500 m. The drop in water temperature starts at this zone. The abyssal zone starts from 1500 m and extends over the entire depth of the ocean. The deep-sea falls in the abyssal zone. (Figure 1.1). Deep-sea is the largest ecosystem on earth. With a mean depth of 3800 m and an average temperature of 4ºC, it is a huge refrigerator, where the usual metabolic activities proceed at a prolonged rate.

Figure 1.1: Details of different zones of the ocean about the depth



Picture courtesy: Brooks/Cole Cengage Learning

Microbial community is the most abundant and ubiquitous community. The biomass of microbes living in the earth's sub-surface outweighed the biomass of microbes

living on the earth's surface (Daniel et al. 2006, Pfiffner et al. 2006). The first reported marine microbe (bacteria) was isolated in 1955 by Morita and Zobell from Pacific; marine fungi were isolated from Atlantic waters by Roth et al. in 1964. Fungi, along with bacteria, are prominent decomposers in the environment (Romani et al. 2006, Stursova et al. 2012) degrading lignin (Dashtban et al. 2010), the second most abundant polymer (Thakur et al. 2014).

The first report of fungus cultured from the deep-sea was in 1992 by Raghukumar et al. In that study, marine fungi were isolated from the calcareous shells. The shells were obtained from the Bay of Bengal at a depth of 960 m. Numerous filamentous marine fungi, as well as yeasts, were later isolated from deep-sea. Jones et al. (2015) have described a total of 1112 species of marine fungi which included 47 species of *Aspergillus* and 37 species of *Penicillium*. While plenty of molecular studies have revealed the diversity in deep-sea, isolating and obtaining viable cultures has been a challenge. This challenge is owing to the characteristics (physical, chemical and biochemical) of the abyssal zone. A few of them have been mentioned below (Locket 1977, Childress 1995, Danovaro et al. 2014)

i. **Increasing pressure:** Pressure increases by 0.1 MPa (roughly 1 bar or 1 atm) for every 10m increase in the depth due to the force exerted by the water column.

ii. **Low temperature:** The water temperature is similar to the atmospheric temperature at the surface, drops slowly and steadily in euphotic zone depending on the penetration of sunlight; the temperature drops rapidly in the thermocline; and in the abyssal zone is a constant of 2–4^{o}C.

iii. **Oligotrophism:** The source of energy flow in the ocean is by (i) the photosynthetic activity in the euphotic zone, (ii) marine snow, (iii) chemosynthetic activity of microbes near the hydrothermal vents and (iv) release of energy after the decomposition of sizeable dead biomass of large fishes. Based on the rate of sinking and settlement of the dead matter in the ocean floor, the condition in deep-sea could either be a feast or famine for the microbes.

As these conditions are extreme for organisms that survive terrestrially, the organisms isolated from environments such as these are called as extremophiles. Extremophiles are well-known for their unique adaptations to tolerate and live under extreme conditions. Extremophiles are fascinating to study as they should possess a different set of metabolism and pathways to be functional (Dalmaso et al. 2015). Also, studying the physiology of

extremophiles may provide clues on mechanisms for their existence. Studying the cellular components of an organism is essential to understand physiology (Brambl 2009).

In the deep-sea, the exertion of increasing pressure and low temperatures on cell altered the actual volume of the cell and therefore their cellular processes (Ulusu and Tezcan 2001, Shrestha et al. 2015). Most of the studies on pressure adaptations and survival strategies were performed on bacteria and yeasts, *in vitro* and were extrapolated using the principles of thermodynamics (Aertsen et al. 2004). Notable effects of high pressure on organisms were the inactivation/inhibition of enzymes that played crucial role in metabolism (Simpson and Gilmour 1997), reversible changes to pathways that occurred in nucleus (Kobori et al. 1995), breakdown of cell wall structure (Park and Clark 2002), cell membrane (Pagan and Mackey 2000, Ganzle and Vogel 2001) and cellular components/structures/processes (Erijman and Clegg 1998, Niven et al. 1999). With prolonged exposure to an environment, organisms in that environment adapted for survival (Somero and Hochachka 1976).

As a response to these harsh environment, many organisms exhibited a tighter packing of fatty acids in the membranes of cell-wall (Casadei et al. 2002, Nicolini et al. 2006). Deep-sea microbes have more unsaturated fatty acids in the membrane lipids to overcome the effect of temperature and pressure (Simonato et al. 2006). Some organisms changed the membrane composition under pressure (De Freitas et al. 2012). Water potential and uptake of nutrients were achieved by the accumulation of specific osmolytes such as trehalose and mannitol (Blomberg and Adler 1993). Intra-cellular solute (mostly amino acids) was accumulated by cells to survive under the increased presence of salts (Martin et al. 2002). As membrane proteins of deep-sea organisms come in contact with two different surroundings: the hydrophobic interiors and the polar exterior, these organisms possess adaptions in ATPase of Na^+/K^+ pump (Gibbs and Somero 1989). Substitution of an amino acid on unique functional position within the cell altered the stability and sensitivity of proteins to elevated hydrostatic pressure (Morita 2003). It is considered that excessive regulation of stress proteins and chaperones also assist in survival.

Most studies for piezo-tolerance were performed on non-piezophilic organisms (Kish et al. 2012) due to difficulties in sampling, isolating and maintenance of obligate piezophilies. Direct investigation of fungal growth and survival in the deep-sea would not be possible to monitor due to a variety of constraints, a few of them, such as the

microscopic size of hyphae, presence of ocean currents that would drift conidia from the initial site of experimental setup and mycelia/conidia getting deeply buried by sedimentation in the deep-sea. Alternatively, deep-sea conditions can be simulated in the laboratory to study survival strategies and mechanisms at low temperature and elevated pressures that exist in the abyssal zone of the ocean.

Amongst the previous studies reported on deep-sea fungi, Damare (2007) used different media as well as different methods for isolation of fungi from deep subseafloor sediments. The work included, studies on morphology, detection of fungi in deep subseafloor sediments using immunofluorescence staining techniques, comparison in growth in identical environments between deep-sea fungal isolates and their terrestrial counterparts, differentiation of isolates based on molecular methods, studies on deep-sea yeasts, evaluation of fungal growth under simulated deep-sea conditions, and adaptations of microbes to deep-sea conditions by altering conditions of temperature and pressure. Primary protein study of intracellular protein profiles on SDS PAGE, studies on deep-sea fungal proteases and the probable role of fungi in deep-sea sediments were also included.

Singh (2012) had isolated fungi from deep-sea sediments, identified them by both culture-dependent and culture-independent/molecular methods, constructed phylogenetic trees and described evolutionary distance. Protein profiling using SDS-PAGE was performed for yeasts, which were much easier to lyse as compared to filamentous fungi.

1.2 Why was this study carried out?

While existence, isolation, identification and the possible role of fungi in deep-sea were established beyond doubt, the persisting question was to know how fungi survive, grow and adapt themselves to this coarse habitat. Proteins are responsible for the maintenance of life, replication, defence and reproduction (Breda et al. 2006). Protein analysis allows a precise understanding of the cell's response to various environmental conditions (Kim et al. 2008). Therefore protein studies could solve the unanswered questions about tolerance of harsh conditions and growth in such an extreme environment.

Usually, proteomic studies on fungi have been restricted to a particular site of action. Some examples are, studies on secretions of heterologous proteins in submerged and solid-state cultures (Iwashita 2002) for purposes, such as checking appropriate conditions for the production of chimeric proteins, i.e., non-native heterologous proteins (Punt et al. 2011); proteomic studies on fungal organelle for industrial applications (De Oliveira and De Graaff 2011); study of proteins involved in rutin degradation (Medina et al. 2005); change

in secretome proteins by changing media (Suarez et al. 2005). Protein domains were also analysed to check for new anti-fungal targets (Barrera et al. 2014). However, studies on integral intracellular proteins at a specific point in time was yet to be reported. As fungi are eukaryotes, employing them for studies provides insight that could be extrapolated to other eukaryotes (Brambl 2009).

1.3 Objectives

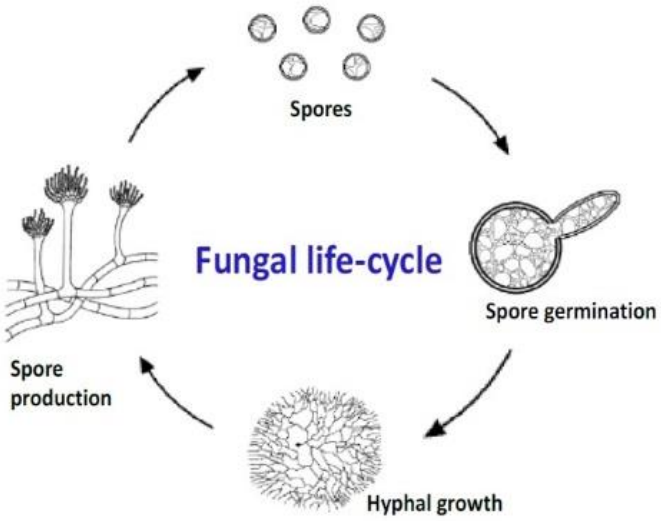
Based on these facts, the objectives of this study were,

1) Optimisation of biomass production by deep-sea fungal isolates at different hydrostatic pressures and low temperature
2) Comparison of protein profiles of deep-sea isolates grown under different hydrostatic pressures and low temperature
3) Identification of proteins expressed under simulated deep-sea condition.

1.4 Outline of the thesis

The fungal life cycle consists of two distinct phases, the mycelium and its growth phase. The spore and its germination phase (Figure 1.2); both these phases were studied for survival strategies in simulated deep-sea conditions.

Figure 1.2: Lifecycle of a fungus



Five fungal cultures were used in this study. One strain of *A. terreus* (#MTCC479 / #IM2) isolated from a terrestrial habitat (obtained from Microbial Type Culture Collection, Chandigarh, India) was included. The four deep-sea fungal isolates used in this study were isolated by Damare et al. 2006 from subseafloor sediments (0–5 cm sections of sediment

core) of the Central Indian Basin (CIB) from the water column depths of 4500–5500 m by dilution plating, particle plating and pressure incubation methods; the samples were incubated for 20–30 days. Dilution plating allowed the isolation of slow-growing microbes after long-term incubation (Jiang et al. 2016), and pressure incubation methods allowed isolation of barotolerant isolates. Of the 181 isolates obtained, *Aspergillus terreus* Thom (#A4636), *Aspergillus flavus* Link (#NIOCC20), *Aspergillus sydowii* Thom and Church (#CH2) and *Penicillium* sp. (#A4623) were selected based on their high abundance in occurrence in the deep subseafloor sediments. *Aspergillus* and *Penicillium* species are globally distributed fungal taxa (Zhang et al. 2014) that displayed abundance in occurrence in the deep subseafloor sediments (Singh et al. 2010, Xu et al. 2014, Jones et al. 2015).

Proteomic studies on mycelial growth were performed for all the five isolates mentioned, whereas proteomic studies on conidial germination were performed for *A. terreus* and *A. flavus* (Table 1.1). The two grounds for this choice were: *A. terreus* and *A. flavus* represented 60% of cultivable isolates obtained. *Aspergillus* genus was most dominant of all the isolates obtained from deep-sea sediments (Damare 2007, Singh 2012); the protein database of *A. terreus* and *A. flavus* had many curated, identified and described proteins as compared to *A. sydowii*. The genus-specific identity of *Penicillium* was discouraging its protein studies on conidial germination. Although competition between microbes exists in all ecosystems, to accurately understand the mechanisms and reason behind the competition, the simplification process is necessary (Melin et al. 2002). Therefore, setting up experimental systems with pure cultures was essential.

Table 1.1: Details of the isolates used in this study

S No	Generic name	Name in experiments	Authorities	Isolated/Obtained from	Mycelial studies	Conidial studies
1	*Aspergillus terreus*	#A4636	Thom 1918	Deep subseafloor sediments (CIB)	✓	✓
2	*Aspergillus terreus* var. *terreus*	#MTCC479	Thom 1918	Culture collection, IMTECH, Chandigarh, India	✓	✓
3	*Aspergillus flavus*	#NIOCC20	Link 1809	Deep subseafloor sediments (CIB)	✓	✓
4	*Penicillium* sp.	#A4623	Link 1809	Deep subseafloor sediments (CIB)	✓	--

S No	Generic name	Name in experiments	Authorities	Isolated/Obtained from	Mycelial studies	Conidial studies
5	*Aspergillus sydowii*	#CH2	Thom and church 1926	Deep subseafloor sediments (CIB)	✓	--

Under each objective, the following studies were planned and performed

Objective 1: Optimisation of biomass production by deep-sea fungal isolates at different hydrostatic pressures and low temperature.

- *Examining the effect of solutions for harvesting conidia and counting of conidia*
- *Optimising incubation period for conidial germination studies*
- *Evaluation of the germination potential of fungal conidia under the simulated deep-sea conditions*
- *Evaluation of the viability of A. terreus (#A4636 and #MTCC479) conidia at low temperatures*
- *Evaluation of the sporulation potential of A. terreus (#A4636, #MTCC 479), A. flavus (#NIOCC20) at 4^{o}C in submerged condition*
- *Optimising conditions for conducting studies on mycelial growth*
- *Finalising the method for growing fungal mycelia under the simulated deep-sea condition*

Objective 2: Comparison of protein profiles of deep-sea isolates grown under different hydrostatic pressures and low temperature

- *Comparison of protein profile obtained from wet and lyophilised biomass*
- *Standardising protein extraction*
- *Checking out the best-suited media for cultivation of fungi*
- *Standardising salt-removal protocols for the extracted protein*
- *Confirmation of finalised methods*
- *Standardising in-gel digestion and in-solution digestion protocols*
- *Analysis of digested protein by LC-MS-QToF*
- *Evaluation of the two-dimensional separation of proteins.*

Objective 3: Identification of proteins expressed under the simulated deep-sea condition

- *Generation of phylogenetic trees to finalise the protID for species-specific database search*
- *Processing the spectral data with data extractor*
- *Species-specific MS/MS search in NCBI and Swiss-Prot databases*
- *Autovalidation of the generated spectra*
- *Generation of peptide and protein lists*
- *Statistical analysis to check for the regulation of proteins*
- *Checking for the placement of commonly regulated proteins in the KEGG pathway database*

Notes

1) All medium used for growing fungi, in this study, were prepared in sea-water obtained from CIB.
2) High-pressure incubations were carried out in tailor-made autoclavable plastic pouches.
3) Mycelial studies were conducted before fungal mats sporulated. The growth phase of mats was verified by viewing under a microscope. Analysing sporulated mycelial mat for protein study would provide details of proteins involved in sporulation, which was not within the ambit of this study. Isolates used in this study are not known to sporulate under submerged conditions.
4) Standardisation/optimisation experiments that dealt with biomass were carried out with two or three strains due to physical limitations in the availability of pressure cultivation chambers. Based on the first set of results, the setup was repeated for all strains for uniformity as well as to confirm the outcome.
5) After incubation, biomass (mycelia and spores) was harvested by filtration in 0.45 µm filter papers.
6) Biomass was washed thoroughly in distilled water (at least three times) to remove remnants of salt and media.
7) Collected biomass was stored at –80^{o}C, was desiccated in freeze-dryer, within 8 hours post-harvest.
8) Nano-pure water preparation: Obtaining fresh water at 18.5 mΩ at 25^{o}C, filtered on 0.22 µm filter paper, autoclaved (15 psi, 121^{o}C, 20 minutes). The storage unit containing this water was opened only at sterile conditions (LAF). Gloves and forceps were used for preparation.
9) Chemicals used for protein work were of MS grade only; work was always carried out in sterile conditions (LAF) with the utmost care
10) Buffers (such as running buffers for electrophoresis) were always prepared fresh.
11) Stock solutions (1M Tris-HCl (pH 8.0), 10 X electrophoresis buffers, etc.) were made in bulk (10 L, where ever applicable) for dilution.
12) Resolving and stacking buffers of SDS PAGE were prepared in large volume (3 L) for uniformity and consistency, aliquoted (in 50 ml tubes) and were stored in -20^{o}C. pH was regularly checked.
13) Molecular and microbiology work had separate sets of regularly calibrated micropipettes.

14) DNase-RNase-protease free microfuge tubes were used for proteomic work. These were used directly without autoclaving.

15) Master mix was prepared (e.g., PCR) where ever it was applicable for uniformity.

16) Proteome work was always carried in ice/low temperature (4^{o}C).

Chapter TWO

OPTIMISATION OF BIOMASS PRODUCTION BY DEEP-SEA FUNGAL ISOLATES AT DIFFERENT HYDROSTATIC PRESSURES AND LOW TEMPERATURE

Chapter 2 – Objective 1

Optimisation of biomass production by deep-sea fungal isolates at different hydrostatic pressures and low temperature

The deep-sea ecosystem is the environment with high pressures and low temperatures (Danovaro et al. 2014), characterising drastic reduction in metabolic activities (Dover 2000), harbouring diverse fungal communities (Nagano and Nagahama 2012) adapted to such environment. Deep-sea sediments play a significant role in organic carbon turnover, where fungi may persist as spores (Zhang et al. 2014) and mycelia.

Fungal spores not only remain dormant (Dango et al. 2011) but also lose viability at low temperatures (Damare et al. 2008, Singh et al. 2010). When directly observed under a microscope, along with fungal mycelia, a few fungal spores were seen in deep-sea sediments (Damare 2007) though they did not display germination. As fungi reported in this study are not known to sporulate under submerged conditions, the presence of fungal spores in deep-sea could be attributed to drifting of spores by wind (Smith et al. 1996) and sedimentation of spores by terrestrial run-offs (Lorenz and Molitoris 1997). There is no universal mechanism for controlling germination of spores, but germination could be initiated by a variety of physical and chemical additives (Cochrane 1974, Ruan et al. 1995, Damare et al. 2006). Hydrothermal vents, marine snow and sinking dead materials could be the possible sources of physical and chemical additives in the deep-sea environment (Haymon 2005, Tivey 2007, Smith et al. 2015, Bilenker et al. 2016). While dead material and marine snow are organic sources, inorganic acids (Bilenker et al. 2016) occurs in spots of deep-sea mining. It is essential to know if there is any change in a total number of germinating conidia in simulated deep-sea condition (high hydrostatic pressures and low temperature) by varying incubation time in presence/absence of external parameters such as additives.

Presence of microbial biomass indicates the role played by microbes in an ecosystem (Graham et al. 2016). Investigation of fungal spore germination and mycelial growth can be the initial steps in understanding the presence of fungi and role played by them in nutrient dynamics in the deep-sea ecosystem (Damare et al. 2008). For this, it was necessary to quantify the period required for conidial germination in simulated deep-sea conditions in the laboratory. While it is not possible to exactly replicate an ecosystem in the laboratory, *in*

vitro conditions can be provided. The fungal isolates studied were isolated from depths of 4500–5500 m. Therefore the upper limit for studying the effect of pressure was 60 MPa.

In the previous studies, the growth of filamentous fungi was evaluated at an elevated pressure of 20 MPa for 20 days at 30°C (Damare 2007) and 15 days in 4°C (Singh 2012). The growth of yeasts was recorded in 20 MPa at 30°C and 4°C; germination of spores was checked at 0.1, 20, 30 MPa at 30°C, 4°C and 40, 50 MPa at 30°C (Singh 2012). These studies confirmed the presence of fungi in deep-sea sediments and described the fungal diversity of the sampled region, the CIB. Also, the effects of elevated pressures, nutrient concentration, low temperature, time-period and additives on the deep-sea and terrestrial isolates were described.

For the study of survival and adaptation strategies of fungi in simulated deep-sea conditions, it was essential to standardise conditions and methods that would produce sufficient biomass required for protein analysis. Production of biomass from extremophiles has always been a challenge (Schiraldi and Rosa 2002) which hinders the extrapolation of laboratory findings into pilot scale tests. Additives were added and was evaluated for an increase in biomass yields. Based on the observations of the initial set of experiments, few more experiments were conducted to verify or validate the results obtained.

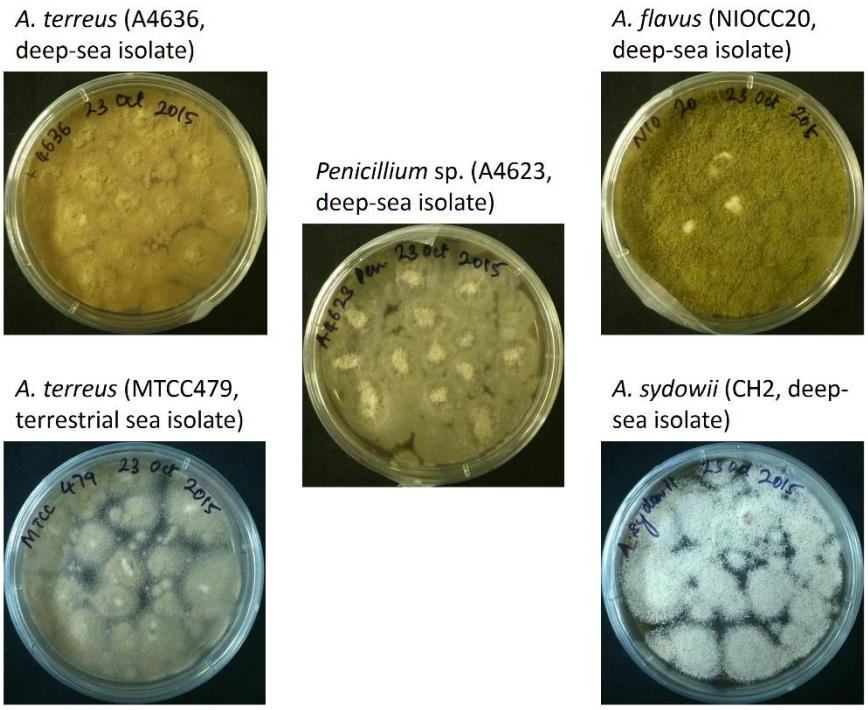
The experiments carried out were:

- *Examining the effect of solutions for harvesting conidia and counting of conidia*
- *Optimising incubation period for conidial germination studies*
- *Evaluation of the germination potential of fungal conidia under the simulated deep-sea conditions*
- *Evaluation of the viability of A. terreus (#A4636 and #MTCC479) conidia at low temperatures*
- *Evaluation of the sporulation potential of A. terreus (#A4636, #MTCC479), A. flavus (#NIOCC20) at 4°C in submerged condition*
- *Optimising conditions for conducting studies on mycelial growth*
- *Finalising the method for growing fungal mycelia under the simulated deep-sea condition*

The five isolates that were used for the study are shown in Figure 2.1. This image was captured after sporulation. The isolates sporulated within three days of incubation at

room temperature and atmospheric pressure. The isolates were grown on 1/5th Malt Extract Broth (MEB). Seawater obtained from CIB was used to prepare the medium.

Figure 2.1: Sporulated images of the five isolates used in this study



2.1 Examining the effect of solutions for harvesting conidia and counting of conidia

On the deep-sea isolate of *A. terreus* (#A4636), various solutions for harvesting conidia were tried. On finalising the solution to harvest conidia, the count of harvested conidia was done on a hemocytometer. This procedure was manual and involved pipetting conidial suspensions and counting. Therefore the standard deviation value was determined. This experiment was performed only for the three isolates (#A4636, #MTCC479, #NIOCC20) that were studied for proteins involved in conidial germination under simulated deep-sea conditions.

Materials and method:

#A4636 was grown on one-fifth strength malt extract agar (MEA), at room temperature (30^{o}C) and atmospheric pressure (0.1 MPa) until visible sporulation occurred (36–48 h). Conidia were harvested in 100% ethanol, 70% ethanol, distilled water, sea water and 0.1% tween 80 (prepared in seawater obtained from CIB) by flooding the plates. The conidial suspensions were observed under a microscope. The viability of the harvested conidia was checked by spread-plating 100 µl from each harvest on one-fifth strength MEA plates. The plates were incubated at room temperature and atmospheric pressure for three days.

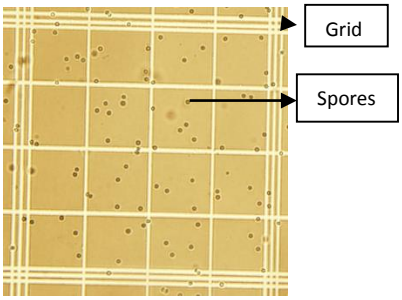
For determining the count of harvested conidia, a 100 µl of the conidial suspension was placed on a hemocytometer, spread over the gratings using coverslip. The total number of conidia were counted in each of the 25 boxes. Counting all conidia in 25 boxes was performed in replicates of 10; mean and standard deviation value for the conidial count was noted down.

Results:

Conidia suspended in all five solutions: absolute and 70% ethanol, distilled, and seawater, and 0.1% tween 80 did not display morphological abnormality when viewed under a light microscope. Harvesting conidia in 0.1% tween 80 were the easiest, followed by harvesting in alcohol suspensions. It was difficult to harvest conidia in distilled water and seawater as these conidia were hydrophobic. Harvesting of conidia in distilled and seawater required either tapping the sporulated plates (which was not advisable as *A. terreus* is an opportunistic pathogen) or scrapping the plates with a glass spreader. While scrapping conidia with a glass spreader, some mycelial filaments were also scrapped. Harvesting mycelium was undesirable. Conidia harvested in alcohols did not show viability on spread-

plating. Conidia harvested in distilled water, sea water and 0.1% tween 80 showed viability. Conidia in 0.1% tween 80 were evenly spread without clumping. Therefore, 0.1% tween 80 was used to harvest spores/conidia for all the further studies. Figure 2.2 shows conidia of *A. terreus* harvested in 0.1% tween 80 spread on a haemocytometer.

Figure 2.2: Conidia of *A. terreus* (#A4636) harvested in 0.1% tween 80 spread on a haemocytometer (100 µl). (at 40X magnification)



The mean value of the count of conidia was 30–50 x 10^6 conidia ml^{-1}. Standard deviation values have been tabulated (Table 2.1).

Table 2.1: Standard deviation values for conidial count

Organism	**Standard Deviation**
Aspergillus terreus (#A4636)	±16.89
Aspergillus terreus (#MTCC 479)	± 6.66
Aspergillus flavus (#NIOCC20)	± 2.18

Close standard deviation values imply that mean/average is tightly spaced. From result, the count for #A4636 is not as tightly spaced as it was for #MTCC479 and #NIOCC20. However, as chances for mechanisation of this experimentation was nil, the standard deviation of the spore/conidia count was performed for all experiments involving studies on germination of conidia and applied where ever it was deemed necessary.

2.2 Optimising incubation period for conidial germination studies

A. terreus isolates (#A4636 and #MTCC479) were chosen for optimising the incubation period.

Materials and method:

#A4636 and #MTCC479 were grown on one-fifth strength MEA at room temperature (30^{o}C) and atmospheric pressure (0.1 MPa) until visible sporulation occurred (36–48 h). These agar plates were flooded with 0.1% tween 80 to harvest conidia. The concentration of conidia in these conidial suspensions were determined by a haemocytometer. Harvested conidia were serially diluted with 0.1% tween 80 solutions to obtain a count of 30–50 x 10^{6} conidia ml^{-1} to be used as the inocula. The conidial suspensions were vortexed to reduce clumping of conidia before enumeration and inoculation. For each enumeration, 100 µl of the conidial suspension was used. The counting was carried out ten times for each conidial suspension, and the average values were reported. The conidial suspensions were placed on ice during inoculation.

For each isolate, a total of 72 plastic pouches were prepared for inoculations and incubations. These 72 pouches were categorised into six sets with each set having 12 pouches (Figure 2.3).

Figure 2.3: Details of inoculations with inducers, for one isolate, at one temperature and one pressure

Legend: *In denoted inocula; MEB denoted 1/5th MEB. Ht denoted heat treatment at 70^{o}C for 60 min. T denoted thiamine. NaP denoted sodium pyruvate. S denoted sucrose. H_2SO_4 denoted sulphuric acid. HCl denoted hydrochloric acid.*

Heat treated (70°C, 1 h) samples	Samples that lacked heat treatment
Pouch 1 (with thiamine): In + MEB + T + Ht	Pouch 7 (with thiamine): In + MEB + T
Pouch 2 (with sodium pyruvate): In + MEB + NaP + Ht	Pouch 8 (with sodium pyruvate): In + MEB + NaP
Pouch 3 (with sucrose): In + MEB + S + Ht	Pouch 9 (with sucrose): In + MEB + S
Pouch 4 (with sulphuric acid): In + MEB + H_2SO_4 + Ht	Pouch 10 (with sulphuric acid): In + MEB + H_2SO_4
Pouch 5 (with hydrochloric acid): In + MEB + HCl + Ht	Pouch 11 (with hydrochloric acid): In + MEB + HCl
Pouch 6 (control, heat treated): In + MEB + Ht	Pouch 12 (control): In + MEB

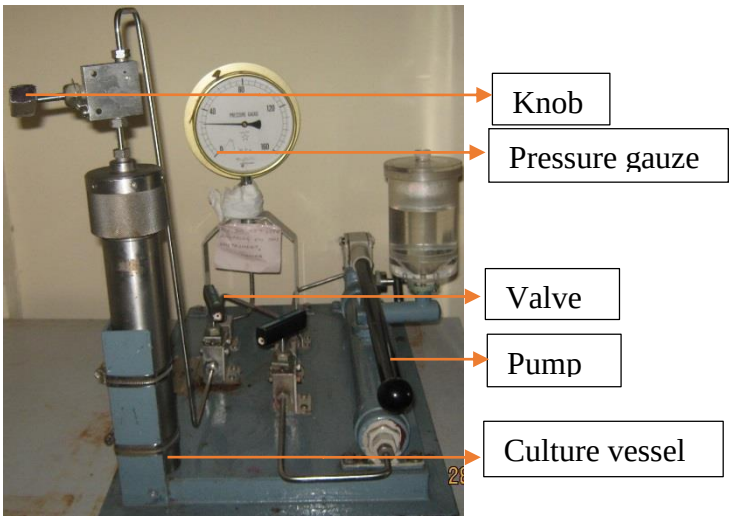
Each pouch had a final volume of 1 ml incubate; 0.8 ml basal medium and 0.1 ml of an additive and 0.1 ml of conidial suspension. Pouches that lacked chemical inducers had 0.9 ml basal medium and 0.1 ml of conidial suspension. Pouches contained conidia with a final concentration of 30–50 x 10^5 conidia ml^{-1}, $1/5^{th}$ strength of MEB and chemical inducers. The chemical inducers (filtered with 0.22µm pore size filter) and their final concentrations used in this study were: hydrochloric acid and sulphuric acid at 0.02 N (Lewis et al. 1991), sodium pyruvate at 0.1 M (Laere et al. 1982), thiamine at 0.1 mg L^{-1} (Siqueira et al. 1982) and sucrose at 4 g L^{-1} (Damare et al. 2008).

Details of the contents (that constituted 1 ml volume) of the pouches for each set (of 12 pouches) were as follows: conidia (final concentration as 30–50 x 10^5 conidia ml^{-1}) and $1/5^{th}$ strength MEB were common to all the pouches; thiamine was added to pouch 1 and pouch 7; sodium pyruvate to pouch 2 and pouch 8; sucrose to pouch 3 and pouch 9; sulphuric acid to pouch 4 and pouch 10; hydrochloric acid to pouch 5 and pouch 11; no chemical inducers were added to pouch 6 and pouch 12.

While preparing the pouches, care was taken not to introduce air gap as the bags might rupture during de-pressurisation because of air bubbles. In a previous study (Damare et al. 2008), heat treatment as germination trigger was provided for 15 min at 50, 60, 80, and 100°C. Pouches 1–6, with incubates, were placed at 70°C for 60 min. Pouches 7–12, without heat treatment, served as the control group for pouches 1–6. After heat treatment, from the six sets of pouches for each isolate, one set of the pouch was incubated at 0.1 and 10, 20, 30, 40 MPa elevated hydrostatic pressures respectively at 4°C. For elevated pressure incubation, pressure cultivation chambers (Tsurumi & Seiki Co., Japan) were used (Figure 2.4). Conidia were also incubated at 0.1 MPa at room temperature (30°C) to check for their viability and germination potential. This study was carried out for 5, 8, 10, 15 and 30 days.

After the incubation period was over, the plastic pouches were cut open, and the content was mixed with calcofluor to stain the conidia for visualisation (Raghukumar and Raghukumar 1998). The suspension was observed under an ultraviolet light filter (excitation wavelength 330–385 nm and barrier filter BA 420) on an epifluorescence microscope. The total number and the germinated conidia were counted, and the percentage of germinated conidia was calculated. Three subsamples from each pouch were counted to obtain an average of the counts. Each counting was carried out by pipetting 100 µl from the harvested incubate.

Figure 2.4: High-pressure cultivation chamber (Tsurumi & Seiki Co., Japan)



Results:

Both strains of *A. terreus* incubated at 30ºC, and 0.1 MPa had conidia germinating after 15–18 h incubation**.** For both the strains of *A. terreus* (#A4636 and #MTCC479), at 4ºC, no germinated conidia were observed in all the pressure conditions (0.1, 10, 20, 30 and 40 MPa) after five and eight-day incubation.

Ten-day incubation at 4ºC had swollen conidia at 30 MPa (less than 5%, only for #MTCC479, in the control condition that lacked any inducers). By the end of 12-day incubation at 0.1 MPa and 4ºC, 10% conidia of both the isolates had germinated. Fifteen-day incubation at 4ºC had less than 10% germinating conidia only with heat treatment in the presence of inorganic acids at 30 and 40 MPa for both the isolates.

Thirty-day incubation at 4ºC had germination in a total of six different conditions for both #A4636 and #MTCC479. Heat-hydrochloric acid treated conidial suspension was the only condition where both the strains had germinated conidia at 30 MPa.

For #A4636, 11% conidia were germinating in heat treated pouch conidial suspension in 1/5th strength MEB and 14% conidia germinated in sucrose-treated conidial suspension at 10 MPa; 22% conidia were germinating in heat-sodium pyruvate treated conidial suspension at 20 MPa; 19% conidia were germinating in heat-hydrochloric acid treated conidial suspension and 92% conidia were germinating in sodium pyruvate treated conidial suspension at 30 MPa; 23% conidia were germinating in heat-treated conidial suspension in basal medium at 40 MPa.

For #MTCC479, 22% conidia were germinating in heat-thiamine treated conidial suspension and 9% conidia germinated in heat-hydrochloric acid treated conidial suspension at 10 MPa; 25% conidia were germinating in heat-sulphuric acid treated conidial suspension at 20 MPa; 21% conidia were germinating in heat-hydrochloric acid treated conidial suspension at 30 MPa; 8% conidia were germinating in thiamine treated conidial suspension at 30 MPa, and 26% conidia were germinating in sucrose-treated conidial suspension at 40 MPa.

In rest of the treatment conditions, germinating conidia were not observed. From these results, it was concluded to incubate conidia/spores of all five is for a minimum of 30 days at elevated pressures and low temperature to evaluate their germination potential.

2.3 Evaluation of the germination potential of fungal conidia under the simulated deep-sea conditions

Materials and methods:

As mention above, the exact procedure, from the preparation of conidial suspension to enumeration after incubation was carried out for conidia of all the five isolates. Samples were incubated for 30 days in 480 plastic pouches (96 plastic pouches for each isolate, categorised into eight sets with each set having 12 pouches) at 0.1 and 10, 20, 30, 40, 50 and 60 MPa elevated hydrostatic pressures at 4°C. Conidia of all the five isolates were also incubated at 0.1 MPa at room temperature (30°C). After 30 days incubation, the samples were stained with calcofluor, visualised and counted.

Factorial analysis of variance (ANOVA) was used to investigate the statistical significance of the different treatments of inducers for the pressure incubated conidia. Newman-keul's test (McHugh 2011) was performed with probabilities for post hoc tests (Statistica 6.0)

Results:

For all the isolates, conidia germinated at 0.1 MPa at room temperature (30°C) in less than 24 hours and developed a thick mycelial mat by the end of 30-day incubation. Conidia in pouches incubated at 4°C had germinated conidia from the 12th day and developed slender mycelial filaments by the end of 30-day incubation. Morphologically, there was no difference between the germinating conidia exposed to simulated deep-sea conditions and those exposed to 0.1 MPa at 4°C and 30°C (Figures 2.5 and 2.6). However, mycelia that had developed in simulated deep-sea had an abnormality in the morphological feature such as bloating (Figure 2.7). Forty MPa was observed as the upper limit for conidial germination for a 30-day incubation.

Figure 2.5: *A. terreus* (#A4636) conidia with 100% germination at 40 MPa, after heat treated, in hydrochloric acid and basal medium, stained with calcofluor and viewed in both UV and visible light (at 40X magnification). Legend: 1 - Germinating conidia

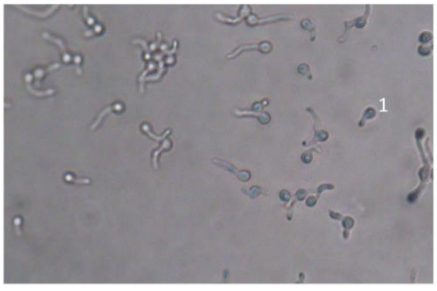


Figure 2.6: *A. terreus* (#MTCC479) conidia grown under normal conditions of temperature (30°C) and pressure (0.1 MPa), stained with calcofluor, showing mycelia and germinating conidia (at 40X magnification). Legend: 1 - Germinating conidia; 2 - Mycelium grown at 0.1 MPa at 30°C.

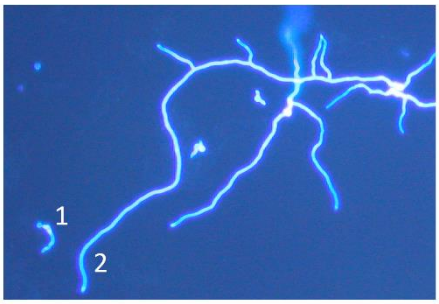
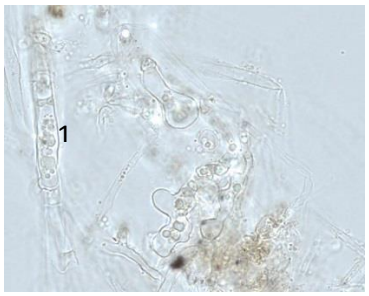


Figure 2.7: Bloated mycelium developed by conidia of *Penicillium* sp. (#A4623) at the end of 30-day incubation at 10 MPa, in control set (absence of both physical and chemical inducers) (at 100X magnification, oil immersion). Legend: 1 - Bloated mycelia



At 4°C, for both isolates of *A. terreus*, over 50% of conidia germinated in some of the pressure pouches. For the deep-sea isolate #A4636, the conidia germinated in heat-hydrochloric acid treated conidial suspension at 40 MPa; in sodium pyruvate treated conidial suspension at 30 MPa; in sucrose-treated conidial suspension and sulphuric acid treated conidial suspensions at 40 MPa. For the terrestrial isolate #MTCC479, conidia germinated in heat-hydrochloric acid treated conidial suspension at 10 MPa and 40 MPa; in heat-sulphuric acid treated conidial suspension at 30 MPa; in sucrose-treated conidial suspension and sulphuric acid treated conidial suspension at 30 MPa, in sulphuric acid treated conidial suspension at 40 MPa. No germination or a low germination percentage (< 25%) was observed for the rest of the additives for *A. terreus* (Table 2.2).

On the contrary, at 4°C, the conidia of *A. flavus*, *Penicillium* sp. and *A. sydowii* had almost 100% germination under all four incubation pressures except a few conditions that had less than 33% germination. For *A. flavus*, 21% conidia germinated in heat-sodium

pyruvate treated conidial suspension at 30 MPa; there were no germinating conidia in two cases: in 1/5th MEB at 10 MPa and in heat-sulphuric acid treated conidial suspension at 30 MPa. For *Penicillium* sp. 30% conidia germinated in heat-thiamine treated conidial suspension at 30 MPa; 18% conidia germinated in heat treated conidial suspension in 1/5th MEB at 40 MPa. For *A. sydowii*, 30% conidia germinated in heat-sucrose treated conidial suspension at 30 MPa; there were no germinating conidia in three cases: in 1/5th MEB conidial suspensions not exposed to heat at 20, 30 and 40 MPa. (Table 2.2)

It was observed that the mycelia developed from conidia not subjected to heat treatment when incubated at 10, 20 and 30 MPa at 4^{o}C. Conidia of *A. flavus* developed into mycelia in sodium pyruvate treated conidial suspension and sucrose treated conidial suspension at 20 MPa. Conidia of *Penicillium* sp. germinated into mycelia in thiamine treated conidial suspension, sodium pyruvate treated conidial suspension, sucrose treated conidial suspension and in 1/5th MEB at 10 MPa; in thiamine treated conidial suspension, in sodium pyruvate treated conidial suspension and in sucrose-treated conidial suspension at 20 and 30 MPa; in hydrochloric acid treated conidial suspension at 30 MPa. Conidia of *A. sydowii* developed into mycelia at 10 MPa in all six treatments of conidial suspension.

No inducer was found to be a stimulant for germination in all the conditions evaluated (Table 2.2). Results of factorial ANOVA with Newman-Keul's test (Table 2.3) showed that there was no statistically significant effect of any inducer on germination.

Table 2.2: Percentage of germination of conidia at different hydrostatic pressures after 30 days incubation at 4^{o}C

Ht indicates heat treatment (70^{o}C for an hour); M indicates visible slender mycelia; conidia were difficult to see through this mycelia; H indicates hyphae growth; –implies no germination

			% germination of conidia					
Pressure (MPa)	**Heat treatment (70^{o}C, one h)**	**Isolate**	**Thiamine (0.1 mg l^{-1})**	**Pyruvate (0.1 M)**	**Sucrose (4 g l^{-1})**	**H_2SO_4 (0.02N)**	**HCl (0.02N)**	**Control**
10	Yes	*A. terreus*(#A4636)	--	--	--	--	--	--
		A. terreus (#MTCC479)	25±1	--	--	--	100	--
		A. flavus (#NIOCC20)	100	100	100	100	100	100
		Penicillium sp. (#A4623)	100	100	100	100	100	100
		A. sydowii (#CH2)	100	100	100	100	100	100
	No	*A. terreus*(#A4636)	9±10.4	6±12.0	--	--	--	--
		A. terreus (#MTCC479)	--	--	--	--	--	--
		A. flavus (#NIOCC20)	100	100	100	100	100	--
		Penicillium sp. (#A4623)	M	M	M	100	100	M
		A. sydowii (#CH2)	M	M	M	M	M	M

Pressure (MPa)	**Heat treatment (70°C, one h)**	**Isolate**	**Thiamine (0.1 mg l^{-1})**	**Pyruvate (0.1 M)**	**Sucrose (4 g l^{-1})**	**H_2SO_4 (0.02N)**	**HCl (0.02N)**	**Control**
20	Yes	*A. terreus*(#A4636)	--	--	--	--	--	--
		A. terreus (#MTCC479)	--	--	9±3.5	--	--	--
		A. flavus (#NIOCC20)	100	100	100	100	100	100
		Penicillium sp. (#A4623)	100	100	100	100	100	100
		A. sydowii (#CH2)	100	100	100	100	100	100
	No	*A. terreus*(#A4636)	--	--	--	--	--	--
		A. terreus (#MTCC479)	--	--	--	--	--	--
		A. flavus (#NIOCC20)	13.69±4.2	M	M	100	100	100
		Penicillium sp. (#A4623)	M	M	M	100	100	100
		A. sydowii (#CH2)	100	100	100	100	100	--
30	Yes	*A. terreus*(#A4636)	--	--	--	--	--	--
		A. terreus (#MTCC479)	--	--	--	100	--	--
		A. flavus (#NIOCC20)	100	21±0.57	100	--	100	100
		Penicillium sp. (#A4623)	30±2	100	--	100	100	100
		A. sydowii (#CH2)	100	100	30±2.5	100	100	100

	Heat treatment (70°C, one h)	**Isolate**	**Thiamine (0.1 mg l^{-1})**	**Pyruvate (0.1 M)**	**Sucrose (4 g l^{-1})**	**H_2SO_4 (0.02N)**	**HCl (0.02N)**	**Control**
30	No	*A. terreus*(#A4636)	--	50±5.8	--	--	--	--
		A. terreus (#MTCC479)	--	--	100	50±7.8	--	--
		A. flavus (#NIOCC20)	100	100	100	100	100	100
		Penicillium sp. (#A4623)	M	M	M	100	M	100
		A. sydowii (#CH2)	100	100	100	100	100	--
40	Yes	*A. terreus*(#A4636)	--	--	--	--	100	--
		A. terreus (#MTCC479)	--	11±9.1	--	25±8.1	50±10.0	--
		A. flavus (#NIOCC20)	100	100	100	100	100	100
		Penicillium sp. (#A4623)	100	100	100	100	100	18±1
		A. sydowii (#CH2)	100	100	100	100	100	100
	No	*A. terreus*(#A4636)	1±4.7	--	100	50±24.5	--	--
		A. terreus (#MTCC479)	--	--	--	100	--	--
		A. flavus (#NIOCC20)	100	100	100	100	100	100
		Penicillium sp. (#A4623)	100	100	100	100	100	100
		A. sydowii (#CH2)	100	100	100	100	100	--

Pressure (MPa)	**Heat treatment (70°C, one h)**	**Isolate**	**Thiamine (0.1 mg l^{-1})**	**Pyruvate (0.1 M)**	**Sucrose (4 g l^{-1})**	**H_2SO_4 (0.02N)**	**HCl (0.02N)**	**Control**
0.1	Yes	*A. terreus*(#A4636)	H	H	H	H	H	H
		A. terreus (#MTCC479)	H	H	H	H	H	H
		A. flavus (#NIOCC20)	H	H	H	H	H	H
		Penicillium sp. (#A4623)	H	H	H	H	H	H
		A. sydowii (#CH2)	H	H	H	H	H	H
	No	*A. terreus*(#A4636)	H	H	H	H	H	H
		A. terreus (#MTCC479)	H	H	H	H	H	H
		A. flavus (#NIOCC20)	H	H	H	H	H	H
		Penicillium sp. (#A4623)	H	H	H	H	H	H
		A. sydowii (#CH2)	H	H	H	H	H	H

Table 2.3: Results of factorial ANOVA (Univariate tests of significance with Sigma - restricted parameterisation) with Newman-Keuls test

Null hypothesis: No inducer plays a pivotal role in triggering germination
With a p-value lesser than 0.01, the hypothesis gets highly significant
All F values mentioned here are lesser than F statistic. Hence the null hypothesis is accepted.

Isolate	**Final F and p-value for two varied parameters (inducer and pressure), in all incubated conditions**	
	F-value (critical)	**p-value**
A. terreus (#A4636)	3.78703	0.000018
A. terreus (#MTCC479)	7.24944	< 0.000000
A. flavus (#NIOCC20)	4.704	0.000001
Penicillium sp. (#A4623)	20.667	< 0.000000
A. sydowii (#CH2)	2.500	0.003634

From the results of Table 2.2, 30-day incubation of conidia in 1/5th MEB, with heat-treatment for conidial suspensions was finalised as the condition to obtain germinating conidia for conducting protein studies on conidial germination.

2.4 Evaluation of *the viability of A. terreus (#A4636 and #MTCC479) conidia at low temperatures*

From the results of "Evaluation of the germination potential of fungal conidia under the simulated deep-sea conditions" study, strains of *A. terreus* were the slowest growers. Therefore, the viability of conidia of these two strains when stored at 4°C was checked. Also, *A. terreus* was industrially essential isolate (Lopez et al. 2004, Kumar et al. 2013, Huang et al. 2014, Farag et al. 2015), and extremophiles have higher probabilities of nurturing novel metabolites (Mosier et al. 2013). Hence, the effect of other low-temperature storages (Freeze dried and stored at 4°C, [ii] –20°C and [iii] Liquid nitrogen treated followed by storage at –80°C.) that would be available in industries was also checked. This study was carried out for 33 days.

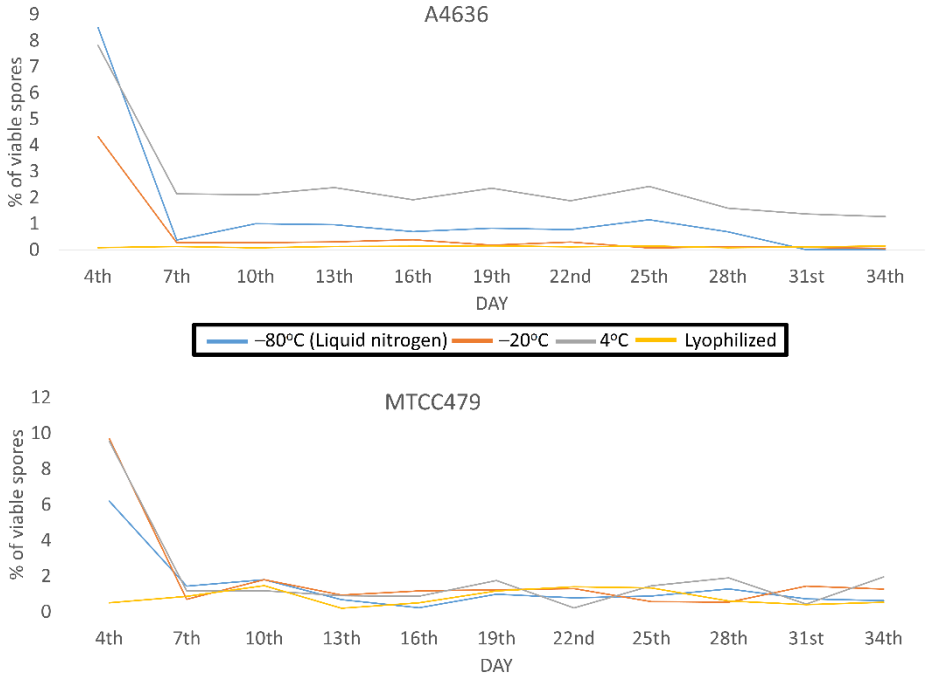
Materials and methods:

A total of 200 vials (100 vials each for #A4636 and #MTCC479), with one ml of conidial suspension (in 0.1% tween 80) of *A. terreus* isolates were stored at four different conditions as follows: [i] flash frozen with liquid nitrogen and stored at –80°C, [ii] stored at –20°C, [iii] stored at 4°C, and [iv] freeze-dried and stored at 4°C. Before subjecting to the different storage treatments, the viability of the conidia was considered 100%. After every three days, i.e. on the 4th day, one sample (for each isolate) was removed from each of the storage condition. The viability of the conidia was checked by spread-plating 100 µl of conidia suspension on MEA (1/5th strength) and by counting the number of colonies produced on the plates. For the freeze-dried samples, 200 µl of 0.1% tween 80 was added, and the dried conidia were re-suspended for a few minutes before 100 µl of this conidial suspension was spread-plated. Each conidium was considered to give rise to one fungal colony and counted as a colony forming unit. This experiment was carried out for 33 days.

Result and discussion:

Conidia of *A. terreus* stored at various low temperatures showed viability on spread-plating on MEA medium (1/5th strength) in the form of colony forming units. For #A4636 and #MTCC479, the percentage of viable conidia dropped steadily, reaching less than 10% at the end of 3-day storage. It can be concluded that only about 0.1–2% conidia retained viability at all low-temperature storages evaluated by the end of 33-day incubation (Figure 2.8)

Figure 2.8: Results of viability studies of conidia of *A. terreus* (#A4636 and #MTCC479) placed at different storage conditions



It is speculated that slow growers may have evolved mechanisms to evade pressure stress.

2.5 Evaluation of the sporulation potential of A. terreus (#A4636, #MTCC479), A. flavus (#NIOCC20) at 4°C in submerged condition

The fungal isolates used in this study was not known to sporulate under submerged conditions. Sporulation potential of *A. terreus* and *A. flavus* in submerged conditions was conducted to assert the claim. Studies of proteins involved in conidial germination were performed only for *A. terreus* and *A. flavus*. Therefore this study was carried out only for the three isolates.

Materials and methods:

The conidial suspension (1 ml, prepared in 0.1% tween 80) of the three isolates were placed individually in a sterile 15 ml microfuge tube with 1/5th MEB as a basal medium (14 ml). The conidial suspension was placed till brim, and the tube was sealed with paraffin before shutting with a lid so that it lacked air. The possibility of an air gap was checked by inverting the tube. The samples were placed horizontally, at a low temperature of 4°C, and was checked periodically for germination of conidia, development of mycelia and sporulation in the submerged state since August 2014.

Result and discussion:

No sporulation was observed in all the three isolates as of October 2017 (Table 2.4). It should also be noted that usually, the lifespan of fungi is very short ranging from a few hours to a month until they sporulate. Therefore, data collected at 202nd and further days of incubation is invalid. From Table 2.4, the fact that these isolates do not sporulate in submerged conditions is reiterated.

Table 2.4: Fate of fungal conidia at 4°C and 0.1 MPa, over a period of 45 days

Incubation days	***A. terreus* (#A4636)**	***A. terreus* (#MTCC479)**	***A. Flavus* (#NIOCC20)**
16 days	Less than 5% germinating conidia		
33 days	Less than 20% germinating conidia. conidia were clumped		
41 days	Thin mycelia	--	Swollen conidia
47 days	Mycelia	Germinating conidia, thin mycelia	
202 days	Dense mat. No sporulation		
518 days	Dense mat. No sporulation		
1122 days	Dense mat. No sporulation (As on 30th Sep 2017)		

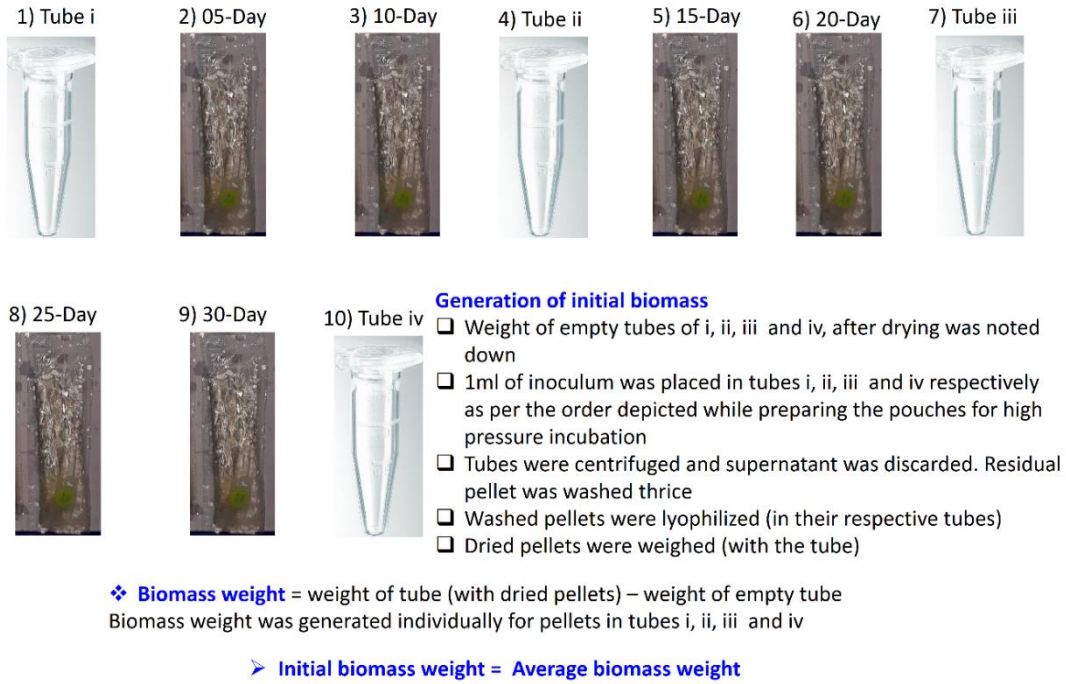
2.6 Optimising conditions for conducting studies on mycelial growth

Materials and methods:

Experiments for optimising incubation period to study mycelial growth was conducted at room temperature (30°C) and 30 MPa. Conidia of *A. terreus* (#A4636 and #MTCC 479) and *A. flavus* (#NIOCC20) were inoculated in 20 ml of 1/5th MEB in 100 ml flasks. Each isolate was grown in 10 flasks to generate a considerable quantity of biomass. The flasks were placed at room temperature until isolates flourished (24–48 h). For each isolate, mycelia from 10 flasks were pulled into a sterile conical flask with glass beads. This mycelium was broken by agitating glass beads (1 mm Ø). An ml of the broken mycelia was used as inoculum.

Plastic pouches with 1/5th MEB was prepared for pressure incubation. Each pouch had nine ml of medium and one ml of inoculum. One ml of the mixture of inoculum was collected by pipetting at regular intervals in oven-dried, pre-weighed microfuge tubes. The procedure of preparing pouches for high-pressure incubation and initial biomass generation, for an isolate, has been depicted in Figure 2.9.

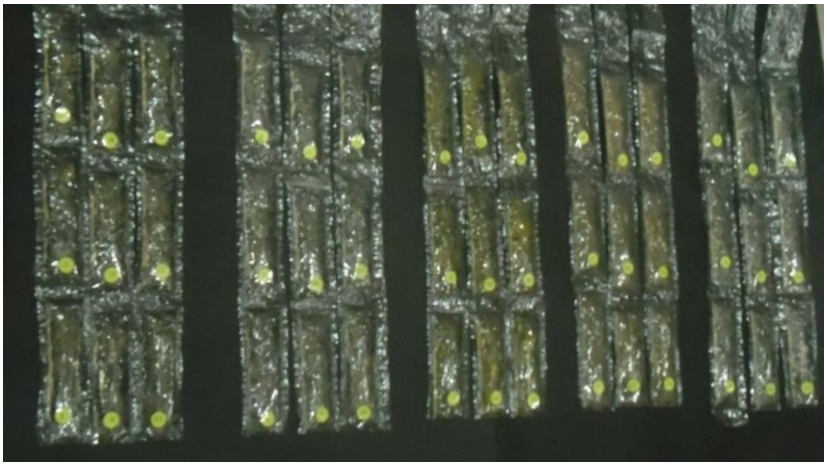
Figure 2.9: Experimental set up for mycelial growth at 30 MPa and 30°C



After preparing the pouches (for all three isolates under study, Figure 2.10) they were incubated at 30 MPa and 30°C. Initial biomass (for each isolate) was noted down on day 0 (before high-pressure cultivation). The biomass generated was monitored every five days. Once the quantity of biomass generated was measured by de-pressurising and drying the

sample, that sample was not re-incubated or used for biomass generation studies. The increase in biomass was calculated by weighing the dried fungal mycelia obtained after incubation.

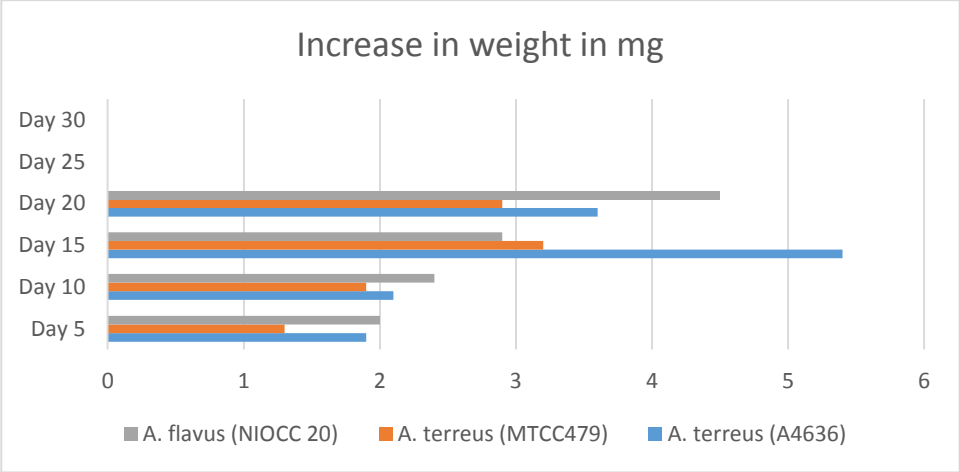
Figure 2.10: Plastic pouches with fungal mycelia (for one isolate)



Results:

The biomass increase in mg has been presented as a graph in the figure below (Figure 2.11). On 25th and 30th-day incubation, there was no increase in biomass for all three isolates.

Figure 2.11: Results of 5, 10, 15, 20, 25 and 30 day incubations at 30 MPa, 30^{o}C



For the three isolates, at some incubation point, the quantity of biomass recorded at incubation completion was lesser than the initial biomass. This phenomenon could either be due to mycelial breakdown during high-pressure incubation (Chisti and Young 1986) or due to fungal lysosomes (Wilson et al. 1970) released from broken mycelia that were used as inocula or due to the loss of mycelial biomass during pipetting, washing and handling of the sample.

2.7 Finalising the method for growing fungal mycelia under the simulated deep-sea condition

Materials and methods:

To overcome the hurdle of loss of biomass as mentioned above, conidia were used as inocula instead of broken mycelia. The procedure followed for obtaining mycelial biomass for protein studies was:

In plastic pouches with 1/5th MEB (9 ml), conidia of individual isolates (1 ml with final concentration as 30–50 x 10^5 conidia ml^{-1}) were inoculated. The inoculated plastic pouches were placed horizontally at room temperature and atmospheric pressure for 24 hours (which allowed conidia to germinate and develop mycelia). After 24 h incubation, the plastic pouches with mycelium were placed in hydrostatic pressure chambers for elevated pressure conditions (10, 20 and 45 MPa) for 30 days (as finalised from the results of spore/conidia germination studies) at a low temperature (4^{o}C) and room temperature (30^{o}C, control set).

After incubation at elevated pressures, mycelium in plastic pouches was harvested by filtering on 0.45 µm nitrocellulllose filter papers, washed with distilled water (to remove remnants of salt and medium), lyophilised and placed at room temperature until further use.

Results:

Each condition had samples cultivated in triplicates. Replicates from each condition was harvested, dried and weighed in different filter papers. This procedure was done to obtain the average increase in biomass yield at a particular growth condition. For protein studies, biomass from each triplicate sets was pulled together as a single unit. Elevated pressure conditions above 40 MPa was detrimental to the growth of mycelia from germinating conidia (as observed in conidia germination experiments). Hence, 45 MPa was set as an upper limit for mycelial biomass cultivation as the growth of mycelia was very slow at 40 MPa (Krishnaswamy and Damare, unpublished results). Also, these deep-sea isolates came from a minimum depth of 4500 m.

Summary of chapter 2

For objective one, "Optimisation of biomass production by deep-sea fungal isolates at different hydrostatic pressures and low temperature" the details and conclusion on the conditions finalised for cultivating fungi were:

- ***Examining the effect of solutions for harvesting conidia***

0.1% tween 80 was found to be the best to harvest conidia. The percentage error during conidial counting of *A. terreus* (#A4636, #MTCC479), and *A. flavus* (#NIOCC20) was ± 16.89, ± 6.66 and ± 2.18 respectively.

- ***Optimising incubation period for conidia germination studies***

Period: 30 days (5, 8, 10, 15 and 30-day incubations were checked).

Pressures: 10, 20 and 30 MPa for conidia (0.1, 40, 50 and 60 MPa were checked).

Temperature: 4^{o}C and 30^{o}C (as control for samples cultivated at 4^{o}C).

Additives: Heat treatment for conidial germination studies (at 70 ^{o}C, for one h). Pouches were also maintained without heat treatment as a control for samples sets that were heat treated

Inoculum: Conidia were suspended in 0.1% tween 80.

- ***Examining the germination potential of fungal conidia under the simulated deep-sea conditions***

Conidia of all five isolates germinated at 0.1 MPa and 30^{o}C within 24 h; and at 0.1, 10, 20, 30 and 40 MPa at 4^{o}C after a 30-day incubation.

- ***Examining the viability of A. terreus (#A4636 and #MTCC479) conidia at low temperatures***

The percentage of viable conidia decreased steadily and dropped to less than 10% by the end of the third day in all low-temperature storages. Less than 0.1–2 % conidia of both the strains retained viability by the end of 33-days in the four low-temperature storages tested ([i] flash frozen with liquid nitrogen and stored at –80^{o}C, [ii] stored at –20^{o}C, [iii] stored at 4^{o}C, and [iv] freeze-dried and stored at 4^{o}C).

- ***Evaluation of the sporulation potential of A. terreus (#A4636, #MTCC479), A. flavus (#NIOCC20) at 4^{o}C in submerged condition***

None of the isolates sporulated in submerged conditions in the 47-day study.

- ***Optimising conditions for conducting studies on mycelial growth***

This study was inconclusive as biomass was lost during experimentation. The study was conducted at 5, 10, 15, 20, 25 and 30-day incubations at 30 MPa, 30^{o}C.

- ***Finalising the method for growing fungal mycelia under the simulated deep-sea condition***

Period: 30 days

Pressures: 0.1, 10, 20 and 45 MPa (was also checked).

Temperature: 4^{o}C and 30^{o}C (as control for 4^{o}C).

Inoculum: Conidia were incubated in basal medium (1/5th MEB), incubated at optimal conditions (0.1 MPa and 30^{o}C) for 24 h and were allowed to develop mycelia (for mycelial growth studies). After mycelia developed (by the end of 24 h incubation), the samples were placed under elevated pressures

Conidia incubated in 0.1 MPa, 4^{o}C germinated and developed mycelia by the end of 30-day incubation. So proteins from ungerminated/dormant conidia were used as the control for protein studies on germinating conidia. Although additives did not play any role in improving germination percentage for a 30-day incubation, heat treatment was retained to check if chaperons (heat shock proteins) were produced only in the presence of heat treatment. The entire set of biomass that was generated was lyophilised and stored at room temperature before protein studies commenced. Based on the results for the optimisations of conditions for biomass generation, a total of 79 samples were processed for protein analysis. The details of the samples have been presented in Tables 2.5 and 2.6.

Table 2.5: Details on samples of mycelia that were cultivated for protein studies

Sample number	Mycelia_isolate	Temperature	Pressure (MPa)
1	*A. terreus*_A4636	30°C	0.1
2	*A. terreus*_MTCC479	30°C	0.1
3	*A. flavus*_NIOCC20	30°C	0.1
4	*Penicillium* sp_A4623	30°C	0.1
5	*A. sydowii*_CH2	30°C	0.1
6	*A. terreus*_A4636	30°C	10
7	*A. terreus*_MTCC479	30°C	10
8	*A. flavus*_NIOCC20	30°C	10
9	*Penicillium* sp_A4623	30°C	10
10	*A. sydowii*_CH2	30°C	10
11	*A. terreus*_A4636	30°C	20
12	*A. terreus*_MTCC479	30°C	20
13	*A. flavus*_NIOCC20	30°C	20
14	*Penicillium* sp_A4623	30°C	20
15	*A. sydowii*_CH2	30°C	20
16	*A. terreus*_A4636	30°C	45
17	*A. terreus*_MTCC479	30°C	45
18	*A. flavus*_NIOCC20	30°C	45
19	*Penicillium* sp_A4623	30°C	45
20	*A. sydowii*_CH2	30°C	45
21	*A. terreus*_A4636	4°C	0.1
22	*A. terreus*_MTCC479	4°C	0.1
23	*A. flavus*_NIOCC20	4°C	0.1
24	*Penicillium* sp_A4623	4°C	0.1
25	*A. sydowii*_CH2	4°C	0.1

Sample number	Mycelia_isolate	Temperature	Pressure (MPa)
26	*A. terreus*_A4636	4^{o}C	10
27	*A. terreus*_MTCC479	4^{o}C	10
28	*A. flavus*_NIOCC20	4^{o}C	10
30	*A. sydowii*_CH2	4^{o}C	10
31	*A. terreus*_A4636	4^{o}C	20
32	*A. terreus*_MTCC479	4^{o}C	20
33	*A. flavus*_NIOCC20	4^{o}C	20
34	*Penicillium* sp_A4623	4^{o}C	20
35	*A. sydowii*_CH2	4^{o}C	20
36	*A. terreus*_A4636	4^{o}C	45
37	*A. terreus*_MTCC479	4^{o}C	45
38	*A. flavus*_NIOCC20	4^{o}C	45
39	*Penicillium* sp_A4623	4^{o}C	45
40	*A. sydowii*_CH2	4^{o}C	45

Table 2.6: Details on samples of conidia that were cultivated for protein studies

NA-Not applicable; Heat treatment (70^{o}C for 60 mins)

Sample number	Spore_Isolate	Temperature	Pressure (MPa)	Heat treatment
41	*A. terreus*_A4636 (Ungerminated)	NA	NA	NA
42	*A. terreus*_MTCC479 (Ungerminated)	NA	NA	NA
43	*A. flavus*_NIOCC20 (Ungerminated)	NA	NA	NA
44	*A. terreus*_A4636	4^{o}C	10	Yes
45	*A. terreus*_MTCC479	4^{o}C	10	Yes
46	*A. flavus*_NIOCC20	4^{o}C	10	Yes
47	*A. terreus*_A4636	4^{o}C	20	Yes

Sample number	Spore_Isolate	Temperature	Pressure (MPa)	Heat treatment
48	*A. terreus*_MTCC479	4ºC	20	Yes
49	*A. flavus*_NIOCC20	4ºC	20	Yes
50	*A. terreus*_A4636	4ºC	30	Yes
51	*A. terreus*_MTCC479	4ºC	30	Yes
52	*A. flavus*_NIOCC20	4ºC	30	Yes
53	*A. terreus*_A4636	4ºC	10	No
54	*A. terreus*_MTCC479	4ºC	10	No
55	*A. flavus*_NIOCC20	4ºC	10	No
56	*A. terreus*_A4636	4ºC	20	No
57	*A. terreus*_MTCC479	4ºC	20	No
58	*A. flavus*_NIOCC20	4ºC	20	No
59	*A. terreus*_A4636	4ºC	30	No
60	*A. terreus*_MTCC479	4ºC	30	No
61	*A. flavus*_NIOCC20	4ºC	30	No
62	*A. terreus*_A4636	30ºC	10	Yes
63	*A. terreus*_MTCC479	30ºC	10	Yes
64	*A. flavus*_NIOCC20	30ºC	10	Yes
65	*A. terreus*_A4636	30ºC	20	Yes
66	*A. terreus*_MTCC479	30ºC	20	Yes
67	*A. flavus*_NIOCC20	30ºC	20	Yes
68	*A. terreus*_A4636	30ºC	30	Yes
69	*A. terreus*_MTCC479	30ºC	30	Yes
70	*A. flavus*_NIOCC20	30ºC	30	Yes
71	*A. terreus*_A4636	30ºC	10	No
72	*A. terreus*_MTCC479	30ºC	10	No
73	*A. flavus*_NIOCC20	30ºC	10	No
74	*A. terreus*_A4636	30ºC	20	No

Sample number	Spore_Isolate	Temperature	Pressure (MPa)	Heat treatment
75	*A. terreus*_MTCC479	30°C	20	No
76	*A. flavus*_NIOCC20	30°C	20	No
77	*A. terreus*_A4636	30°C	30	No
78	*A. terreus*_MTCC479	30°C	30	No
79	*A. flavus*_NIOCC20	30°C	30	No

Each of the 79 samples was cultivated in triplicates, harvested, lyophilised, weighed, pulled together and stored at room temperature for protein analysis.

Prelude to chapter 3

Details of standardising the protein extraction methods would be discussed. The focus of standardisation was to extract maximum proteins and also to obtain a good protein profile on polyacrylamide gels. After the method was finalised, proteins were extracted from these 79 samples and analysed.

Chapter THREE

COMPARISON OF PROTEIN PROFILES OF DEEP-SEA ISOLATES GROWN UNDER DIFFERENT HYDROSTATIC PRESSURES AND LOW TEMPERATURE

Chapter 3 – Objective 2

Comparison of protein profiles of deep-sea isolates grown under different hydrostatic pressures and low temperature

Extraction, identification and classification of proteins are essential not only to ascertain the species-specific presence of a protein moiety but also for harnessing functional properties. Studying the alterations occurring at the proteomic level is indispensable for recognising the changes that occur at different ecologies. In filamentous fungi, protein extraction has been challenging due to chitinous cell-wall. Traditionally, liquid nitrogen was the medium of choice to lyse fungal hyphae for extracting proteins. However, it was an inefficient choice to lyse cells when biomass was less than a gram (Gibbons et al. 2014, Leite et al. 2012).

Homogenisation/lysis using various kinds of beads in a bead beater is fast gaining popularity as an alternative to traditional techniques of lysing the cells. In this method, the type and the size of the beads used for extraction are important while standardising parameters for molecular extraction (Gibbons et al. 2014). Constituents of the buffer used during lysing maintain not only the stability of extracted molecules (Urbansky and Schock 2000) but also solubility. There are numerous methods to classify proteins, based on their source, shape, composition, biological function and solubility; based on solubility, they can be classified as either water soluble or water insoluble proteins (Jain 2005). It is necessary to design extraction buffer that would extract both water-soluble and water-insoluble proteins.

The extraction of proteins, in general, could be in single step, where only one buffer is used, or it could be multi-step where more than one buffer is used. The single buffer system is designed either to get water soluble or water insoluble (lipid soluble) proteins (Sattayasai 2012) and in some cases, to extract both the types of proteins. In the multi-step extraction, generally, the first buffer is designed to solubilise water-soluble proteins; subsequent buffers are designed to obtain increasingly difficult-to-solubilise proteins.

Biomass build-up under elevated hydrostatic pressures is negligible, a few milligrams, which makes protein studies in these conditions intriguing. Despite the odds, an improved method of extraction of intracellular proteins from biomass as less as 5 mg (dry weight) without the need for liquid nitrogen based homogenisation has been described. Various lysing methods and extraction buffers were evaluated to extract proteins.

It is a well-known fact that medium composition altered biomass production (Cooke 1968). Organic ingredients in a complex medium such as yeast extract or peptone have undefined compounds; synthetic media has every ingredient defined (Basu et al. 2015). Biomass grown on synthetic medium could provide better protein profile as compared to biomass grown on complex medium. Therefore, total biomass generated (in milligrams) and protein profile of fungal cultures, when grown in different media were checked.

Nano-chip LC-MS-QToF system was used for peptide mass fingerprinting. Capillary electrophoresis complemented liquid chromatography. Mass spectrometers work by ionising molecules and then sorting and identifying the ions according to their mass-to-charge (m/z) ratios. The two critical components in this process are the ion source and mass analyser. Ion source generates ions, and mass analyser sorts the ions (Basics of LC/MS, Primer, Agilent technologies). The ion source of this instrument was dual electron spray ionisation (ESI), and the mass analyser was quadrupole time-of-flight (QToF). The QToF operated in scanning mode. Dissociation of ions was collision induced with multiple-stage MS.

This instrument had a sensitivity of 10 pg in MS/MS mode; mass accuracy error in MS mode was less than 1 ppb; the mass resolution was 25,000 at 322 m/z and 40000 at 1522 m/z. MS/MS scan/sec (100-3200 m/z) was 10 scan/sec with a dynamic range of 5.0 orders. Agilent 1260 infinity series HPLC. This instrument had a sensitivity of detecting molecules in its atto (10^{-18}) concentration (Agilent 6538 UHD Manuals).

The use of this instrument for protein studies nullified the disadvantage of 2DE and protein microarray (Shah and Damare 2018). This instrument separated peptides in LC followed by a split in ion based on mass by charge (m/z) ratio in the MS. The detection of protein by the instrument was based on abundance. Therefore, different gradient patterns were tried to get a good elution profile for maximum coverage of proteins present in the sample.

The simulated deep-sea conditions to generate biomass (20 mg for mycelia and 5mg for conidia) for protein studies were standardised in the previous objective. Under this objective, the studies carried out were:

- *Comparison of protein profiles obtained from wet and lyophilised biomass/*
- *Standardising method for protein extraction*
- *Optimising the medium for cultivation of fungi to obtain sufficient biomass for protein extraction*
- *Standardising salt-removal protocols for the extracted protein*

- *Confirmation of optimised protein extraction and salt-removal methods on other fungal isolates*
- *Standardising in-gel digestion and in-solution digestion protocols*
- *Analysis of digested protein by LC-MS-QToF*
- *Separation of proteins by 2-DE*

Protein profiles were generated for all these studies, which were compared with one another to finalise protocols for standardisation.

3.1 Comparison of protein profiles obtained from wet and lyophilised biomass

This comparison of protein profile between lyophilised and wet biomass was performed using conidia of the deep-sea isolate, *A. terreus* (#A4636).

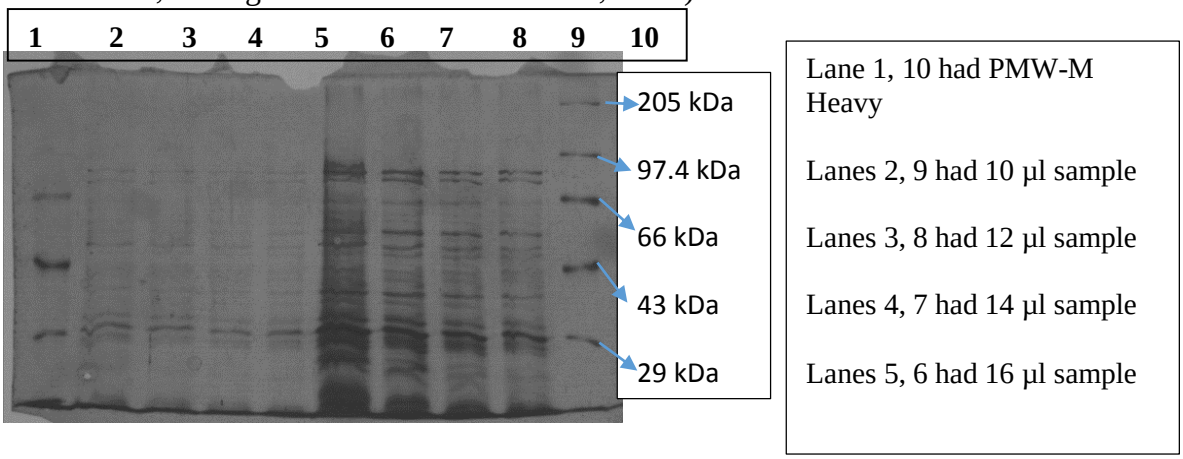
Materials and methods:

Conidia of *A. terreus* (#A4636) were harvested by flooding the agar plates of sporulating mycelia with 0.1% tween 80. The first set of harvested conidia was washed off 0.1% tween and lyophilised. The second set was washed off 0.1% tween, suspended in distilled water and was placed at –80ºC until protein extraction. Conidia from both the sets were scooped and placed in a separate lysate tube (Lysing Matrix A) for lysis. 500 µl of YBB buffer and 50 µl of 10 X protease inhibitor was added to the lysate tubes. The contents were homogenised at 6.0 m s^{-1} for 40 s with an intermittent gap of 5 min, followed by high-speed centrifugation (14000 rpm, at 4ºC for 15 min) and the supernatant was collected. The lysate tubes were placed in ice during the intermittent gap. Profiling of the extracts was done on the SDS-gel at a constant voltage of 90 V; electrophoresis was carried out for one h and 45 min. Silver staining (Heukeshovan and Dernick 1985) was performed to view the protein profile.

Result and discussion:

The profile of proteins harvested in 0.1% tween 80 is shown in Figure 3.1.

Figure 3.1: Protein profile of *A. terreus*, #A4636 conidia harvested in 0.1% tween 80 (in YBB buffer, homogenised at 6.0 m s^{-1} for 40 s, twice)



Lanes 2–4 had samples of proteins extracted from wet biomass (conidia stored at –80ºC). Lanes 6–9 had samples of proteins extracted from lyophilised biomass. From Figure 3.1, it can be concluded that for the same quantity/volume of protein sample loaded on wells, proteins extracted from lyophilised biomass was better as compared to proteins extracted from wet biomass. Therefore, only lyophilised biomass was used for protein extractions.

3.2 Standardising method for protein extraction

The entire standardisation procedure was carried out on mycelia of *A. terreus* (#A4636). The mycelium was grown in 1/5th MEB, at room temperature and atmospheric pressure for two days. The finalised protocol after examining with #A4636 was checked on other isolates before establishing its efficiency.

Materials and methods:

To evaluate the best lysing condition (that would yield maximum proteins), different methods of homogenisation like sonication and vortexing with glass beads, mechanical disruption using FastPrep homogeniser (Figure 3.2) with lysing matrix A and zirconium beads were tried (Table 3.1). For FastPrep homogenising, the disruption was performed at 6.0 m s^{-1} for 40 s and 6.5 m s^{-1} for 60 s. During all conditions of lysis, biomass was suspended in extraction buffer.

Table 3.1: Homogenisation methods and beads tried for protein extraction

Technique	Observation and inference
Liquid nitrogen	This method was not successful in every attempt when the quantity of biomass taken for crushing is less than 1g.
Sonication	The technique did not homogenise the isolates used in this study. Chitinous cell wall did not rupture with sonication.
Vortexing	Required longer duration for homogenisation. Excess duration for homogenisation implied loss of proteins due to degradation.
High-speed bead beating homogenisation	Homogenisation occurred in very less time. An excess amount of heat generated was the only shortfall that was managed by performing the homogenisation in dry-ice or short pulses. During intermittent gaps (a minimum of 5 min), the lysate tube was placed in ice for cooling.
Glass beads	Glass carries a charge which hinders extraction of all cytosolic proteins.
Lysing Matrix A	Protein extracted using this lysing matrix could not be quantified as it was too less for quantification.
Zirconium beads	The quantity and quality of protein extracted were better as compared to other lysing matrices used for homogenisation.

Figure 3.2: MP homogeniser (Picture courtesy: Google)

All extraction buffers (Table 3.2) were prepared fresh. Five hundred µl of each extraction buffer and five µl of 100 X Protease Inhibitor Cocktail (PIC, Serva #39104) were added to the lyophilised biomass for protein extraction individually. In the case of single buffer extractions, homogenisation was performed twice with intermittent cooling in ice, while for multi-buffer extractions (S. No. 6 and 7, Table 3.2), the homogenisation was performed twice for the first buffer and only once for the remaining buffers with the same biomass/pellet. The homogenised lysate tube was centrifuged at 14,000 rpm at 4^{o}C for 15 min and the supernatant collected. In the case of multi-buffer extractions, the supernatant was collected individually.

Quantification of protein was carried-out using Qubit protein assay as per manufacturer's instructions. The proteins were aliquoted in required quantities and stored at -80^{o}C until further use.

Table 3.2: Protein extraction buffers composition

S No	Buffer type	Buffer composition
1	Single	10 mM Tris-HCl/EDTA buffer (TE buffer) + 5 µl of 0.7 M β-mercapto-ethanol.
2	Single	YBB buffer (MP FastPrep protein isolation kit) + 5 µl of 0.7 M β-mercapto-ethanol.
3	Single	Urea buffer with SDS (1.5 % SDS, 9 M Urea, 25 mM Tris-HCl [pH 6.8], 10 mM EDTA and 0.7 M β-mercapto-ethanol). – Modified from Osherov and May 1998.
4	Single	Urea buffer with IPG (7 M urea, 2 M thiourea, 4 % CHAPS, 0.8 % IPG buffer, 20 mM DTT in 20 mM Tris-HCl of pH 8.0). – Bregar et al. 2012.

S No	Buffer type	Buffer composition
5	Single	Phenol buffer (500 mM Tris – HCl, EDTA – 50 mM, Sucrose – 700 mM, KCl – 100 mM. pH adjusted to 8.0) – Faurobert et al. 2007.
6	Combination buffer	Tris-glycine @ pH 8.3 (3 g Tris, 14.4 g glycine in 1L deionised water) - Bridge et al. 2003 , + Urea I buffer (9 M urea, 4 % CHAPS, 100 mM DTT – prepared in 40 mM Tris-HCl pH 8.0) - Modified from Rabilloud et al. 1997, Modified from Epperson and Martin 2011, + Urea II buffer (6 M urea, 3 M thiourea, 4 % CHAPS, 100 mM DTT – prepared in 40 mM Tris-HCl pH 8.0). – Modified from Remelli 2011.
7	Combination buffer	(Buffer 1) Tris – $MgCl_2$ @ pH 8.3 (0.5 M Tris-HCl, 2 % CHAPS, 20 mM $MgCl_2$, 2 % DTT) - Bhadauria and Peng 2010 + (Buffer 2) Urea I buffer (9 M urea, 4% CHAPS, 100 mM DTT – prepared in 40mM Tris-HCl pH 8.0), + (Buffer 3) Urea II buffer (6 M urea, 3 M thiourea, 4 % CHAPS, 100 mM DTT – prepared in 40 mM Tris-HCl pH 8.0). – Modified from Remelli 2011.

The protein samples were electrophoresed on 12% resolving with six % stacking SDS gels respectively; a Tris-glycine buffer was used for sample loading. In the case of silver staining, 250 µg total protein was loaded in each lane, while 500 µg protein was loaded in each lane for colloidal Coomassie blue staining. Sample loading dye was used before loading the samples in the SDS gel. The loading dyes were mixed with ß-mercaptoethanol in the ratio of 20:1. The volume ratio between 2X sample loading dye (added with ß-mercaptoethanol) and protein samples was 1:1. After mixing the protein samples with sample loading dye, the samples were placed in a dry bath set at 95^{o}C for 5 min. Heat treated protein samples were allowed to cool down to room temperature and were loaded within the wells of the polyacrylamide gel. At a constant voltage of 90 V, electrophoresis was carried out for one h and 45 min. The SDS gels were stained either with colloidal Coomassie blue (Neuhoff et al. 1988) or with silver staining method (Heukeshovan and Dernick 1985). For combination buffers, protein profile on SDS gels was determined individually (by storing the protein extracts separately) as well as after mixing the protein extracts obtained from all three buffers

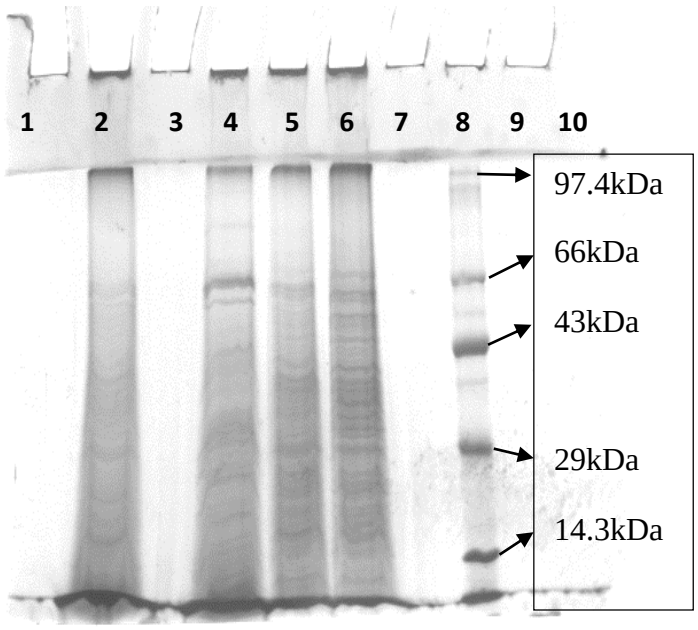
Results:

Dry vortexing followed by addition of extraction buffer resulted in degraded proteins as viewed on the SDS gel; the protein yield was too less to be quantified. (Table 3.3). Homogenisation at high speed (6–6.5 ms^{-1}) for the very short duration of 45–60 s proved

very useful in lysing the fungal cell wall. Homogenisation using zirconium beads was finalised for all protein extractions as it gave the maximum proteins.

In the case of multi-buffer protein extraction, there was no visible change in the protein profile when buffers were mixed (Figure 3.3). Hence protein extracts from combination buffers were collected in the same microfuge tube.

Figure 3.3: Protein profiles of mycelia of *A. terreus*, #A4636 obtained by using Tris-$MgCl_2$ combination buffer (unmixed and mixed)



2 – Combination buffer. Mix of
Tris – $MgCl_2$, 6 .5 m s^{-1}, 60 s, twice
Urea buffer – 9 M, 6.5 m s^{-1}, 60 s, once
Urea buffer – 6 M, 6.5 m s^{-1}, 60 s, once
4 – Combination buffer part I; Tris – $MgCl_2$; 6.5 m s^{-1}, 60 s, twice
5 – Combination buffer part II; Urea buffer – 9 M; 6.5 m s^{-1}, 60 s, once
6 – Combination buffer part III; Urea buffer – 6 M; 6.5 m s^{-1}, 60 s, once
8 – Protein molecular weight marker, medium

Table 3.3: Protein quantity obtained using different lysing methods and matrices

S. No.	Method of lysing	Biomass type	Beads used	The buffer used for extraction	Biomass weight	Protein quantity
1	Liquid nitrogen	Conidia	Glass (0.125 – 0.25 mm Ø)	TE buffer	Approx. the weight of biomass taken was 900 mg – 1g	Not detected
2	Liquid nitrogen + dry vortexing (20 min)	Conidia	Glass	TE buffer		Not detected
3	Liquid nitrogen + wet vortexing – with buffer (30 min)	Conidia	Glass	TE buffer		Not detected
4	Sonication	Mycelia	Glass	TE buffer		Too-less to be quantified ($< 2 \mu g\ ml^{-1}$)
5	Dry vortexing (45 min)	Conidia	Glass	TE buffer		Degraded protein was viewed on SDS gel. Too-less to be quantified ($<2\ \mu g\ ml^{-1}$)
6	High-speed homogenisation	Conidia	Zirconium (0.1 mm Ø)	TE buffer		$296\ \mu g\ ml^{-1}$
7	High-speed homogenisation	Mycelia	Lysing Matrix A (6.223 mm Ø)	YBB buffer		Not detected

S. No.	Method of lysing	Biomass type	Beads used	The buffer used for extraction		Protein quantity
8	High-speed homogenisation	Mycelia	Zirconium	YBB buffer	Approx. the weight of biomass taken was 900 mg – 1g	186 µg ml^{-1}
9	High-speed homogenisation	Mycelia	Zirconium	Urea buffer (with SDS)		549 µg ml^{-1}
10	High-speed homogenisation	Mycelia	Zirconium	Urea buffer (with IPG buffer)		5.2 mg ml^{-1}*
11	High-speed homogenisation	Mycelia	Zirconium	Phenol buffer		Beyond quantification range # (> 200 µg $µl^{-1}$)
12	High-speed homogenisation	Mycelia	Zirconium	Tris-glycine combinational buffer		251.2 µg ml^{-1}
13	High-speed homogenisation	Mycelia	Zirconium	Tris-$MgCl_2$ combination buffer (Standardised buffer)		772 µg ml^{-1}

Beyond quantification range implied that protein was serially diluted for performing qubit quantification assay, but to no avail. Although qubit does not mention phenol as an interfering compound, de-salting protocols were carried out, and the pellet was re-dissolved in 10 mM TE buffer for quantification. Despite this, quantification did not yield result.

* Even though Urea-IPG buffer gave a maximum quantity in protein extraction, it was suspected to provide the false positive result as it was with IPG buffer that contains ampholytes. This claim was verified by measuring the protein quantity from a control sample (buffer without proteins).

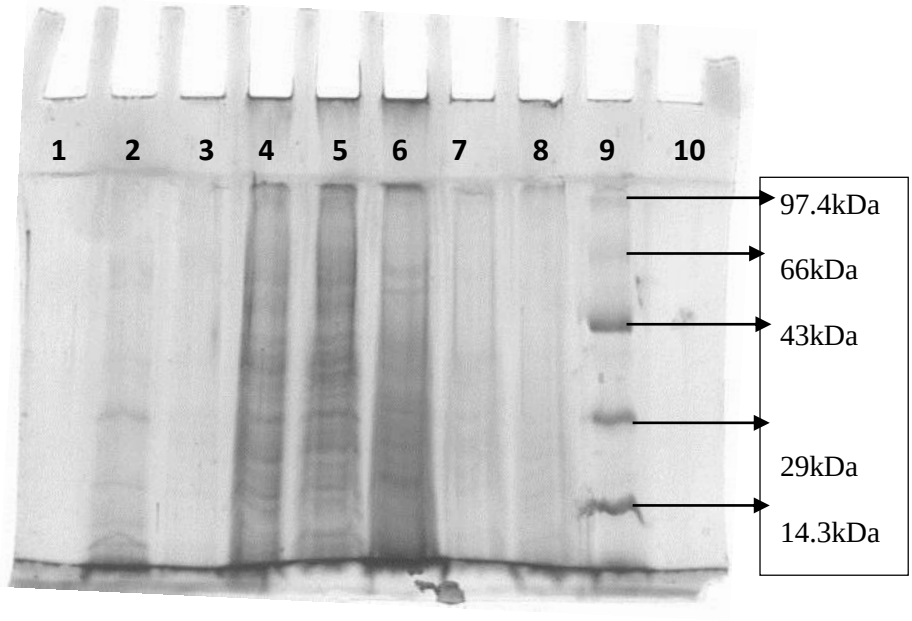
Quantification of proteins in urea buffer produced false positive results that may be eliminated by measuring the reading of blank (standardised extraction buffer + PIC, without proteins) followed by subtracting the blank value from the QUBIT readings of other samples before proceeding with calculations (as mentioned in the QUBIT manual).

The traditional method of using liquid nitrogen was not very successful if the quantity of biomass employed for crushing was around 1–9 mg. Even 1 g of biomass was insufficient to recover adequate amounts of protein (Table 3.3). High-intensity sonication broke the mycelia into smaller filaments (as viewed under a microscope) but did not rupture the cell wall. Homogenisation with zirconia beads at 6.5 m s^{-1} for 60 s was superior to 6.0 m s^{-1} for 40 s. Protein extracted in phenol buffer (Table 3.2, S. No 5) left residues on the SDS gel. The protein profile of all buffers mentioned in Table 3.2 (except phenol-buffer extracted proteins), when homogenised with zirconia beads, has been depicted in Figure 3.4.

Combination of buffers was found to yield better protein profile on the SDS gel; the buffer that was finalised for protein extraction was Tris-$MgCl_2$ combination buffer. This buffer had three parts. Buffer part 1 was 0.5 M Tris-HCl at pH 8.3, 2 % CHAPS, 20 mM $MgCl_2$, 2 % DTT ; buffer part 2 was 9 M urea, 4 % CHAPS, 100 mM DTT – prepared in 40 mM Tris-HCl of pH 8.0; buffer part 3 was 6 M urea, 3 M thiourea, 4 % CHAPS, 100 mM DTT – prepared in 40 mM Tris-HCl of pH 8.0. (Table 3.3). Homogenisation (at 6.5 m s^{-1} for the 60 s) was performed twice for the first buffer with an intermittent gap of 5 minutes (the lysate tubes were placed in ice for that duration) whereas for the second and third buffers, it was performed only once. In conclusion, high-speed (6.5 m s^{-1}, 60 s) homogenisation along with zirconia beads in combinational buffer system could extract good quantity and quality of proteins, as viewed on the SDS gel.

Figure 3.4: Protein profiles of mycelia of *A. terreus*, #A4636 using different buffers*

**excluding protein extracted using phenol buffer*



1 – 10 mM TE Buffer
2 – YBB buffer
3 – Urea buffer (with SDS)
4 – Urea buffer (with IPG)
5 – Combination buffer – mix of
Tris-$MgCl_2$ 6.5 m s^{-1}, 60 s
Urea buffer – 9 M
Urea buffer – 6 M
6 – Combination buffer – mix of
Tris-Glycine 6.5 m s^{-1}, 60 s
Urea buffer – 9 M
Urea buffer – 6 M
7 – Combination buffer – mix of
Tris-$MgCl_2$ 6.0 m s^{-1}, 40 s
Urea buffer – 9 M
Urea buffer – 6 M
9 – Protein molecular weight marker, medium

3.3 Optimising the medium for cultivation of fungi to obtain sufficient biomass for protein extraction

Although high-speed (6.5 m s^{-1}, 60 s) homogenisation and Tris-$MgCl_2$ combination buffer was finalised for protein extraction, the resultant protein profile on gel had high background and did not qualify as a noise-free image. It was speculated that a part of this problem could be due to the organic residues that may accumulate in the mycelia grown in an undefined/complex medium (MEB in this case). As background noise was prominent despite washing the mycelium during harvest, various media were checked for cultivating the fungal isolates to obtain a protein profile with least noise. Therefore, mycelia of all five isolates were cultivated in different media (Czapek Dox broth: CDB wherein sucrose was replaced with 5% glucose, $1/5^{th}$ MEB, MEB, Potato dextrose broth: PDB, Soybean casein digest: SBCD), proteins from mycelia grown on each medium was extracted and profile was checked on SDS gel.

Materials and method:

One ml of conidia suspension (30–50 x 10^6 spores ml^{-1} in 0.1% tween 80) of all five isolates were inoculated in 9 ml of each media (CDB, $1/5^{th}$ MEB, MEB, PDB, SBCD respectively prepared in CIB sea water) in plastic pouches and were incubated at 0.1 MPa, room temperature for 24 h. After 24 h incubation, one set of pouches were placed at 45 MPa; while the other set of pouches were placed at 0.1 MPa (Figure 3.5). The experiment was carried out for 30 days at 30°C to generate maximum biomass.

On completion of incubation, the biomass from replicates of each pouch was harvested on a single dried, pre-weighed 0.45 µm nitrocellulose filter paper. Salts and excess media were removed from the filter papers by washing thrice with five ml of distilled water. The mycelium was preserved in –80°C until lyophilisation. Subsequently, after lyophilisation, the samples were stored at room temperature in airtight containers; the weight of triplicate, dried mycelial sample in each filter paper was recorded.

Protein extraction (in finalised conditions) and profiling (in Coomassie stain) was carried out from all the mycelial samples obtained from all the media studied, as mentioned in the previous section. Quality of protein extract was checked on the SDS gel.

Figure 3.5: Plastic pouches with different media and inoculum

Results:

Biomass generated in each medium has been tabulated in Table 3.4. The summary of the results is recorded in Table 3.5.

Table 3.4: Biomass produced in each medium (in mg)

Pressure	Isolates	Details of media				
		MEB	MEB(1/5th)	CDB*	PDB	SBCD
0.1MPa	*A. terreus* (#A4636)	33.8	7.4	127.9	93.9	**131.9**
	A. terreus (#MTCC479)	121.1	33.7	120.0	131.3	**148.0**
	A. flavus (#NIOCC20)	83.6	21.3	**152.8**	106.5	115.0
	Penicillium sp. (#A4623)	139.2	23.1	**177.8**	123.4	139.8
	A. sydowii (#CH2)	92.5	17.1	**138.3**	115.2	126.2
45MPa	*A. terreus* (#A4636)	12.1	6.1	11.4	11.4	**50.3**
	A. terreus (#MTCC479)	**13.9**	6.1	4.3	8.4	7.6
	A. flavus (#NIOCC20)	9.2	9.4	**12.1**	11.9	10.0
	Penicillium sp. (#A4623)	9.7	9.3	10.1	14.3	**22.5**
	A. sydowii (#CH2)	5.3	5.3	6.4	12.4	**16.1**

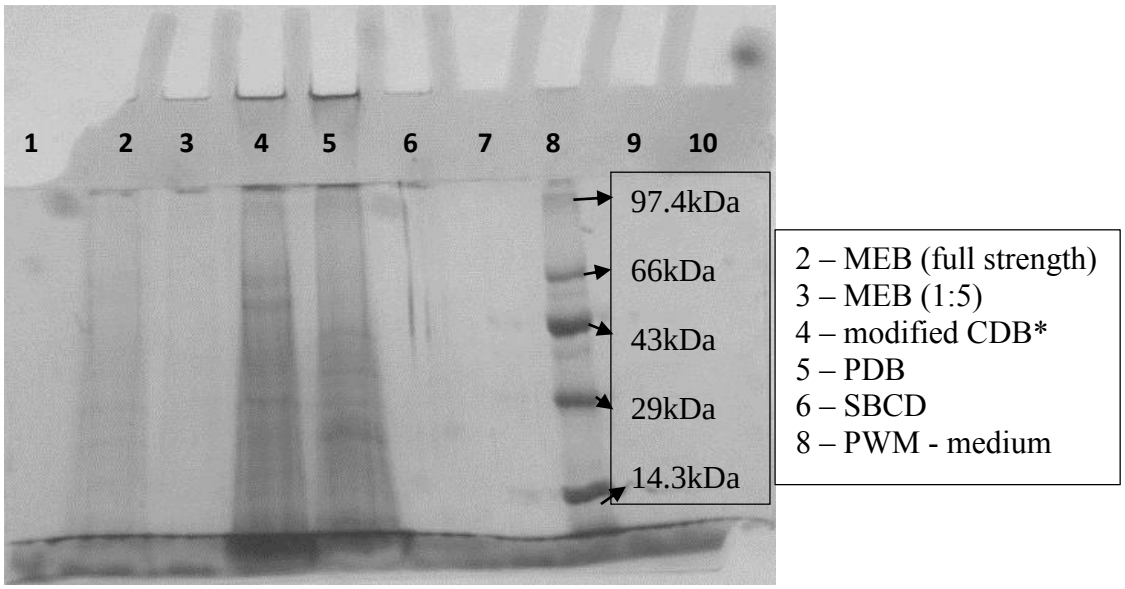
Table 3.5: Summary of Table 3.4

Isolate	Best suited media with biomass (mg)	
	At 0.1MPa	At 45MPa
A. terreus (#A4636)	SBCD - 131.9	SBCD - 50.3
A. terreus (#MTCC479)	SBCD - 148	MEB - 13.9
A. flavus (#NIOCC20)	CDB* - 152.8	CDB* - 12.1
Penicillium sp. (#A4623)	CDB* - 177.8	SBCD - 22.5
A. sydowii (#CH2)	CDB* - 138.3	SBCD - 16.1

CDB* - sucrose replaced with 5% glucose.

Both the tables state that there was no medium that commonly and consistently supported growth at high pressure. Protein profile as viewed on SDS gel extracted from *A. terreus* (A3636) cultivated in different media is represented in Figure 3.6.

Figure 3.6: Protein profile of *A. terreus* (#A4636) cultivated at 45 MPa in different media



CDB* - sucrose replaced with 5% glucose.

CBD* demonstrated as the best medium for fungal cultivation by leaving least residue over polyacrylamide gels. As this study involves protein analysis, media that yielded the least residue was preferred for the cultivation of samples under elevated hydrostatic pressures; consequently, modified CDB (sucrose replaced with 5% glucose) was finalised for the cultivation of fungal isolates for protein studies involving mycelia. Glucose, a monosaccharide, would be easily broken down than sucrose, a disaccharide.

3.4 Standardising salt-removal protocols for the extracted protein

Protein solubility is affected by ions (Duong-Ly and Gabelli 2014), and so desalting is vital for the study of proteins as salts hinder analysis. Therefore, various salt removal protocols were tried on proteins that were extracted in Tris-$MgCl_2$ three-buffer-system.

Materials and method:

Various salt removal protocols were tried on protein samples extracted from *A. terreus* (A4636) isolate cultivated at room temperature and atmospheric pressure. Details of desalting methods tried, along with observation and inference has been recorded in Table 3.6.

Table 3.6: Salt removal protocols used for desalting

Desalting methods	**Details**	**Observation/Remarks**
PAGE PERFECT	G-Biosciences, USA, Cat # 786 – 123	The intensity of protein bands on SDS gels was faint
PERFECT FOCUS	G-Biosciences, USA, Cat # 786 – 124	The intensity of protein bands on SDS gels was faint. This method was used for de-salting proteins before IEF.
Trichloroacetic acid (TCA) – Acetone precipitation method	13.3% TCA + 0.3% DTT were dissolved in 100% ice-chilled Acetone. The protein (in solution) was incubated over-night, at –20°C, in this suspension (3 times the volume) followed by washing thrice with ice-cold acetone and air drying the protein pellet. (Bregar et al. 2012)	Residual TCA in protein pellets caused streaks on SDS gel. Excess washing was required to remove TCA.
Saturated TCA precipitation method	Protein (in solution) was incubated overnight in thrice its volume of saturated TCA at 4°C followed by washing thrice with ice-cold acetone and drying the protein pellet. (Modified from Stoyanov et al. 2001)	Residues of TCA were seen on SDS gels.
Methanol precipitation method	Protein (in solution) was incubated overnight at –20°C in 9 times the volume with 100% methanol followed by washing with 90% methanol and drying the protein pellet. (Modified from Fic et al. 2010)	The intensity of protein bands on SDS gels was better as compared to the previous methods. Worked best for in-solution digestion, for samples to be analysed in LC-MS-QToF system.

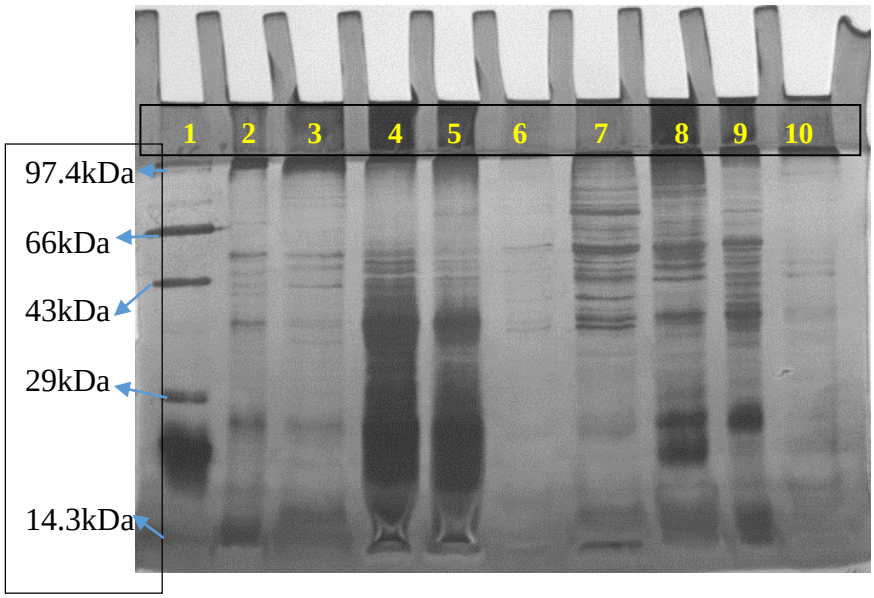
Desalting methods	Details	Observation/Remarks
Acetone precipitation	The protein sample (in solution) was incubated overnight (–20°C) 9 times the volume of ice-cold acetone; this was followed by washing thrice with ice-cold acetone and air drying the pellet. (Modified from Stoyanov et al. 2001)	This method required lots of washing and drying before the protein pellet could be used.
BioRad micro spin columns	Kit (Cat # 732 – 6221), USA	Standardised for SDS gels.
Ammonium acetate precipitation	0.1 M ammonium acetate in ice-cold methanol Faurobert et al. 2007	The procedure was long and tedious and hence was discontinued.

The salted out protein pellets were electrophoresed on SDS PAGE as detailed in the previous section. If the pellets were sturdy, it was solubilised in a minimum quantity of 0.2 M sodium hydroxide for electrophoresis. The gels were silver stained.

Results:

Biorad's micro spin columns were standardised for salting out proteins before electrophoresing on SDS gels. Methanol precipitation was found to be best suited for in-solution digestion. Primarily, all these were carried out to precipitate proteins for further studies. The protein pellet so obtained could be re-dissolved in the buffer of interest, such as rehydration buffer in case of IEF or sample loading buffer in case of PAGE, or dissolution buffer in case of MS analysis based on the forthcoming experimentation. The alkali did not cause any residue on SDS gel. The SDS gel image of protein samples extracted by homogenisation (at 6.5 m s^{-1} for the 60 s) in Tris-$MgCl_2$ three-buffer-system after salting out using Biorad's micro spin column has been depicted in Figure 3.7. The initial quantity of biomass used for extracting the proteins to obtain this protein profile (as shown in Figure 3.7) was approximately 20 mg.

Figure 3.7: Mycelial protein profiles of *A. terreus*, #A4636, and #MTCC479 obtained in Tris-$MgCl_2$ three-buffer-system (after desalting with Biorad's micro spin column)



1 – Protein molecular weight marker – medium

2 - YBB buffer, #A4636

3 - YBB buffer, #MTCC479

4 - Urea buffer, #A4636 (with SDS)

5 - Urea buffer, #A4636 (with IPG)

6 - Tris-glycine combination buffer, #A4636

7 - **Standardized buffer, #A4636**

8 - **Standardized buffer, #MTCC479**

9 - Urea buffer, #MTCC479, (with IPG)

10 - Tris-glycine combination buffer, #MTCC479

3.5 Confirmation of optimised protein extraction and salt-removal methods on other fungal isolates

The optimised method for homogenisation and salting out with zirconia beads in Tris-$MgCl_2$ three-buffer-system and salting out was tested for consistency and efficiency by trying the established protocol on eight randomly chosen marine isolates from 8 different genera.

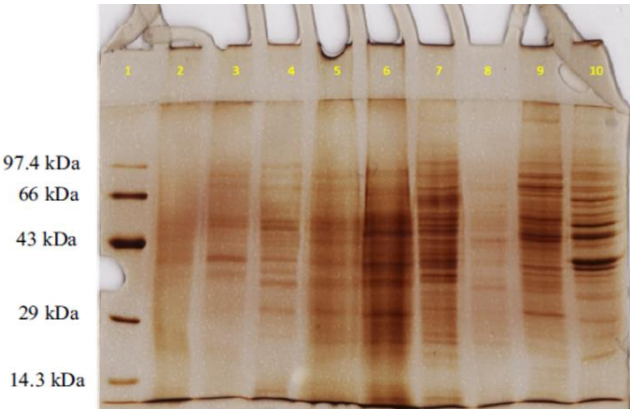
Materials and methods:

The isolates were grown on modified CDB (sucrose replaced with 5% glucose) for 6–7 days at room temperature (30^{o}C, static conditions). Mycelia were harvested individually on 0.45 µm nitrocellulose filter papers, washed, lyophilised and stored in airtight containers at room temperature. Five mg of dried biomass of each strain was weighed and used for homogenisation. Five hundred µl of each buffer-part 1 (Tris-$MgCl_2$) and five µl of 100 X PIC were added to the lyophilised biomass for protein extraction individually. The contents of the tube were homogenised twice at 6.5 m s^{-1} for 60 s. A gap of 5 min was maintained between the homogenisation and during the gap period, the lysate tubes were placed in ice. The homogenised-lysate tube was centrifuged at 14,000 rpm at 4^{o}C for 15 min and the supernatant collected. The procedure of homogenising, centrifugation and collection of the supernatant was repeated for the remaining two buffer-parts with the addition of 5µl of 100 X PIC in each buffer part. For each isolate, the supernatants from three-buffer parts containing protein extracts were collected in a single tube, and the protein samples were desalted with Biorad's micro spin column. A total of eight protein extracts were electrophoresed using SDS PAGE to view the protein profiles after silver staining.

Results:

The protein profile from 5 mg dry biomass of 8 different isolates is shown in Figure 3.8.

Figure 3.8: Protein profile of eight different fungal cultures using the optimised Tris–$MgCl_2$ buffer, with FastPrep homogenisation using zirconia beads



Lane1: PMW (Medium)
Lane 2: *Sarocladium strictum*
Lane 3: *Cladosporium cladosporioides*
Lane 4: *Hortaea werneckii*
Lane 5: *Acremonium alternatum*
Lane 6: *Purpureocillium lilacinum*
Lane 7: *Penicillium citrinum*
Lane 8: *Engyodontium* sp.
Lane 9: *Aspergillus flavus*
Lane 10: Unidentified filamentous fungal isolate

The quantity of protein extracted from each of 5mg dried biomass was quantified using Qubit assay kit. The details of the quantification are mentioned in Table 3.7

Table 3.7: Fungal isolates used in the study and proteins obtained from the lyophilised biomass (~5mg) by the finalised method* as quantified by Qubit assay kit

Fungal isolates used	**Protein concentration (mg ml^{-1})**
Sarocladium strictum	3.14
Cladosporium cladosporioides	2.78
Hortaea werneckii	3.6
Acremonium alternatum	3.66
Purpureocillium lilacinum	2.48
Penicillium citrinum	1.04
Engyodontium sp.	1.028
Aspergillus flavus	2.42
Unidentified filamentous fungus	2.35

*****Lysing with zirconium beads using FastPrep homogeniser and Tris-$MgCl_2$ 3-buffer system.

From Figure 3.8 and Table 3.7, FastPrep homogenisation (6.5 m s^{-1} for 60 s) using zirconia beads in Tris–$MgCl_2$ buffer multi-buffer system was standardised to extract proteins. The salts in the buffer systems were removed by desalting with Biorad's micro spin column. The results were consistent, and the protein profile obtained after performing these procedures showed clarity, as seen in Figures 3.7 and 3.8. Also, the quantity of protein obtained was good, as inferred from Table 3.7 considering the initial biomass quantity (5 mg dry weight) that was used for extraction. The efficiency of these methods of protein extraction and de-salting have been reported by Krishnaswamy et al. 2018.

3.6 Standardising in-gel digestion and in-solution digestion protocols

As a continuation of protein studies, protein samples were analysed in LC-MS-QToF. This analysis involved the digestion of proteins. The sample preparation methods for in-gel and in-solution digestion has been described below.

Materials and methods:

In-gel digestion was performed by excising protein bands/spots. The protein gel was washed thoroughly (thrice, 20 minutes each time) in nano-pure water to remove external contaminants. After washing, protein bands/spots of interest were excised. Excised bands were de-stained with destainers. For in-solution digestion, processing the protein sample started with the dissolution of protein in chaotropic agents. Dissolution of protein pellet or de-staining of excised protein spot/band was followed by reduction, alkylation, cleavage (using enzymes), over-night incubation (for enzymatic action) and pH adjustment (for long-term preservation). The accuracy of in-gel digestion protocol was checked by excising two marker bands of known protein molecular weight marker (medium sized 97.4 kDa – 14.3k Da, PMW-M) and processing them as per the standardised protocol; in-solution protocol's digestion accuracy was checked by processing the solution of bovine serum albumin (BSA) as per the standardised protocol.

Results:

The procedure for in-gel and in-solution were standardised on viewing confirmed results from positive controls. BSA (66 kDa) was detected at the end of in-solution analysis and marker bands of phosphorylase B (97.4 kDa), and BSA (66 kDa) were detected at the end of in-gel analysis in LC-MS-QToF. The standardised protocols of in-gel digestion and in-solution digestion have been described. All solutions used in the procedure were prepared fresh before the start of the analysis.

In-gel digestion **(**Modified from Kinter and Sherman 2000):

Destaining solution: 1:1 ratio of 30 mM potassium ferricyanide and 100 mM of sodium thiosulphate. This mixture was destainer.

Washing solution: 50 µl of 500 mM ammonium bicarbonate in 450 µl of nanopure water (1:10 dilution). (named as mixture A). Mixture A was mixed with acetonitrile in ratio 1:1 to prepare the washing solution.

Ten mM DTT (named as mixture B was prepared in 50 mM ammonium bicarbonate)

55 mM IAA (named as mixture C was prepared in 50 mM ammonium bicarbonate)

Trypsin was prepared as mentioned in the technical bulletin catalogue (T6567, Sigma). Briefly,

90 µl of Acetonitrile in 910 µl of nano-pure water gave 9% acetonitrile (1000 µl) (mixture D)

100 µl of mixture D was added to 4 mg of Ammonium bicarbonate (0.004 g) (mixture E)

72 µl of mixture E in 828 µl of mixture D gave 900 µl of mixture F

900 µl of Mixture F was mixed with 100 µl of 1 mM HCl that gave an ml of mixture G (was vortexed well)

Mixture G (1 ml) was added to the trypsin vial; the vial was incubated in ice for 15–20 min and was named mixture H

The required quantity of mixture H was used for digestion.

PROTOCOL

Day 1

1) Spots were excised using cut tips, blade, or surgical knife; excised spots were placed in 0.7 ml tube
2) 100 µl of nano-pure water was added to the tube; harsh vortexing for 5–10 min followed
3) After vortexing, 100 µl of nano-pure water was discarded. Steps 2 and three were repeated.
4) 100 µl of destainer was added, and the samples were incubated at room temperature for as long as the stain visibly disappears (> 15 min)
5) As the excised spot got transparent, destainer was discarded.
6) 100 µl of washing solution was added; harsh vortexing for 5–10 min followed; after vortexing, the washing solution was discarded
7) Step 6 was repeated
8) 100 µl of acetonitrile was added; harsh vortexing for not more than five min followed, and acetonitrile was discarded
9) 100 µl of mixture B (10 mM DTT) was added to the tube containing the processed spot and was incubated for 45 min to 1 h at 56^{o}C; following incubation, mixture B was discarded.
10) 100 µl of mixture C (55 mM IAA) was added to the same tube and was incubated for 30 min in the dark; after incubation, mixture C was discarded
11) The washing solution was added, (enough to cover the gel); harsh vortexed for ten min and discarded; this procedure was repeated.
12) 100 µl of acetonitrile was added, vortexed generally for a few moments; the tube was incubated at room temperature until the gel got opaque white after which acetonitrile was discarded.
13) 20 µl of 20 µg ml^{-1} trypsin (prepared as specified) was added and incubated overnight at 37^{o}C.

Day 2

14) 2:1 ratio of formic acid: acetonitrile was added to the digested protein sample to adjust the pH to 2–3 (by checking in pH paper)
15) Harsh vortexing followed for 30 min to an hour
16) The digested, pH adjusted sample was sonicated using the liquid bath for 45s, thrice, with a gap of 3 min between each sonication.
17) The extract was collected in a separate tube
18) Ten µl of this extract was taken for sample analysis.
19) Gel pellets and remnant extract were dried in a speed vac and stored at –20^{o}C until next use.
20) To the dried extract, ten µl of 0.1% formic acid prepared in acetonitrile could be added, whenever required for further use.

In-solution digestion **(**Modified from Kinter and Sherman 2000):

Before the start of in-solution digestion, 100 µl of the sample was desalted using methanol precipitation (Table 3.6).

Few notes before the start of the digestion

8.33 µl of 12 N HCl in 991.66 µl of nano-pure water gave 1 ml of 0.1 M HCl (as 1 M HCl = 1 N HCl); From 0.1 M HCl, one µl of 0.1 M HCl was added to 99 µl of nanopure water to make 100 µl of 1 mM HCl

20 µl of 1 mM HCl was added to 20 µg Trypsin vial and incubated for 30 minutes in ice.

Day 1

1) The protein pellet was dissolved in 50 µl of 6 M urea.
2) To the dissolved protein, ten µl of 200 mM DTT (made in 50 mM ammonium bicarbonate) and incubated for an hour at room temperature.
3) After reduction by incubating in DTT, 24 µl of 200 mM IAA (made in 50 mM ammonium bicarbonate) was added and incubated for 30 min, in the dark, at room temperature.
4) 24 µl of 200 mM DTT was again added and incubated at room temperature for 45 min.
5) 500 µl of 50 mM Tris-HCl was added to dilute the 6 M urea into 0.6 M urea.
6) One µg µl^{-1} of trypsin was added to the reaction tube and incubated overnight at 37^{o}C.

Day 2

7) Formic acid was added to the digest to lower the pH to 2–3 (confirmed using a pH paper).
8) The quantity required for MS analysis (60 µl) was removed and placed in the analysis vial while the remnant analyte was stored at –20^{o}C until next use.

The sensitivity of staining procedure and the resolution of proteins were drawbacks of gel-based proteomics. Besides, in-solution digestion had the advantage of entire protein moiety being analysed. Therefore, only in-solution digests were carried out for protein analysis.

3.7 Analysis of digested protein by LC-MS-QToF

Agilent's LC-MS-QToF system was used for protein analysis.

Materials and methods:

The aqueous solvent system for LC was 0.1% formic acid (named A%) while the organic solvent system was acetonitrile: water at 90:10 ratio in 0.1% formic acid (named B%). From trypsin digested in-solution sample, eight µl digest was injected for each analysis. Analytes were placed at 5°C. Various elution profiles were tried varying from 30 minutes to 100 minutes.

Chip-based separation was adopted using Prot ID chip 150 II 300A C18 150mm column. As an internal standard, calibrant POS principal ion of mass 922.010 was included. The control of system and acquisition of data was done using "Mass hunter" data acquisition software version 5.0. The high voltage between 1650–2300 V was set for electron spray ionisation (ESI), depending on the total number of injections and the age of the chip. Time of flight was set to 2 GHz of extended dynamic range. Auto MS/MS was set to the standard formula of $Auto\ \frac{MS}{MS} = \frac{\text{Slope x } (\frac{m}{z})}{100} + offset$ with charge value being 2, slope set to 3.2 and offset value set to 5, in positive mode with 2 ppm accuracy; nitrogen gas was inside the collision cell. The minimum mass range for both MS and MS/MS was set to 100 m/z, and the maximum range was set to 3000 m/z. The flow rate was 4.0 µl min^{-1} for in-organic solvent and 0.5 µl min^{-1} for the organic solvent. Output was generated as spectra, which was employed for further analysis. By trying a different gradient with varying time ranges for the liquid chromatography, different elution profiles were observed. Non-linear gradient worked better than a linear gradient. Although linear gradient should elute all the proteins bound to the column with 98% acetonitrile, it did not happen with the samples. Therefore, varying ranges of non-linear gradients were tried to obtain maximum coverage, and 71-min protocol worked the best. Details for the 71min gradient for the nano-pump of LC are tabulated in Table 3.8.

Table 3.8: Details of the LC gradient (nano-pump)

Time (minutes)	A%	B%	Flow (µl min^{-1})	Max pressure limit (bar)
0.00	98.0	2.0	0.50	280
7.00	98.0	2.0	0.50	200
57.00	10.0	90.0	0.40	200
67.00	10.0	90.0	0.40	200

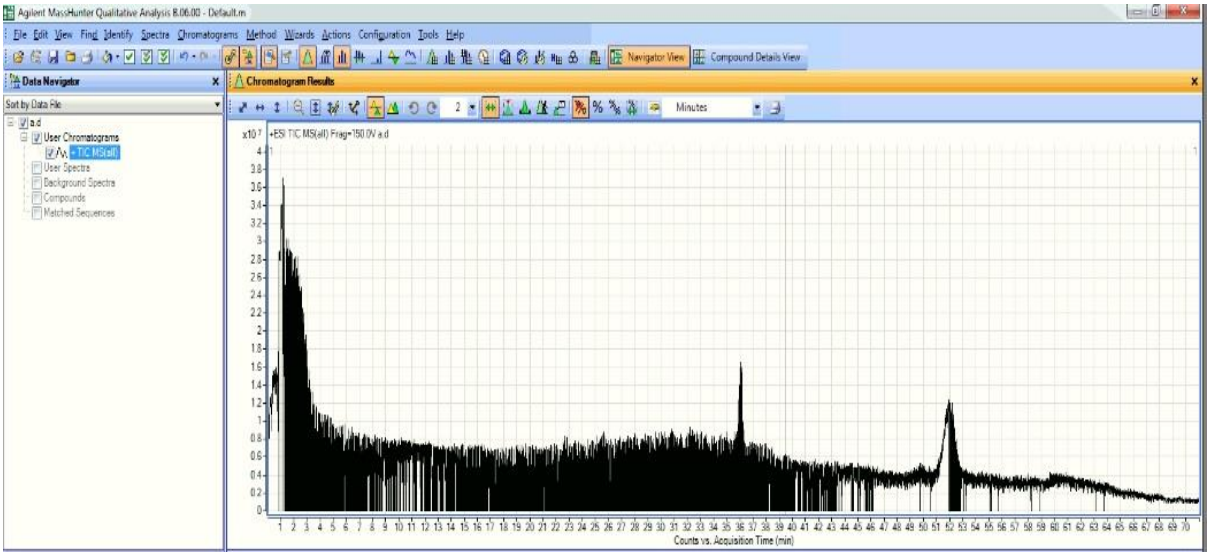
Time (minutes)	A%	B%	Flow (µl min^{-1})	Max pressure limit (bar)
70.00	98.0	2.0	0.50	200
71.00	98.0	2.0	0.50	200

The accuracy of the 71-minute protocol for sample analysis was checked by including internal standards of mass references, which were detected.

Results:

A screen-shot of chromatogram (Figure 3.9) for the 71-minute gradient is presented below (as presented in mass hunter qualitative analysis software)

Figure 3.9: Screenshot of chromatogram generated for the 71-minute gradient (*A. flavus*, grown at 0.1 MPa and 30°C)



There was abundance in the detection of chromatograms in the 71-minute protocol. MS/MS analysis showed that most peptides were eluted and processed as ions. It is best to process technical replicates for high throughput technology (Palsson 2003). Subsamples were injected four times to generate technical replicates.

3.8 Separation of proteins by 2-DE

Proteins can be separated two-dimensionally based on their weight and charge on PAGE. The first-dimensional separation is based on charge and is performed using a technique called Isoelectric focusing (IEF).

Materials and methods:

The analysis was carried out with 500 mg protein (control samples of mycelia) for each rehydration. Various rehydration buffers and IEF protocols were checked for efficiency. Each trial lasted for three days.

Day 1: Passive re-hydration was performed overnight for protein samples; the procedure for rehydration was followed as mentioned in Biorad (163–2105)'s kit. Only re-hydration buffers were varied as mentioned in table 3.10; a total of 150 µl of rehydration buffer was used for each IPG strip for rehydration.

Table 3.9: Details of rehydration buffers tried

IEF rehydration buffer composition
150 µl of 8 M urea, 2% CHAPS, 50 mM DTT, 0.2% w/v 3–10 ampholyte, bromophenol blue (Bio-rad manual for IEF)
150 µl of 7 M urea, 2 M thiourea, 4% w/v CHAPS, 2% v/v IPG buffer (pH 3–10), 20 mM DTT, Bromophenol blue (Bhadauria and Peng 2010)
50 µl of 10 mM Tris-HCl (pH 8.0) + 50 µl of 8 M urea, 4% CHAPS, 100 mM DTT, 0.2% ampholytes (prepared in 40 mM Tris) + 50 µl of 5 M urea, 2 M thiourea, 4% CHAPS, 100 mM DTT, 0.2% ampholytes (prepared in 40 mM Tris) – Modified from APAF protocol (The Australian proteome analysis facility)
150 µl of 7 M urea, 2 M thiourea, 4% CHAPS, 0.8% IPG buffer, 20 mM DTT, 20 mM Tris, 0.002% bromophenol blue (Bregar et al. 2012)
150 µl of 9 M urea, 4% CHAPS, 0.5% triton X-100, 20 mM DTT, 1.2% pharmalytes, bromophenol blue (Faurobert et al. 2007)

Day 2: On every trial of IEF protocol, protein samples were rehydrated with each of the re-hydration buffer mentioned above. IEF was carried out with one protocol at a time as mentioned in table 3.11. On completion of IEF, the IPG strip was stored in –20°C for SDS PAGE. The protocols were changed on the probability of trial and error; gel images were obtained based on second-dimensional separation on SDS gel.

Table 3.10: IEF protocols tried

S. No	IEF Protocol	S. No	IEF Protocol
1	Step 1: 250 V, Linear, 30 min Step 2: 4000 V, Linear, 2 h Step 3: 4000 V, Rapid, 10000 Volt hours Step 4: 50 V, Linear, 1 h	2	Step 1: 250 V, Linear, 1 min Step 2: 2000 V, Linear, 28 min Step 3: 2000 V, Linear, 1 h Step 4: 4000 V, Linear, 3 h Step 5: 4000 V, Rapid, 10000 Volt hours
3	Step 1: 250 V, Linear, 30 min Step 2: 4000 V, Linear, 2 h Step 3: 4000 V, Rapid, 10000 Volt hours	4	Step 1: 250 V, Linear, 45 min Step 2: 4000 V, Linear, 3 h Step 3: 4000 V, Rapid, 10000 Volt hours
5	Step 1: 250 V, Linear, 1 min Step 2: 250 V, Linear, 1 h Step 3: 1000 V, Linear, 1 h Step 4: 2000 V, Linear, 1 h Step 5: 4000 V, Linear, 3 h Step 6: 4000 V, Rapid, 10 000 Volt hours Step 7: 51 V, Linear, 15 min	6	Step 1: 250 V, Linear, 1 h Step 2: 1000 V, Linear, 1 h Step 3: 2000 V, Linear, 2 h Step 4: 4000 V, Linear, 3 h Step 5: 4000 V, Rapid, 10 000 Volt hours

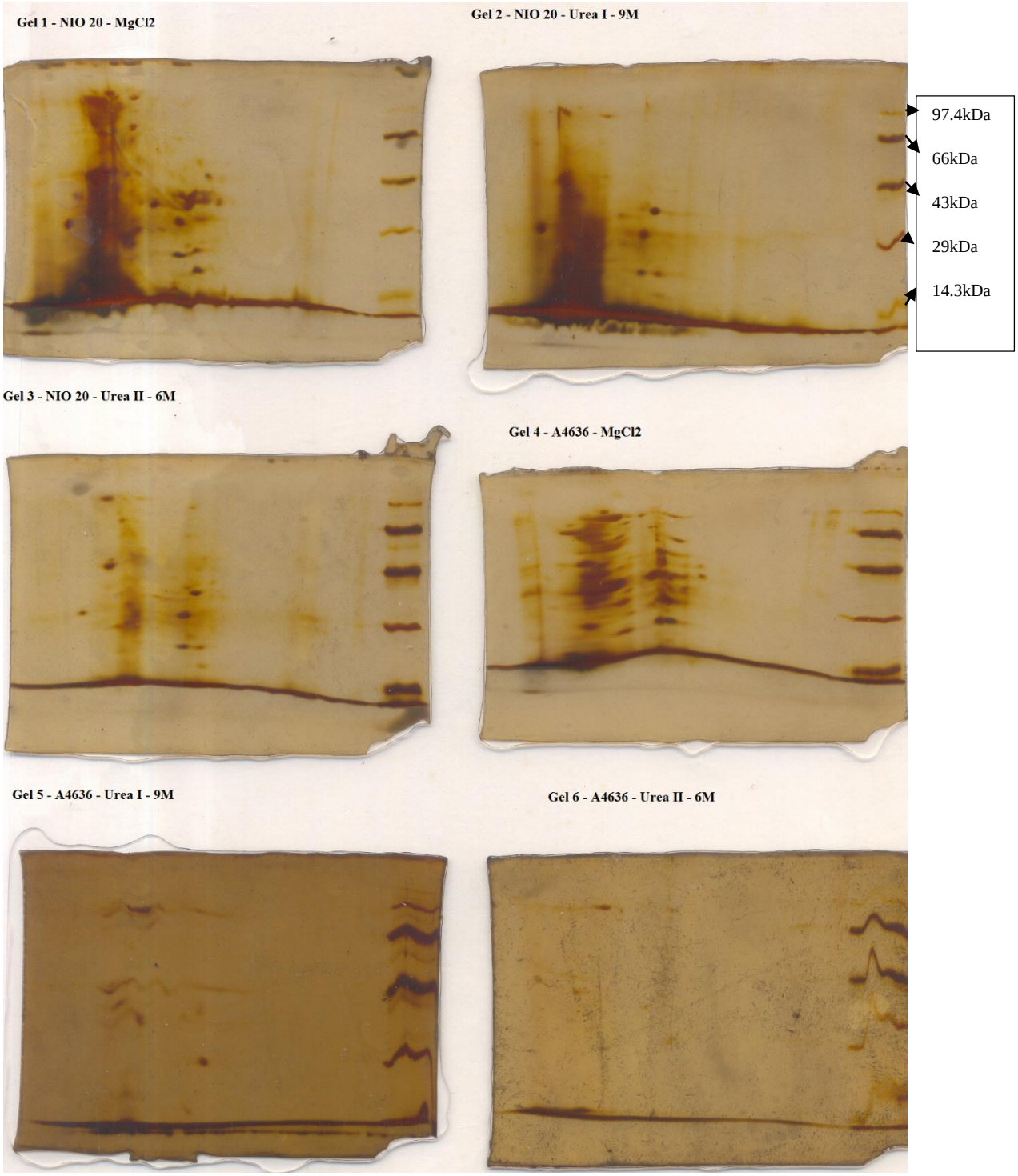
Day 3: IEF-performed IPG strips were thawed at room temperature and were treated with equilibration buffers as mentioned in Biorad's manual. Meanwhile, SDS gel with 12% resolving buffer was cast. Focused IPG strip was inserted between the plates of SDS gel and was sealed using low-melting agarose gel (as mentioned in Biorad's catalogue). Care was taken that there was no air gap between the inserted IPG strip and resolving buffer of SDS gel. A small groove was carved in agarose part of SDS gel at an end to load protein molecular weight marker (medium sized). PAGE and silver staining was performed as mentioned in experiment 3.2

Results and discussion:

For all the rehydration and IEF protocols that were tried, none could produce consistent and good separation of proteins when viewed on SDS gels. The quantity of protein (500 mg) tried for two-dimensional separation was detected to be insufficient to produce sharp protein spots on the gel. Further, obtained gel images could not be reproduced due to

minute differences in dimension during casting of SDS gels. As the protein quantity obtained from biomass grown under simulated deep-sea conditions could never be ≥ 1 g, IEF was discontinued. A set of gel figures obtained after 2-D separation is shown below in Figure 3.10.

Figure 3.10: Image of protein gels after a 2-dimensional separation in 7M rehydration buffer (Table 3.10, no 2) and 7-hour IEF separation (Table 3.11, S. No 1)



Protein extracts in buffer parts of the standardised Tris-$MgCl_2$ buffer for protein extraction were separated two-dimensionally during one of the trails. The depicted figure shows 2-D separation tried on protein extracts (of *A. flavus*, #NIOCC20 and *A. terreus* #A4636) in individual buffer-parts. Protein molecular weight marker of medium size was used.

Summary of chapter 3

For objective two, "Comparison of protein profiles of deep-sea isolates grown under different hydrostatic pressures and low temperature", the particulars of protocols that were standardised are mentioned below.

- ***Comparison of protein profiles obtained from wet and lyophilised biomass***

Lyophilised biomass gave better protein profile as compared to the profile obtained from wet biomass. Hence, only lyophilised biomass was used for obtaining proteins for profiling.

- ***Standardising method for protein extraction***

Protocol for Protein extraction: Ice was required for the entire procedure. Constituents of the buffer have been detailed

Buffer 1: named as Tris – $MgCl_2$

Constituents: 0.5 M Tris-HCl at pH 8.3, 2 % CHAPS, 20 mM $MgCl_2$, 2 % DTT

Buffer 2: named as urea I buffer

Constituents: 9 M urea, 4% CHAPS, 100 mM DTT. This buffer was prepared in 40 mM Tris-HCl (pH 8.0)

Buffer 3: named as Urea II buffer

Constituents: 6 M urea, 3 M thiourea, 4 % CHAPS, 100 mM DTT. This buffer was prepared in 40 mM Tris-HCl (pH 8.0)

Before starting the experiment, a dry bath was set at 37^{o}C; centrifuge was set at 4^{0}C. The pH of Tris-HCl (used in buffer I) was adjusted to 8.300 before the addition of the other buffer constituents; the addition of chaps did not increase the volume of the buffer. Addition of urea and thiourea (used in buffers 2 and 3) increased the net volume of buffer drastically. Therefore, the initial volume of 40mM Tris-HCl (pH 8.0) (used in the preparation of buffers 2 and 3) added was less than half of the total volume of the buffer that was prepared; the constituents were heated at 37^{o}C with frequent vortexing at repeated time intervals. Temperatures higher than 37^{o}C alters the structure of Urea. Heating after addition of chaps did not have any negative effect on the buffers. DTT was added just before the start of the experiment, to the buffers, as DTT has a short half-life. DTT was added once buffers II and III came to room temperature, after the 37^{o}C incubation.

Lyophilised biomass (2–5 mg) was transferred to the screw-cap tubes that were half-filled with zirconia beads (0.1 mm ϕ). This tube was named as lysate tube.

500 µl of Buffer I, five µl of 100 X protease inhibitor was added to the lysate tube. Contents were homogenised at 6.5 m s^{-1}, 60 s, twice, with five min gap in between the homogenisation (the samples were placed in ice during the gap). The homogenised lysate

tube was centrifuged at 14 000rpm, 4^{o}C for 15 min. The supernatant from the lysate tube was transferred to a fresh microcentrifuge tube (named "A").

After adding the specified quantity of DTT to buffer II, 500 µl of Buffer II, 5 µl of 100 X protease inhibitor was added to the original/same lysate tube and was homogenised at 6.5 m s^{-1} for 60 s, centrifuged at 14000 rpm, 4^{o}C for 15 min; the supernatant was transferred to the same microcentrifuge tube ("A").

Similarly, after adding the specified quantity of DTT to buffer III, 500 µl of Buffer III, 5 µl of 100 X protease inhibitor was added to the original/same lysate tube and was homogenised at 6.5 m s^{-1} for 60 s, centrifuged at 14000 rpm, 4^{o}C for 15 min; the supernatant was transferred to the same microcentrifuge tube ("A").

The protein extract was aliquoted and stored at –80^{o}C until further use.

- ***Optimising the best medium for cultivation of fungi to obtain sufficient biomass for protein extractions***

Modified CDB (wherein sucrose was replaced with 5% glucose) was the best-suited media to cultivate fungal mycelia for protein studies

- ***Standardising salt-removal protocols for the extracted protein***

Salting-out procedures were performed immediately after protein extraction and quantification. For protein profiling on SDS gel, Bio-rad's micro spin column purification was done as per the protocol mentioned in the catalogue. Methanol precipitation immediately followed protein extraction to avoid degradation of protein extracts due to repeated freeze-thawing. To the protein extract, 100% methanol (MS grade) was added in the ratio of 1:9 and incubated in the freezer (–20^{o}C), over-night. Next day, the sample was centrifuged at 14000 rpm for 20 min at room temperature; the supernatant was discarded. The remnant protein pellet was washed in 90% methanol, centrifuged at 14000 rpm for 20 minutes at room temperature. Protein pellet thus obtained was dried in a speed vacuum and stored in an air-tight tube, at room temperature until used for in-solution digestion.

- ***Confirmation of optimised protein extraction and salt-removal methods on other fungal isolates***

The optimised methods of protein extraction and salting out displayed consistency when checked with eight different filamentous fungal isolates. Therefore, these methods were standardised to extract and process proteins for further study.

- ***Standardising in-gel digestion and in-solution digestion protocols***

With slight modifications, protocols of Kinter and Sherman 2000 was standardised.

- ***Analysis of digested protein by LC-MS-QToF***

A 71-minute protocol was optimised to analyse the trypsin digests.

- ***Separation of proteins by 2-DE***

Gel images obtained after two-dimensional separation lacked clarity, consistency, and were not reproducible. As gel-free proteomics has advantages in proteomic studies as compared to gel-based proteomics, the latter was discontinued.

Gel-based (single as well as two dimensional) protein profiling of the five fungal isolates was not done. Instead, gel-free protein profiling was carried out for all the isolates by using LC-MS-QToF.

Prelude to chapter 4

The spectra generated after LC-MS-QToF analysis were processed to obtain the final results about proteins produced by the cell when cultivated in simulated deep-sea conditions. This chapter describes the methods that were followed to deconvolute spectra to obtain the results about protein study.

Supplementary notes

3.SN1 Details of SDS-PAGE and gel composition (As mentioned in Mini-PROTEAN Tetra cell, Instruction Manual, Biorad, USA, Catalog number 165-8000)

The buffers used for SDS-PAGE and the composition of gels were detailed below.

1) Acrylamide/Bis-acrylamide at 29.2% : 0.8% distilled water.

Bis-acrylamide was N'N'-bis-methylene-acrylamide.

2) Resolving buffer was 1.5 M Tris-HCl at pH 8.8 (pH was adjusted using 6N HCl)
3) Stacking buffer was 0.5 M Tris-HCl at pH 6.8 (pH was adjusted using 6 N HCl).

While stacking and resolving buffers could be kept at 4^{o}C for long time storage (30 days), 10% APS (ammonium persulphate) was always prepared fresh. For a 10 ml gel volume, 50 µl of 10% APS was used for both stacking as well as resolving gels. Five and 10 µl of TEMED was used for resolving and stacking gels. Table 3.9 provided the composition of chemicals for casting SDS gels

Table 3.11: Composition of chemicals for gel casting (in ml)

% gel	Distilled water	Acrylamide/Bis	Gel volume	The volume of 10% SDS
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	2.4	5.0	2.5	0.1
16	2.1	5.3	2.5	0.1
17	1.7	5.7	2.5	0.1

3.SN2 Protocol for coomassie (G250) staining

Electrophoresed gel was washed twice with distilled water for 10 mins with constant shaking on the rocker. Followed by this, the gel was put in fixative for either an hour or over-night. Fixative for Coomassie staining was prepared by mixing ethanol (12 ml), glacial acetic acid (3 ml), and distilled water (15 ml). Coomassie stain (slightly modified from Neuhoff et al. 1988) was prepared fresh, without changing the sequence of chemicals during preparation. The constituents were ammonium sulphate (3 g), distilled water (30 ml), ethanol (7.5 ml), 5% Coomassie blue stain, G250 (0.6 ml) and orthophosphoric acid (0.36 ml). After fixative treatment, it was discarded; the gel was placed in the Coomassie stain prepared freshly, as described above. The gel was left in the stain overnight. Next day, the stain was discarded, and the gel was de-stained with distilled water until there was no extra dye left over the gel. Once clear bands were observed, the gel was documented by Alpha gel imager.

3.SN3 Protocol for silver staining: (Heukeshovan and Dernick 1985)

Electrophoresed gel was rinsed with fixative for minimum one hour or could be placed in fixative over-night. The fixative was 50% methanol, 12% glacial acetic acid, 0.5% formaldehyde, and distilled water for remaining proportion. The gel was washed with methanol, twice, for 20 mins for each duration of washing and was followed by rinsing in 0.02% sodium thiosulphate until the gel rehydrated; sodium thiosulphate was discarded. The gel was stained with silver stain solution for 30 mins in the dark. Silver stain was 0.2% silver nitrate, 75 µl formaldehyde in 100 ml distilled water. The silver stain was washed; developer was added on the gel until bands start appearing. The developer was 6 gm sodium carbonate, 50 µl formaldehyde, 40 µl of 10% sodium thiosulphate in 100 ml distilled water. Once clear brown bands appeared, the reaction was stopped by discarding the developer, and adding fixative. After rinsing the gel with fixative, fixative was discarded, and the stained gel was stored in distilled water. The gel was documented by Alpha gel imager.

Chapter FOUR

IDENTIFICATION OF PROTEINS EXPRESSED UNDER THE SIMULATED DEEP-SEA CONDITION

Chapter 4 – Objective 3

Identification of proteins expressed under the simulated deep-sea condition

The spectra generated from LC-MS-QToF were analysed for the identification of proteins. A variety of software can be used to process the spectra obtained after MS/MS analysis. A few of them are X!Tandem (Duncan et al. 2005), SEQUEST, Mascot, Byonic (Bern et al. 2007), Myrimatch (Tabb et al. 2007), OpenMS (Rost et al. 2016). Some software were tailor-made and developed based on necessity such as Andromeda (Cox et al. 2011, Max Planck Institute of Biochemistry, Germany), Comet (Eng et al. 2013, University of Washington, USA), Tide (University of Washington, USA), Greylag (Stowers Institute of Medical Research, USA), InsPec T (University of California, USA), MassWiz (Institute of Genomics and Integrative Biology, India), OMSSA (National Center for Biotechnology Information, USA) and Phenyx (Geneva Bioinformatics, Switzerland). Through protein identification, details regarding the characteristics of proteins, such as the name of the protein, molecular weight, pI, species, accession number can be revealed. When proteins obtained from pressure cultivated samples, were compared against proteins obtained from samples grown at normal pressure, proteins unique to pressure conditions were obtained.

The software to be used is most of the times based on the spectral file generated by a specific instrument and mostly are specific for specific types of data files. In this study, "Spectrum Mill MS Proteomics Workbench" rev B.04.01.141. was used for the generation of protein/peptide summary. Spectrum mill summarised datasets across multiple experiments and gave output at the protein level (Agilent Technologies, 5988-9443EN). The steps followed for spectral analysis were data-extraction, MS/MS analysis, autovalidation, generation of protein and peptide lists and generation of files required for statistical analysis. Data extractor performed the spectral preprocessing by extracting the spectra from the RAW data files, eliminated noise and poor quality data from being searched on databases (Agilent Technologies, 5988-9443EN). The processing depended on the data type which included averaging, centroiding, filtering by quality, assigning precursor charge and calculated the spectral features; it also disabled similarity merging to improve coverage and to detect more low-level peptides for simple samples. (Agilent Technologies, G2721-90028). Following data extraction, the total number of spectra generated from MS analysis, the total number of spectra taken for MS/MS analysis, and

the total mzXML file generated were recorded. The MS/MS search performed the search of spectra against MS/MS databases. It had improved dynamic peak thresholding which enabled the identification of more low-abundance and short-chain peptides; the explicit "mass gap" allowed the search of unexpected and unknown modifications; MS/MS auto-validation contributed to the peptide score which was necessary to generate protein/peptide summary; protein summary of the analysed spectra gave the protein grouping based on the shared peptides by summarising, reviewing, filtering and validating results from the MS/MS searches (Agilent Technologies, G2721-90028).

The statistical analysis of the generated proteins for comparison of regulations was performed by "Mass Profiler Professional" (MPP) version 13.0 build 210916 (Agilent Technologies, G3835-90000). MPP had filters for minimum absolute abundance and minimum relative abundance with RT and mass windows for compound alignment. The sample summary was obtained after these processes which gave the details of the compound present/absent when compared with the control experiments. The sample summary was evaluated, and on finding it satisfactory, the data was normalised with an internal standard or external scalar, and a baselining option was chosen to treat all compounds equally regardless of the intensity. This analysis generated the summary report which allowed grouping of the experiments/samples to run filters of compounds that were reported as present, absent or marginally present. The quality control of the experiments were checked on PCA plots. The fold-change cut off and p-value cut off were adjusted based on the control group that gave output as proteins, with details of fold change values. By comparing the values of fold change between control and experimental groups, proteins that were either up or down-regulated were identified.

By determining the placement of commonly regulated proteins in established pathway databases like KEGG, the pathways getting induced/altered/affected at various growth stages could be studied in detail. KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways were networks of protein interaction that includes a variety of cell processes (Kanehisa et al. 2002). This chapter also describes the organisation hierarchy in networks information. Based on this available information, the following analyses were performed

- *Generation of phylogenetic trees to finalise the closest matching organism for species-specific database search*
- *Processing the spectral data with data extractor*
- *Species-specific MS/MS search in NCBI and Swiss-Prot databases*
- *Autovalidation of the generated spectra*
- *Generation of peptide and protein lists*
- *Statistical analysis to check for the regulation of proteins*
- *Checking for the placement of commonly regulated proteins in the KEGG pathway database*

4.1 Generation of phylogenetic trees to finalise the closest matching organism for species-specific database search

Materials and methods:

All five isolates were grown in MEB for 48 h at room temperature and atmospheric pressure. Fresh mycelial mats were harvested. Genomic DNA was extracted using the Zymo Fungal/Bacterial DNA extraction kit as per the manufacturer's protocol. In short, homogenisation using zirconia beads was performed twice in FastPrep Homogeniser at 6.0 m s^{-1} for the 40 s, with an intermittent gap of 5 min during the crushing. Extraction efficiency was checked on 0.6% agarose by gel-electrophoresis with 1X TBE, 1kB ladder at 130 V for 20 min. For *A. terreus* (#A4636, #MTCC479), *A. flavus* (#NIOCC20) PCR was performed for 18s rDNA genes using NS1 (GTAGTCATATGCTTGTCTC) and NS4 (CTTCCGTCAATTCCTTTAAG) universal primers. For *Penicillium* sp (#A4623) and *A. sydowii* (#CH2), PCR was carried out using universal ITS primers (White et al. 1990) (ITS_1 - TCCGTAGGTGAACCTGCGG and ITS_4- TCCTCCGCTTATTGATATGC).

Table 4.1 provides details of the chemicals with the volume used for PCR. Tables 4.2 (a) and 4.2 (b) provide details of standardised PCR cycles.

Table 4.1: Reagent concentration for each reaction is as follows

Components for one PCR reaction	**Volume**
10X Taq buffer with 15mM $MgCl_2$	5 µl
10mM dNTP	1 µl
Forward primer (20pm/µL)	1 µl
Reverse primer (20pm/µL)	1 µl
Taq Polymerase 3U/µL	0.5 µl
DNA (50ng/µL; maximum of 140ng)	2-5 µl
Nano-pure water	36.5-39.5 µl
Total	50 µl

Steps from denaturation to renaturation was repeated 30 times for PCR involving 18s primers and 35 times for PCR involving ITS primers. The PCR product was checked on 1.2% agarose by gel-electrophoresis with 1X TBE, 100bp ladder at 130 V for 20 min.

The PCR product was purified using AxyPrep PCR clean-up kit with final elution performed in nano-pure water instead of the elution buffer of the kit. Quality of PCR product was checked in nanodrop. DNA sequencing was performed by the Genetic analyser 3130*xl* (ABI) based on the Big Dye Terminator v 3.1 (Chain terminator) chemistry, as per the manufacturer's protocol and instructions.

Table 4.2 (a) and 4.2 (b): PCR conditions for 18s and ITS primers respectively

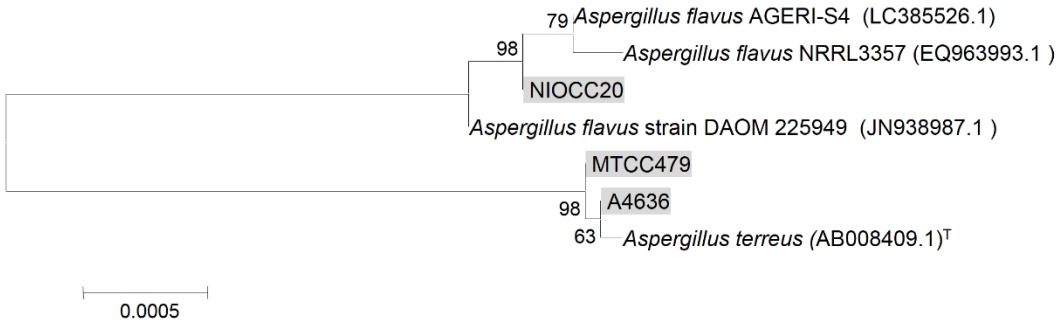
PCR Conditions (18s primer)	**Time**	**PCR Conditions (ITS primer)**	**Time**
Initial denaturation [98^{o}C]	5 min	Initial denaturation [98^{o}C]	5 min
Denaturation [95^{o}C]	1 min	Denaturation [95^{o}C]	1 min
Primer binding (Annealing) [54^{o}C]	45 s	Primer binding (Annealing) [52^{o}C]	45 s
Extension [72^{o}C]	45 s	Extension [72^{o}C]	1 min
Final Extension [72^{o}C]	10 min	Final Extension [72^{o}C]	10 min

Phylogenetic-analysis

The results of sequencing were matched with NCBI's GenBank. Based on the result, sequences of 18s rDNA/ITS genes of the same genus were obtained from libraries of culture collections databases such as NRRL culture collection, CBS database, CCINFO. Mega 5.05 software was used to construct phylogenetic trees (Tamura et al. 2011). Clustal W sequence alignment was used for sequence aligning (Thompson et al. 1994). Algorithms for the Neighbor-Joining method (Saitou and Nei 1987, Tamura et al. 2004) were used for phylogenetic-tree construction with boot-strap values set for 1000 replicates (Felsenstein 1985).

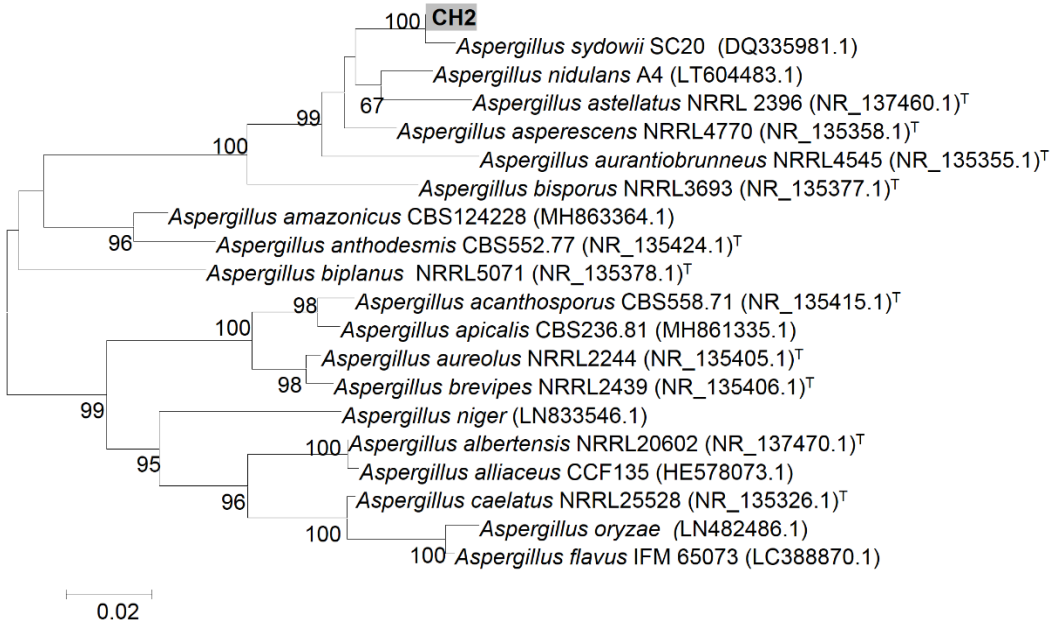
The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) were shown next to the branches. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and were in the units of the number of base substitutions per site. All ambiguous positions were removed for each sequence pair. Evolutionary analyses were conducted in MEGA5.05. Results of the phylogenetic analysis are shown in Figures 4.1, 4.2 and 4.3.

Figure 4.1: Neighbour-joining phylogenetic tree for rDNA 18s sequences (1000 bp)



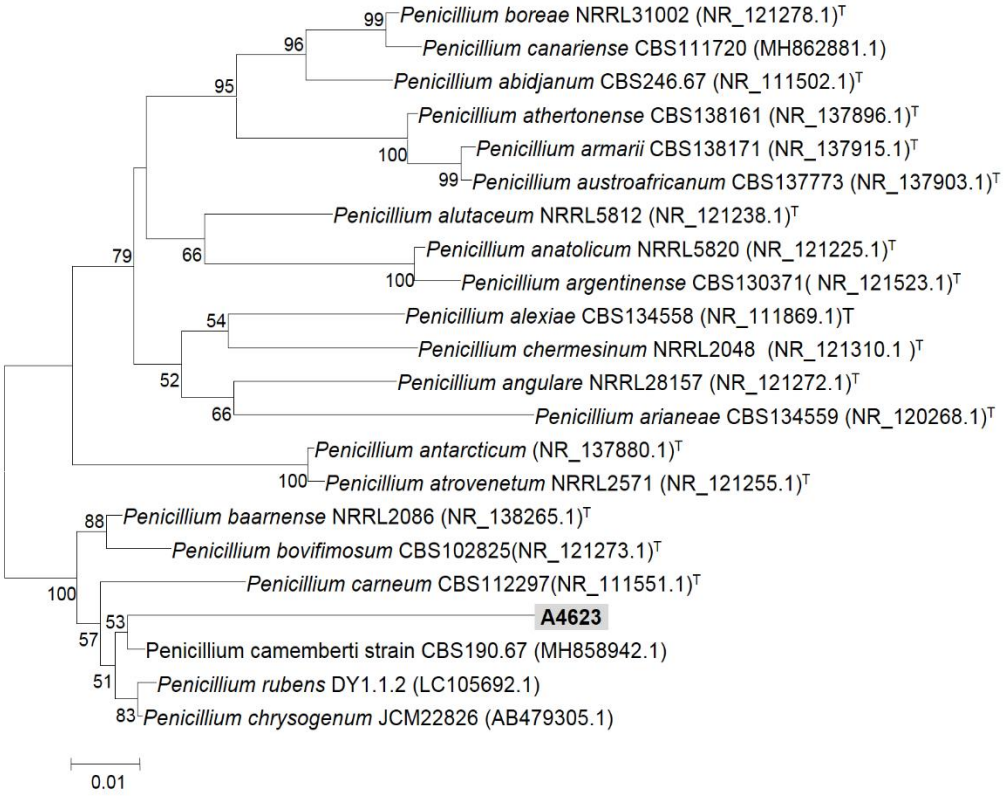
The optimal tree with the sum of branch length = 0.00411708 was shown. The analysis involved seven nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 2512 positions in the final dataset.

Figure 4.2: Neighbour-joining phylogenetic tree for rDNA ITS sequence (1000 bp)



The optimal tree with the sum of branch length = 0.58895381 was shown. The analysis involved 20 nucleotide sequences. There were a total of 679 positions in the final dataset.

Figure 4.3: Neighbour-joining phylogenetic tree for rDNA ITS sequence (1000 bp)



The optimal tree with the sum of branch length = 0.48327188 is shown. The analysis involved 22 nucleotide sequences. There were a total of 730 positions in the final dataset.

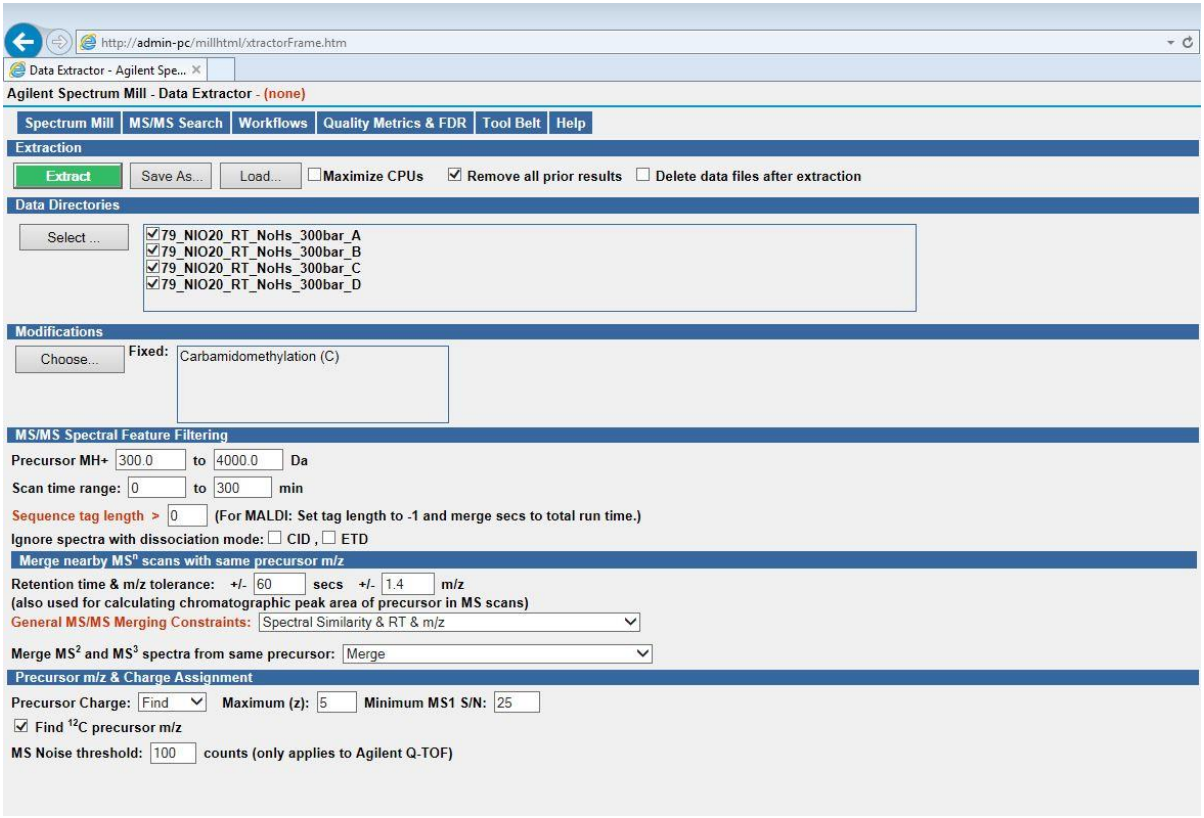
From Figures 4.1, 4.2 and 4.3, it was observed that all the isolates are well placed within the expected clusters having high similarity index. Therefore, the taxonomy ID of the organisms used for MS/MS search was - Swiss-Prot: *A. terreus* NIH2624, *A. flavus* NRRL 3357; NCBI: *A. terreus* txid_33178. *A. flavus* txid_5059. *A. sydowii* has a dedicated database, but the total number of reviewed and unreviewed proteins are low. Therefore, *Penicillium* sp. and *A. sydowii* were checked across the complete genus-specific database.

4.2 Processing the spectral data with data extractor

Methods:

All samples were processed with IAA, so carbamidomethylation was the only modification performed on the protein samples. Formic acid was added as adduct in organic as well as inorganic solvents. Hence, there was high protonation ion yield (MH+) that gave rise to readily detectable ions for MS/MS analysis even under low-energy collisions (Hua and Cole 2000). The MH+ range was set between 300-4000 Da. This analysis generated spectra that were employed for MS/MS analysis. A screenshot of data extraction is presented in Figure 4.4.

Figure 4.4: Screen-shot of data extraction before MS/MS analysis



Results:

The total number of spectra generated from MS analysis, the total number of spectra taken for MS/MS analysis, and the total mzXML file generated were recorded and are presented in Tables 4.3 to 4.10. TRN* represents Technical replicate number.

Table 4.3: Details of the total number of spectra generated for analysis for *A. terreus* (#A4636, mycelia), deep-sea isolate

Pressure	**Temperature**	**Total MS spectra**	**Total MS spectra triggered for MS/MS**	**MS/MS spectral file (mzXML)**	**TRN***
0.1 MPa	30ºC	4818	1634	1556	1
		4806	1615	1506	2
		4813	1783	1663	3
		4712	1580	1451	4
	4ºC	4224	364	177	1
		4245	448	212	2
		4349	360	222	3
		4232	394	167	4
10 MPa	30ºC	4796	1722	1637	1
		Error	Error	error	2
		4663	1401	1300	3
		4721	1537	1430	4
	4ºC	4521	1201	853	1
		4835	1333	1031	2
		4886	1194	1089	3
		4896	1207	1101	4
20 MPa	30ºC	4890	1524	1396	1
		4946	1796	1710	2
		5090	2073	1975	3
		4962	1784	1703	4
	4ºC	4235	531	254	1
		4345	581	451	2
		4315	531	401	3
		4320	579	429	4
45 MPa	30ºC	3383	1801	564	1
		3380	1797	599	2
		3385	1824	607	3
		3384	1899	496	4
	4ºC	4906	1309	1182	1
		4844	1101	989	2
		4922	1166	1088	3
		4918	1153	1084	4

Table 4.4: Details of the total number of spectra generated for analysis for *A. terreus* (#MTCC479, mycelia), terrestrial isolate

Pressure	**Temperature**	**Total MS spectra**	**Total MS spectra triggered for MS/MS**	**MS/MS spectral file (mzXML)**	**TRN***
0.1 MPa	30ºC	4612	1187	1036	1
		5017	1775	1657	2
		5047	2001	1892	3
		4924	1682	1581	4
	4ºC	4641	1022	872	1
		4784	1105	992	2
		4824	1148	1067	3
		4811	1152	1054	4
10 MPa	30ºC	5059	2040	1908	1
		5488	2653	2530	2
		5346	2574	2496	3
		5490	2701	2623	4
	4ºC	4269	414	264	1
		4296	363	253	2
		4309	359	300	3
		4312	413	318	4
20 MPa	30ºC	4676	1558	1417	1
		5096	2064	1992	2
		4937	1753	1694	3
		5030	1939	1876	4
	4ºC	4800	1302	1092	1
		4786	994	865	2
		4899	1220	1090	3
		4894	1172	1082	4
45 MPa	30ºC	3384	1798	494	1
		3384	1800	552	2
		3385	1882	777	3
		3384	1870	729	4
	4ºC	4395	778	590	1
		4537	817	673	2
		4591	924	837	3
		4584	895	812	4

Table 4.5: Details of the total number of spectra generated for analysis for *A. flavus* (#NIOCC20, mycelia), deep-sea isolate

Pressure	Temperature	Total MS spectra	Total MS spectra triggered for MS/MS	MS/MS spectral file (mzXML)	TRN*
0.1 MPa	30ºC	4891	1961	1927	1
		5016	1822	1742	2
		5273	2335	2251	3
		5024	1881	1821	4
	4ºC	4253	474	214	1
		4392	481	357	2
		4317	484	389	3
		4316	495	404	4
10 MPa	30ºC	4390	762	677	1
		4687	1231	1185	2
		4653	1171	1132	3
		4713	1265	1244	4
	4ºC	4273	559	344	1
		4428	688	581	2
		4407	679	586	3
		4424	692	588	4
20 MPa	30ºC	4482	886	772	1
		4557	1003	944	2
		4735	1286	1212	3
		4674	1168	1116	4
	4ºC	4235	384	166	1
		4261	349	221	2
		4263	319	218	3
		4261	342	216	4
45 MPa	30ºC	3385	1897	713	1
		3383	1586	705	2
		3381	1548	716	3
		3383	1528	774	4
	4ºC	4706	1214	974	1
		4749	1042	878	2
		4814	1503	1049	3
		4823	1359	1128	4

Table 4.6: Details of the total number of spectra generated for analysis for *Penicillium* sp. (#A4623, mycelia), deep-sea isolate

Pressure	Temperature	Total MS spectra	Total MS spectra triggered for MS/MS	MS/MS spectral file (mzXML)	TRN*
0.1 MPa	30ºC	3384	1468	991	1
		3385	1563	967	2
		3385	1651	1071	3
		3385	1660	1075	4
	4ºC	4277	434	272	1
		4258	379	225	2
		4211	525	43	3
		4210	601	46	4
10 MPa	30ºC	3385	1664	883	1
		3385	1691	1015	2
		3382	1707	1035	3
		3384	1792	1058	4
	4ºC	4389	774	490	1
		4393	764	555	2
		4398	777	569	3
		4383	787	549	4
20 MPa	30ºC	3384	1819	631	1
		3385	1812	1086	2
		3384	1744	959	3
		3385	1901	862	4
	4ºC	4716	1191	932	1
		4725	1146	971	2
		4686	1498	967	3
		4714	1098	956	4
45 MPa	30ºC	3385	2032	605	1
		3385	1706	670	2
		3384	1858	761	3
		3384	1844	730	4
	4ºC	4568	954	761	1
		4568	947	793	2
		4568	951	800	3
		4539	950	745	4

Table 4.7: Details of the total number of spectra generated for analysis for *A.sydowii* (#CH2, mycelia), deep-sea isolate

Pressure	Temperature	Total MS spectra	Total MS spectra triggered for MS/MS	MS/MS spectral file (mzXML)	TRN*
0.1 MPa	30ºC	3384	1612	1010	1
		3384	1949	640	2
		3385	1735	1008	3
		3385	1745	1139	4
	4ºC	4859	1380	960	1
		4951	1384	1082	2
		4958	1457	1128	3
		4964	1426	1111	4
10 MPa	30ºC	3384	1781	767	1
		3385	1639	1024	2
		3384	1739	1090	3
		3385	1796	1000	4
	4ºC	4206	1009	61	1
		4195	994	47	2
		4428	703	551	3
		4199	801	56	4
20 MPa	30ºC	3385	1797	568	1
		3384	1700	824	2
		3385	1696	545	3
		3385	1846	515	4
	4ºC	4785	1252	1003	1
		4800	1238	1030	2
		4788	1269	1021	3
		4795	1240	1008	4
45 MPa	30ºC	3385	1854	563	1
		3384	1773	746	2
		3384	1891	813	3
		3384	1928	807	4
	4ºC	4737	1138	994	1
		4739	1153	996	2
		4746	1115	1003	3
		4748	1092	981	4

Table 4.8: Details of the total number of spectra generated for analysis for *A. terreus* (#A4636, germinating conidia), deep-sea isolate

Heat treatment	Temperature	Pressure	Total MS spectra	Total MS spectra triggered for MS/MS	MS/MS spectral file (mzXML)	TRN
Ungerminated/dormant spore			5079	1949	1756	1
Ungerminated/dormant spore			Error	Error	error	2
Ungerminated/dormant spore			Error	Error	error	3
Ungerminated/dormant spore			Error	Error	error	4
Yes	4°C	10MPa	4641	1383	1351	1
			4605	1262	1245	2
			4613	1274	1254	3
			4590	1221	1206	4
		20MPa	4508	1010	993	1
			4390	702	691	2
			4496	970	958	3
			4498	937	919	4
		30MPa	4555	1135	1118	1
			4517	1095	1082	2
			4521	1090	1078	3
			4526	1233	1104	4
	30°C	10MPa	4512	1068	944	1
			4560	1139	1029	2
			4516	1098	1023	3
			4516	1062	997	4
		20MPa	4412	825	786	1
			4390	794	735	2
			4392	794	752	3
			4349	724	689	4
		30MPa	4375	733	718	1
			4347	668	632	2
			4352	676	649	3
			4341	637	598	4
No	4°C	10MPa	4294	433	405	1
			4317	407	386	2
			4306	397	383	3
			4297	423	412	4
		20MPa	4411	767	735	1
			4372	566	513	2
			4350	540	487	3
			4351	527	484	4
		30MPa	4377	680	672	1
			4386	535	501	2
			4342	499	475	3
			4351	499	483	4

Heat treatment	Temperature	Pressure	Total MS spectra	Total MS spectra triggered for MS/MS	MS/MS spectral file (mzXML)	TRN
No	30ºC	10MPa	4565	1268	1143	1
			4379	650	504	2
			4339	727	625	3
			4334	659	568	4
		20MPa	4466	1027	874	1
			4359	581	453	2
			4327	594	491	3
			4265	175	132	4
		30MPa	4563	1262	1094	1
			4366	627	482	2
			4352	685	558	3
			4454	974	802	4

Table 4.9: Details of the total number of spectra generated for analysis for *A. terreus* (#MTCC479, germinating conidia), terrestrial isolate

Heat treatment	Temperature	Pressure	Total MS spectra	Total MS spectra triggered for MS/MS	MS/MS spectral file (mzXML)	TRN
Ungerminated/dormant spore			5086	1341	1079	1
Ungerminated/dormant spore			5064	1365	1131	2
Ungerminated/dormant spore			5079	1404	1156	3
Ungerminated/dormant spore			5096	1388	1184	4
Yes	4ºC	10MPa	4596	1229	1213	1
			4389	855	746	2
			4362	768	670	3
			4395	843	722	4
		20MPa	4675	1564	1451	1
			4371	839	671	2
			4467	1189	976	3
			4507	1089	994	4
		30MPa	4685	1440	1381	1
			4500	1129	1025	2
			4495	1108	1018	3
			4501	1100	1009	4
	30ºC	10MPa	4727	1590	1419	1
			4667	1352	1219	2
			4648	1219	1119	3
			4567	909	848	4
		20MPa	4951	1933	1828	1
			4713	1219	1144	2
			4790	1531	1427	3
			4687	1118	1036	4

Heat treatment	Temperature	Pressure	Total MS spectra	Total MS spectra triggered for MS/MS	MS/MS spectral file (mzXML)	TRN
Yes	30°C	30MPa	4658	1366	1288	1
			4495	948	875	2
			4503	1028	963	3
			4519	924	866	4
No	4°C	10MPa	4374	605	600	1
			4492	767	751	2
			4272	243	211	3
			4283	280	274	4
		20MPa	4254	63	61	1
			4332	551	531	2
			4327	538	524	3
			4328	534	519	4
		30MPa	4509	841	810	1
			4487	923	912	2
			4496	950	941	3
			4487	924	914	4
	30°C	10MPa	4858	1694	1542	1
			NA	NA	NA	2
			NA	NA	NA	3
			NA	NA	NA	4
		20MPa	4902	1783	1594	1
			NA	NA	NA	2
			NA	NA	NA	3
			NA	NA	NA	4
		30MPa	4842	1654	1451	1
			NA	NA	NA	2
			NA	NA	NA	3
			NA	NA	NA	4

Table 4.10: Details of the total number of spectra generated for analysis for *A. flavus* (#NIOCC20, germinating conidia), deep-sea isolate

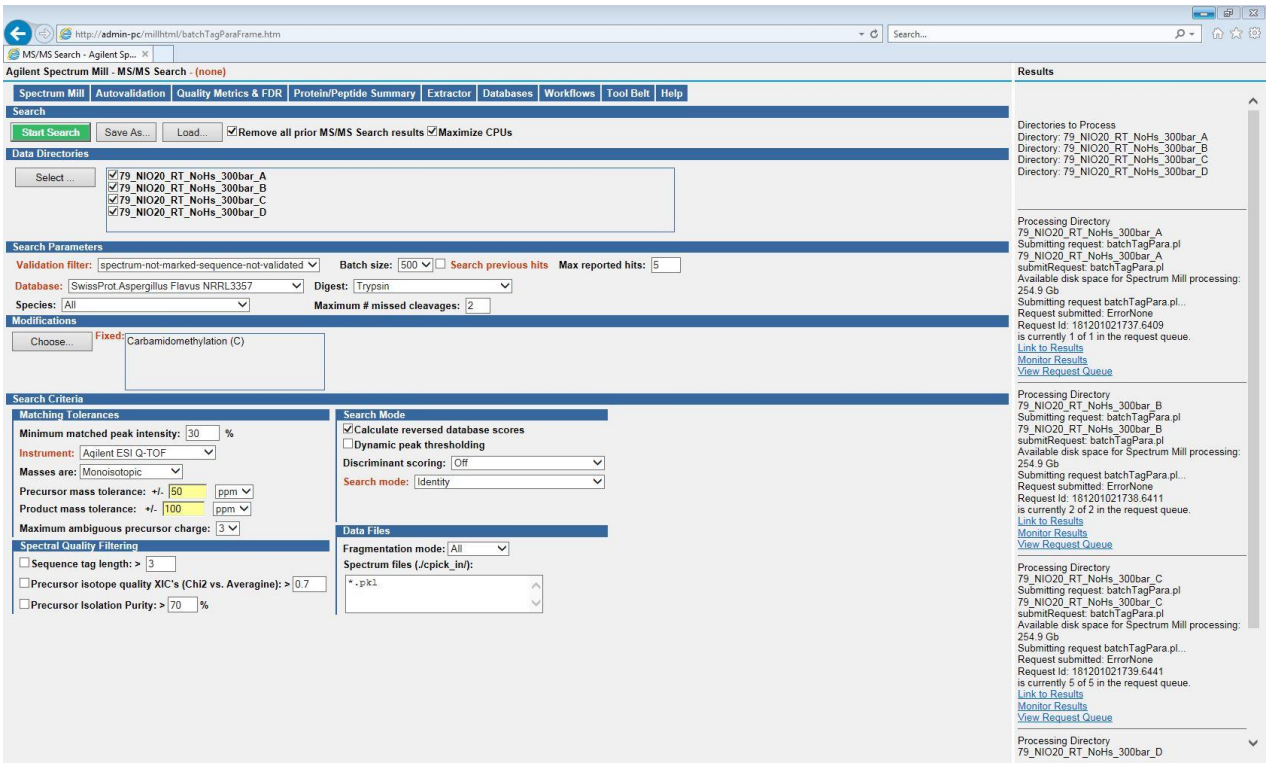
Heat treatment	Temperature	Pressure	Total MS spectra	Total MS spectra triggered for MS/MS	MS/MS spectral file (mzXML)	TRN
Ungerminated/dormant spore			4908	1046	959	1
Ungerminated/dormant spore			4896	1031	946	2
Ungerminated/dormant spore			4905	1021	940	3
Ungerminated/dormant spore			4905	1023	973	4
Yes	4°C	10MPa	4623	1296	1281	1
			4540	1052	1038	2
			4546	1072	1056	3
			4554	1087	1072	4

Heat treatment	Temperature	Pressure	Total MS spectra	Total MS spectra triggered for MS/MS	MS/MS spectral file (mzXML)	TRN
Yes	4ºC	20MPa	4642	1252	1241	1
			4435	774	764	2
			4566	1090	1077	3
			4554	1060	1045	4
		30MPa	4562	1141	1121	1
			4475	930	914	2
			4471	922	917	3
			4472	907	899	4
	30ºC	10MPa	4826	1685	1600	1
			4635	1123	1015	2
			4669	1292	1200	3
			4658	1233	1152	4
		20MPa	4677	1408	1320	1
			4534	945	892	2
			4575	1105	1044	3
			4570	1079	1021	4
		30MPa	4335	612	586	1
			4293	327	309	2
			4294	405	381	3
			4289	393	367	4
No	4ºC	10MPa	4463	807	713	1
			4426	771	740	2
			4460	844	813	3
			4441	816	793	4
		20MPa	4281	224	197	1
			4274	261	256	2
			4268	248	244	3
			4271	247	245	4
		30MPa	4309	355	313	1
			4296	369	353	2
			4286	354	340	3
			4291	347	336	4
	30ºC	10MPa	4862	1699	1548	1
			4833	1627	1428	2
			4894	1699	1539	3
			4912	1713	1551	4
		20MPa	5019	2007	1881	1
			4878	1624	1498	2
			4937	1717	1596	3
			4918	1692	1573	4
		30MPa	4548	1072	950	1
			4493	840	715	2
			4524	1025	905	3
			4447	897	751	4

4.3 Species-specific MS/MS search in NCBI and Swiss-Prot databases

MS/MS search was possible only when the sample had been digested by an enzyme whose cleavage sites were known; each spectrum corresponded to a single peptide/protein; the values of MS/MS search was set based on the results, analysis, and discussions of Cottrell 2011. The taxonomy ID of the organism as per Swiss-Prot was *A. terreus* NIH2624, *A. flavus* NRRL 3357; as per NCBI was *A. terreus* txid_33178 and *A. flavus* txid_5059. *Penicillium* sp. and *A. sydowii* were checked across the complete genus-specific database for all the searches that were performed as their number of reviewed proteins were less than 100 (as on 5th Oct 2017). MS/MS search was conducted five times for each replicate of every analyte. While the first two times were against the entire database of NCBI and Swiss-Prot, third and fourth searches were against organism-specific database search. The first four searches had precursor and product mass tolerance of 50 and 100 ppm respectively, the fifth search was against an organism-specific database with precursor and product mass tolerance of 100 and 200 ppm respectively. During each MS/MS search, all the previous MS/MS search results were removed to avoid the inter-mixing of the results. Screenshot of the MS/MS analysis is presented in Figure 4.5.

Figure 4.5: Sample screen-shot of MS/MS analysis



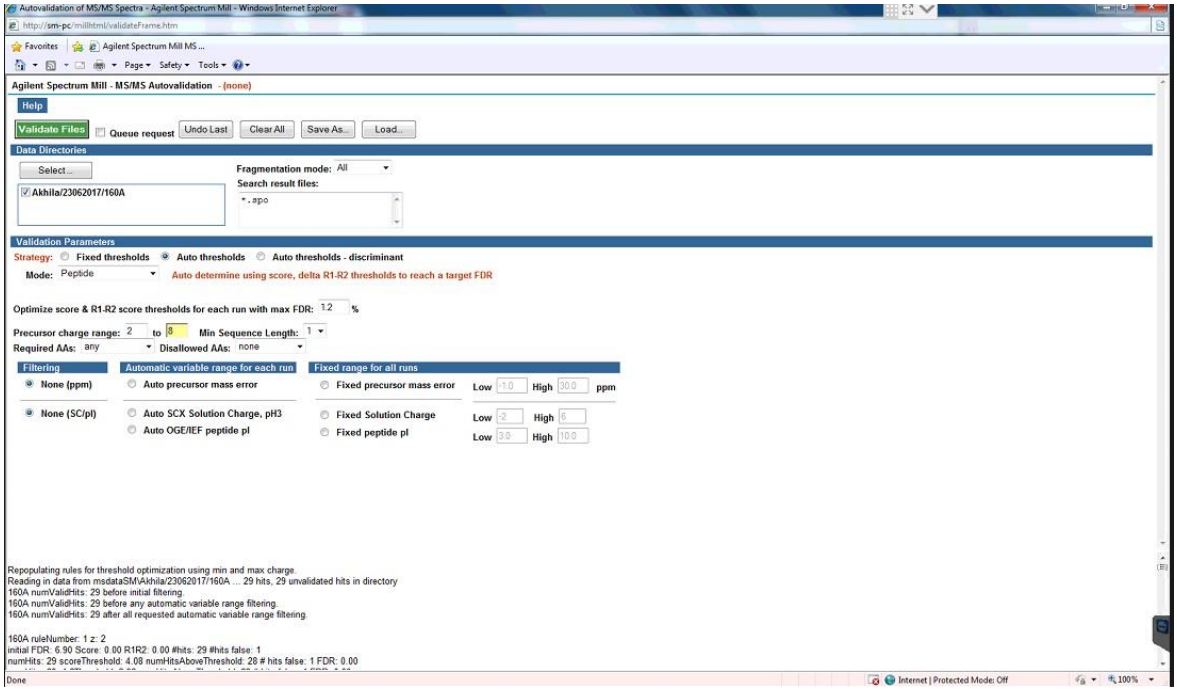
On comparison of results obtained as peptide and protein summary, an organism-specific database search for *A. terreus* (A4636, MTCC 479), *A. flavus* (NIOCC20) and genus-specific database search for *Penicillium* sp. (A4623) and *A. sydowii* (CH2) were standardised with precursor and product mass tolerance set between 50 and 100 ppm.

Reviewed proteins implied that the proteins were manually annotated before being uploaded in UniProtKB/Swiss-Prot whereas unreviewed proteins implied that they were computer-annotated (The UniProt Consortium, 2011). As projects for removing similar proteomes that were causing redundancy were implemented in UniProtKB Swiss-Prot, accession number provided a function for any specific protein (The UniProt Consortium, 2017). Swiss-Prot organism-specific MS/MS search was finalised for *A. terreus* (#A4636, #MTCC479) and *A. flavus* (#NIOCC20); Swiss-Prot genus-specific MS/MS search was standardised for *Penicillium* sp. (#A4623), and *A. sydowii* (#CH2) as the accession number and the function of protein were linked.

4.4 Autovalidation of the generated spectra

Autovalidation was performed for technical replicates of mycelial as well as spore samples. The total number of validated protein hits, false protein hits, and false discovery rate (FRD) per cent have been included for better understanding. FDR was the expected number of false-positive results that could be anticipated from experiments. FDR rates below 0.05 implied that the false positive results were below 5% which validates the analysis. In the case of "autovalidation," the spectra generated were checked for the accuracy and FDR. "False hits" numbers implied the total number of spectra that cannot be validated and hence cannot be checked for FDR. (Agilent G2721AA/G2733AA Spectrum Mill MS Proteomics Workbench). Figure 4.6 was a screenshot while performing autovalidation

Figure 4.6: Screenshot while performing autovalidation



Results of autovalidation for all the samples have been tabulated from Tables 4.11 to 4.18. TDR represented Technical replicate number, and FDR represented false discovery rate.

Table 4.11: Details of autovalidation of *A. terreus* (#A4636, mycelia), a deep-sea isolate, under different pressure and temperature conditions

Pressure	**Temperature**	**No of validated hits**	**False hits**	**FDR (%)**	**TRN***
0.1 MPa	30°C	250	0	0	1
		234	0	0	2
		292	0	0	3
		231	0	0	4
	4°C	22	0	0	1
		29	0	0	2
		18	0	0	3
		11	0	0	4
10 MPa	30°C	469	1	0.43	1
		0	0	0	2
		301	0	0	3
		324	1	0.62	4
	4°C	270	0	0	1
		416	1	0.48	2
		473	2	0.85	3
		480	2	0.83	4
20 MPa	30°C	376	1	0.53	1
		330	1	0.61	2
		438	2	0.91	3
		314	0	0	4
	4°C	31	0	0	1
		50	0	0	2
		54	0	0	3
		55	0	0	4
45 MPa	30°C	9	0	0	1
		6	0	0	2
		9	0	0	3
		7	0	0	4
	4°C	151	0	0	1
		163	0	0	2
		199	0	0	3
		175	0	0	4

Table 4.12: Details of autovalidation of *A. terreus* (#MTCC479, mycelia), a terrestrial isolate, under different pressure and temperature conditions

Pressure	Temperature	No of validated hits	False hits	FDR (%)	TRN*
0.1 MPa	30°C	215	0	0	1
		291	0	0	2
		266	0	0	3
		176	0	0	4
	4°C	134	0	0	1
		130	0	0	2
		178	0	0	3
		158	0	0	4
10 MPa	30°C	622	2	0.64	1
		691	2	0.58	2
		615	2	0.65	3
		649	2	0.62	4
	4°C	8	0	0	1
		8	0	0	2
		11	0	0	3
		10	0	0	4
20 MPa	30°C	356	0	0	1
		380	1	0.53	2
		244	0	0	3
		345	0	0	4
	4°C	439	2	0.91	1
		419	2	0.95	2
		523	2	0.76	3
		502	2	0.8	4
45 MPa	30°C	18	0	0	1
		8	0	0	2
		6	0	0	3
		7	0	0	4
	4°C	108	0	0	1
		197	0	0	2
		180	0	0	3
		167	0	0	4

Table 4.13: Details of autovalidation of *A. flavus* (#NIOCC20, mycelia), a deep-sea isolate, under different pressure and temperature conditions

Pressure	**Temperature**	**No of validated hits**	**False hits**	**FDR (%)**	**TRN***
0.1 MPa	30°C	395	1	0.51	1
		443	2	0.9	2
		543	2	0.74	3
		332	0	0	4
	4°C	9	0	0	1
		24	0	0	2
		15	0	0	3
		19	0	0	4
10 MPa	30°C	131	0	0	1
		158	0	0	2
		174	0	0	3
		204	0	0	4
	4°C	38	0	0	1
		85	0	0	2
		83	0	0	3
		95	0	0	4
20 MPa	30°C	127	0	0	1
		99	0	0	2
		141	0	0	3
		127	0	0	4
	4°C	11	0	0	1
		15	0	0	2
		13	0	0	3
		17	0	0	4
45 MPa	30°C	48	0	0	1
		24	0	0	2
		24	0	0	3
		21	0	0	4
	4°C	156	0	0	1
		146	0	0	2
		167	0	0	3
		185	0	0	4

Table 4.14: Details of autovalidation of *Penicillium* sp. (#A4623, mycelia), a deep-sea isolate, under different pressure and temperature conditions

Pressure	Temperature	No of validated hits	False hits	FDR (%)	TRN*
0.1 MPa	30ºC	62	0	0	1
		64	0	0	2
		73	0	0	3
		59	0	0	4
	4ºC	22	0	0	1
		21	0	0	2
		1	0	0	3
		1	0	0	4
10 MPa	30ºC	2	0	0	1
		6	0	0	2
		6	0	0	3
		6	0	0	4
	4ºC	76	0	0	1
		84	0	0	2
		91	0	0	3
		101	0	0	4
20 MPa	30ºC	4	0	0	1
		3	0	0	2
		1	0	0	3
		0	0	0	4
	4ºC	205	0	0	1
		192	0	0	2
		210	0	0	3
		204	0	0	4
45 MPa	30ºC	2	0	0	1
		1	0	0	2
		4	0	0	3
		3	0	0	4
	4ºC	119	0	0	1
		114	0	0	2
		128	0	0	3
		99	0	0	4

Table 4.15: Details of autovalidation of *A. sydowii* (#CH2, mycelia), a deep-sea isolate, under different pressure and temperature conditions

Pressure	Temperature	No of validated hits	False hits	FDR (%)	TRN*
0.1 MPa	30ºC	22	0	0	1
		0	0	0	2
		25	0	0	3
		32	0	0	4
	4ºC	140	0	0	1
		163	0	0	2
		150	0	0	3
		160	0	0	4
10 MPa	30ºC	7	0	0	1
		4	0	0	2
		11	0	0	3
		7	0	0	4
	4ºC	2	0	0	1
		0	0	0	2
		51	0	0	3
		1	0	0	4
20 MPa	30ºC	7	0	0	1
		11	0	0	2
		10	0	0	3
		10	0	0	4
	4ºC	76	0	0	1
		65	0	0	2
		88	0	0	3
		69	0	0	4
45 MPa	30ºC	4	0	0	1
		5	0	0	2
		5	0	0	3
		3	0	0	4
	4ºC	91	0	0	1
		82	0	0	2
		95	0	0	3
		98	0	0	4

Table 4.16: Details of autovalidation of *A. terreus* (#A4636, germinating conidia), deep-sea isolate, under different pressure and temperature conditions

Heat treatment	Temperature	Pressure	No of validated hits	False hits	FDR (%)	TRN*
Ungerminated/dormant spore			116	0	0	1
Ungerminated/dormant spore			--	0	0	2
Ungerminated/dormant spore			--	0	0	3
Ungerminated/dormant spore			--	0	0	4
Yes	4°C	10 MPa	25	0	0	1
			33	0	0	2
			23	0	0	3
			24	0	0	4
		20 MPa	22	0	0	1
			12	0	0	2
			15	0	0	3
			18	0	0	4
		30 MPa	16	0	0	1
			16	0	0	2
			11	0	0	3
			6	0	0	4
	30°C	10 MPa	71	0	0	1
			84	0	0	2
			81	0	0	3
			77	0	0	4
		20 MPa	0	0	0	1
			0	0	0	2
			0	0	0	3
			0	0	0	4
		30 MPa	40	0	0	1
			32	0	0	2
			37	0	0	3
			33	0	0	4
No	4°C	10 MPa	3	0	0	1
			3	0	0	2
			4	0	0	3
			4	0	0	4
		20 MPa	8	0	0	1
			2	0	0	2
			2	0	0	3
			3	0	0	4

Heat treatment	Temperature	Pressure	No of validated hits	False hits	FDR (%)	TRN*
No	4°C	30 MPa	7	0	0	1
			6	0	0	2
			6	0	0	3
			10	0	0	4
	30°C	10 MPa	68	0	0	1
			77	0	0	2
			82	0	0	3
			63	0	0	4
		20 MPa	55	0	0	1
			61	0	0	2
			55	0	0	3
			15	0	0	4
		30 MPa	88	0	0	1
			66	0	0	2
			60	0	0	3
			64	0	0	4

Table 4.17: Details of autovalidation of *A. terreus* (#MTCC479, germinating conidia), terrestrial isolate, under different pressure and temperature conditions

Heat treatment	Temperature	Pressure	No of validated hits	False hits	FDR (%)	TRN*
Ungerminated/dormant spore			429	2	0.93	1
Ungerminated/dormant spore			394	1	0.51	2
Ungerminated/dormant spore			397	1	0.5	3
Ungerminated/dormant spore			423	2	0.95	4
Yes	4°C	10 MPa	18	0	0	1
			22	0	0	2
			17	0	0	3
			13	0	0	4
		20 MPa	31	0	0	1
			16	0	0	2
			9	0	0	3
			29	0	0	4
		30 MPa	12	0	0	1
			46	0	0	2
			28	0	0	3
			40	0	0	4
	30°C	10 MPa	209	0	0	1
			163	0	0	2
			196	0	0	3
			166	0	0	4
		20 MPa	302	0	0	1
			239	0	0	2
			246	0	0	3
			203	0	0	4
		30 MPa	133	0	0	1
			103	0	0	2
			100	0	0	3
			109	0	0	4
No	4°C	10 MPa	9	0	0	1
			4	0	0	2
			0	0	0	3
			2	0	0	4
		20 MPa	0	0	0	1
			4	0	0	2
			3	0	0	3
			2	0	0	4
		30 MPa	23	0	0	1
			3	0	0	2
			6	0	0	3

Heat treatment	Temperature	Pressure	No of validated hits	False hits	FDR (%)	TRN*
No	4ºC	30 MPa	8	0	0	4
	30ºC	10 MPa	216	0	0	1
			117	0	0	2
			107	0	0	3
			74	0	0	4
		20 MPa	257	0	0	1
			110	0	0	2
			149	0	0	3
			150	0	0	4
		30 MPa	128	0	0	1
			91	0	0	2
			53	0	0	3
			46	0	0	4

Table 4.18: Details of autovalidation of *A. flavus* (#NIOCC20, germinating conidia), a deep-sea isolate, with details of protein hits under different pressure and temperature conditions

Heat treatment	Temperature	Pressure	No of validated hits	False hits	FDR (%)	TRN*
Ungerminated/dormant spore			433	2	0.92	1
Ungerminated/dormant spore			382	1	0.52	2
Ungerminated/dormant spore			369	1	0.54	3
Ungerminated/dormant spore			359	1	0.56	4
Yes	4ºC	10 MPa	35	0	0	1
			31	0	0	2
			27	0	0	3
			16	0	0	4
		20 MPa	33	0	0	1
			23	0	0	2
			31	0	0	3
			32	0	0	4
		30 MPa	10	0	0	1
			8	0	0	2
			12	0	0	3
			13	0	0	4
	30ºC	10 MPa	241	0	0	1
			186	0	0	2
			224	0	0	3
			199	0	0	4
		20 MPa	132	0	0	1
			128	0	0	2
			141	0	0	3
			115	0	0	4
		30 MPa	17	0	0	1
			10	0	0	2
			10	0	0	3
			9	0	0	4
No	4ºC	10 MPa	10	0	0	1
			2	0	0	2
			7	0	0	3
			5	0	0	4
		20 MPa	4	0	0	1
			1	0	0	2
			2	0	0	3
			2	0	0	4
		30 MPa	5	0	0	1
			1	0	0	2
			3	0	0	3

Heat treatment	Temperature	Pressure	No of validated hits	False hits	FDR (%)	TRN*
No	4ºC	30 MPa	1	0	0	4
	30ºC	10 MPa	147	0	0	1
			100	0	0	2
			111	0	0	3
			114	0	0	4
		20 MPa	356	0	0	1
			274	0	0	2
			276	0	0	3
			271	0	0	4
		30 MPa	95	0	0	1
			61	0	0	2
			59	0	0	3
			63	0	0	4

4.5 Generation of peptide and protein lists

The total number of protein hits that were generated against MS/MS search of organism-specific databases for *A. terreus*, *A. flavus* (#A4636, #MTCC479, #NIOCC20) and genus-specific database for *Penicillium* sp. and *A. sydowii* (#A4623, #CH2) have been tabulated in Tables 4.19, 4.20.

As seen from table 4.20, some results of spore germination studies had few protein hits, ranging in a single digit numeral. This reduction in the number of protein hits could be due to the lack of protein level information (Cottrell 2013), as corroborated from the results of spectral numbers during data extraction. Figure 4.7 is a representative screen shot of generating protein summary.

Figure 4.7: Screenshot while generating protein summary

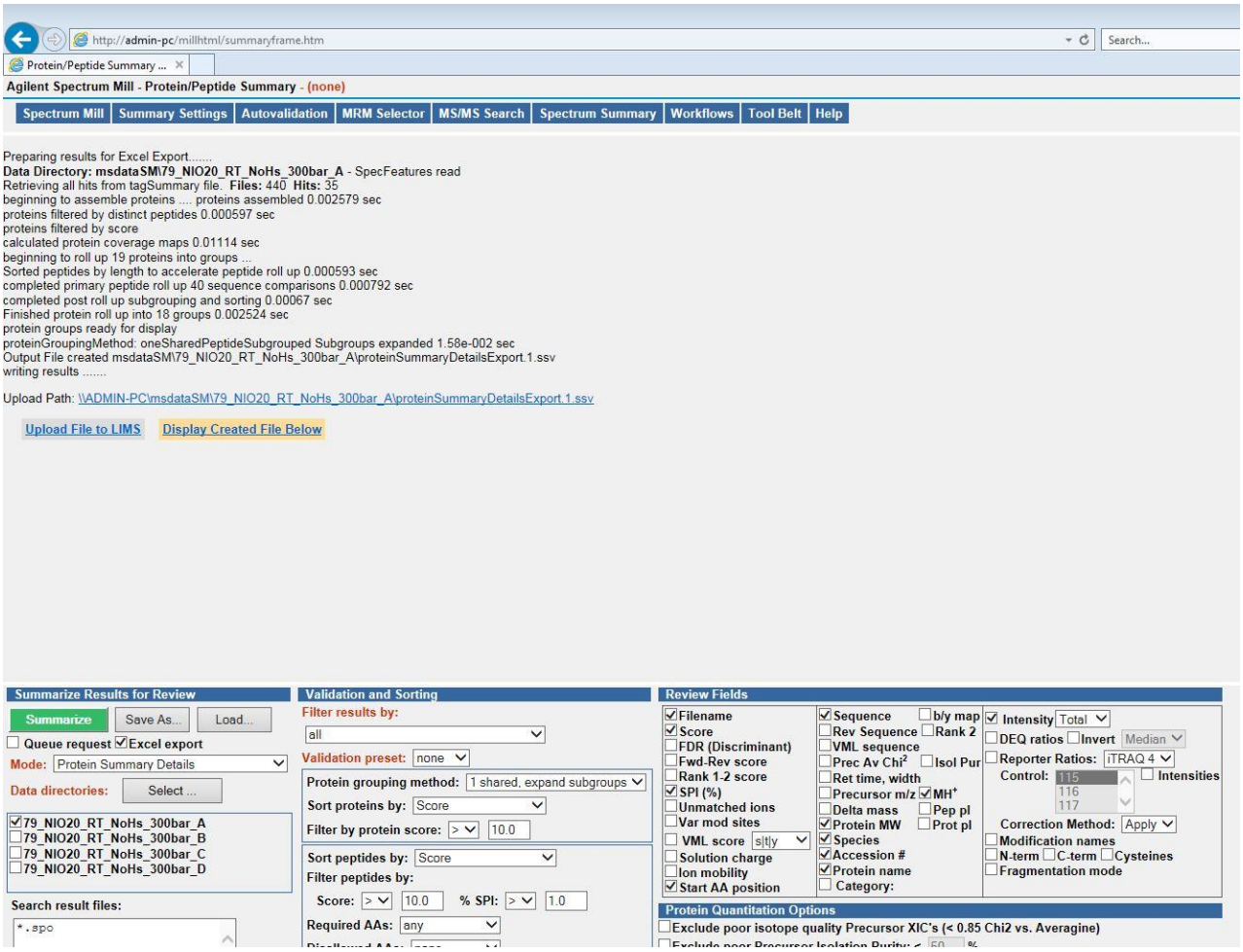


Table 4.19: Protein hits obtained for each mycelial sample

Sample number	Description	Temperature	Pressure (MPa)	Total number of protein hits
1	*A. terreus*_A4636	30°C	0.1	211
2	*A. terreus*_MTCC479	30°C	0.1	169
3	*A.flavus*_NIOCC20	30°C	0.1	236
4	*Penicillium* sp_A4623	30°C	0.1	147
5	*A.sydowii*_CH2	30°C	0.1	126
6	*A.terreus*_A4636	30°C	10	133
7	*A.terreus*_MTCC479	30°C	10	273
8	*A.flavus*_NIOCC20	30°C	10	64
9	*Penicillium* sp_A4623	30°C	10	116
10	*A.sydowii*_CH2	30°C	10	198
11	*A.terreus*_A4636	30°C	20	84
12	*A.terreus*_MTCC479	30°C	20	155
13	*A.flavus*_NIOCC20	30°C	20	72
14	*Penicillium* sp_A4623	30°C	20	22
15	*A.sydowii*_CH2	30°C	20	62
16	*A.terreus*_A4636	30°C	45	14
17	*A.terreus*_MTCC479	30°C	45	20
18	*A.flavus*_NIOCC20	30°C	45	44
19	*Penicillium* sp_A4623	30°C	45	23
20	*A.sydowii*_CH2	30°C	45	26
21	*A.terreus*_A4636	4°C	0.1	32
22	*A.terreus*_MTCC479	4°C	0.1	244
23	*A.flavus*_NIOCC20	4°C	0.1	61
24	*Penicillium* sp_A4623	4°C	0.1	41
25	*A.sydowii*_CH2	4°C	0.1	355
26	*A.terreus*_A4636	4°C	10	209
27	*A.terreus*_MTCC479	4°C	10	48
28	*A.flavus*_NIOCC20	4°C	10	91
29	*Penicillium* sp_A4623	4°C	10	147
30	*A.sydowii*_CH2	4°C	10	68
31	*A.terreus*_A4636	4°C	20	50
32	*A.terreus*_MTCC479	4°C	20	198
33	*A.flavus*_NIOCC20	4°C	20	45
34	*Penicillium* sp_A4623	4°C	20	411
35	*A.sydowii*_CH2	4°C	20	503
36	*A.terreus*_A4636	4°C	45	404
37	*A.terreus*_MTCC479	4°C	45	116
38	*A.flavus*_NIOCC20	4°C	45	285
39	*Penicillium* sp_A4623	4°C	45	284
40	*A.sydowii*_CH2	4°C	45	363

Table 4.20: Protein hits obtained for each spore sample

Sample number	Description	Temperature	Pressure (MPa)	Heat treatment	Total number of protein hits
41	*A.terreus*_A4636_ Ungerminated	NA	NA	NA	107
42	*A.terreus*_MTCC479_ Ungerminated	NA	NA	NA	268
43	*A.flavus*_NIOCC20_ Ungerminated	NA	NA	NA	234
44	*A.terreus*_A4636_spore	4ºC	10	Yes	94
45	*A.terreus*_MTCC479_spore	4ºC	10	Yes	62
46	*A.flavus*_NIOCC20_spore	4ºC	10	Yes	74
47	*A.terreus*_A4636_spore	4ºC	20	Yes	61
48	*A.terreus*_MTCC479_spore	4ºC	20	Yes	74
49	*A.flavus*_NIOCC20_spore	4ºC	20	Yes	61
50	*A.terreus*_A4636_spore	4ºC	30	Yes	41
51	*A.terreus*_MTCC479_spore	4ºC	30	Yes	84
52	*A.flavus*_NIOCC20_spore	4ºC	30	Yes	38
53	*A.terreus*_A4636_spore	4ºC	10	No	8
54	*A.terreus*_MTCC479_spore	4ºC	10	No	10
55	*A.flavus*_NIOCC20_spore	4ºC	10	No	20
56	*A.terreus*_A4636_spore	4ºC	20	No	10
57	*A.terreus*_MTCC479_spore	4ºC	20	No	6
58	*A.flavus*_NIOCC20_spore	4ºC	20	No	7
59	*A.terreus*_A4636_spore	4ºC	30	No	19
60	*A.terreus*_MTCC479_spore	4ºC	30	No	32
61	*A.flavus*_NIOCC20_spore	4ºC	30	No	5
62	*A.terreus*_A4636_spore	30ºC	10	Yes	72
63	*A.terreus*_MTCC479_spore	30ºC	10	Yes	125
64	*A.flavus*_NIOCC20_spore	30ºC	10	Yes	144
65	*A.terreus*_A4636_spore	30ºC	20	Yes	39
66	*A.terreus*_MTCC479_spore	30ºC	20	Yes	158
67	*A.flavus*_NIOCC20_spore	30ºC	20	Yes	111
68	*A.terreus*_A4636_spore	30ºC	30	Yes	36
69	*A.terreus*_MTCC479_spore	30ºC	30	Yes	102
70	*A.flavus*_NIOCC20_spore	30ºC	30	Yes	17
71	*A.terreus*_A4636_spore	30ºC	10	No	38
72	*A.terreus*_MTCC479_spore	30ºC	10	No	69
73	*A.flavus*_NIOCC20_spore	30ºC	10	No	84
74	*A.terreus*_A4636_spore	30ºC	20	No	45
75	*A.terreus*_MTCC479_spore	30ºC	20	No	73
76	*A.flavus*_NIOCC20_spore	30ºC	20	No	151
77	*A.terreus*_A4636_spore	30ºC	30	No	57
78	*A.terreus*_MTCC479_spore	30ºC	30	No	60
79	*A.flavus*_NIOCC20_spore	30ºC	30	No	47

4.6 Statistical analysis to check for the regulation of proteins

The variability in threshold levels checks the expression of the gene; as threshold depends on experimental accuracy and precision (Mariani et al. 2003), only technical replicates were used in this study. Therefore, by checking for fold-change value between samples with one variable parameter, the unique protein to a particular condition can be detected. The minimum threshold for the fold-change increase was kept at 2.0 so that no protein would be missed. To perform this statistical analysis, "mass profiler professional" (MPP) version 13.0 build 210916 was used. A comparison was made against control samples to identify proteins occurring at a specific condition, control-group of samples were varied as described in Table 4.21. In the case of spores, the comparative analysis is detailed in Table 4.22.

Table 4.21: Proteins expressed by mycelia grown at different pressures versus the control sample

Mycelia cultivated at 0.1, 10, 20 MPa Vs. mycelia cultivated at 45 MPa
Mycelia cultivated at 0.1, 10, 45 MPa Vs. mycelia cultivated at 20 MPa
Mycelia cultivated at 0.1, 20, 45 MPa Vs. mycelia cultivated at 10 MPa
Mycelia cultivated at 10, 20, 45 MPa Vs. mycelia cultivated at 0.1 MPa

Table 4.22: Proteins expressed by spores grown at different pressures versus the control sample

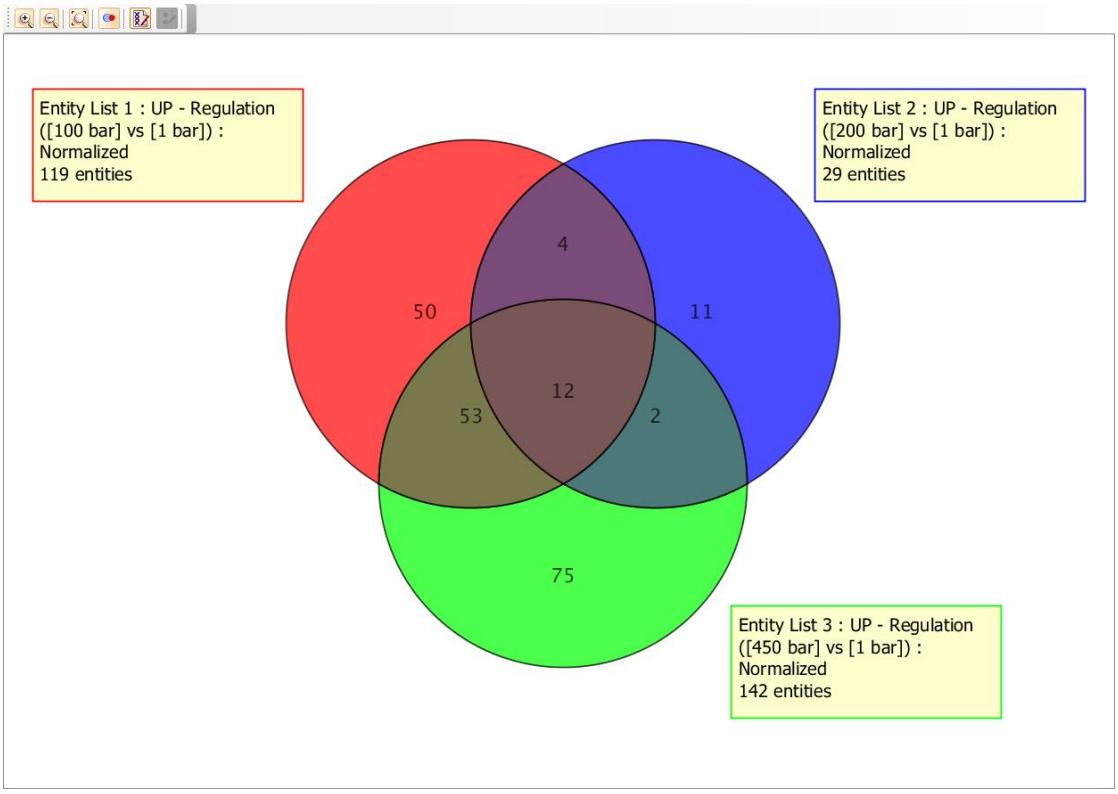
Spores incubated at 10, 20, 30 MPa Vs. Ungerminated spores
Spores incubated at 20, 30 MPa Vs. Spores incubated at 10 MPa
Spores incubated at 30 MPa Vs. Spores incubated at 20 MPa

For both the mycelial and spore protein samples, the comparison was restricted within one temperature set and physical inducer, if applicable. For example, mycelia cultivated at 0.1, 10, 20 MPa, 4^{o}C was compared against mycelia cultivated at 45 MPa, 4^{o}C; heat-shocked spores incubated at 20, 30 MPa, 4^{o}C was compared against heat-shocked spores incubated at 10 MPa, 4^{o}C.

While checking for the regulation of protein samples, up- as well as down- regulations were checked. By up-regulation, proteins that have a higher than 2-fold increase in expression is said to be up-regulated; similarly, those proteins that have a higher than 2-fold

decrease in expression is down-regulated. The necessity and significance of studying only the commonly regulated proteins by addition of confidence limits were detailed by Kim et al. 2008. In this statistical analysis, confidence limits were set as follows: experiment type was kept as combined, which included identified and unidentified proteins; data source was kept as generic; for mass filtering and retention time filtering, all available data were used as there were significant number of unknown proteins generated in protein summary; retention time correction was not performed to avoid assumptions; there was no normalization algorithm, and the baseline option was set. On obtaining the initial summary report based on these set standards, experiment was grouped based on physical and numerical parameters; during filtration of flags and frequency, care was taken that no entity was missed by setting probabilities; after obtaining the quality control report by principal component analysis (PCA), significance analysis was carried out using fold change values that generated entity list along with commonly regulated proteins with specific inputs on fold change values for each condition tested. Figure 4.8 is a representative picture of MPP analysis for one regulation at one condition of control set.

Figure 4.8: Representative picture MPP analysis for up-regulation of proteins from mycelial samples of *A. terreus* (#A4636, deep-sea isolate) cultivated at 4°C



From Figure 4.8, it can be understood that there are a total of 119 proteins that have been up-regulated at 10 MPa when compared against 0.1 MPa. Of these 119 entities, 50, 11 and 75 proteins are exclusively up-regulated at 10, 20 and 45 MPa respectively, when compared against 0.1 MPa. 53, 4 and two proteins are up-regulated commonly between 10 and 45 MPa; 10 and 20 MPa; 20 and 45 MPa respectively, when compared against 0.1 MPa. Twelve proteins have been commonly up-regulated across pressure conditions when compared to 0.1 MPa.

These 12 protein entities that were commonly up-regulated across pressure conditions were the ones of interest as they denote proteins regulated across all elevated pressure conditions. While proteins responsible for activities exclusively to one pressure may point out proteins unique to that pressure condition, studying proteins commonly regulated point to proteins that are necessarily regulated for survival/adaptation/growth under elevated pressure. Therefore, only commonly up- and down-regulated proteins/entities were studied for adaptations after the completion of the statistical analysis. The total number of commonly up-regulated proteins for mycelial and spore studies have been numbered in Table 4.23 and Table 4.24.

Table 4.23: Total number of proteins up-regulated commonly in all pressure conditions for mycelial samples

Temperature	**Comparative analysis**	**#A4636** ***(A. terreus*****)**	**#MTCC479** ***(A. terreus*****)**	**#NIOCC20** ***(A. flavus*****)**	**#A4623** ***(Penicillium* sp.)**	**#CH2** **(*A. sydowii*)**
30^{o}C	10, 20, 45 Vs 0.1 MPa	0	2	2	6	2
	0.1, 20, 45 Vs 10 MPa	3	0	5	1	2
	0.1, 10, 45 Vs 20 MPa	0	2	10	2	1
	0.1, 10, 20 Vs 45 MPa	33	36	14	0	1
4^{o}C	10, 20, 45 Vs 0.1 MPa	12	4	4	17	4
	0.1, 20, 45 Vs 10 MPa	1	29	6	5	31
	0.1, 10, 45 Vs 20 MPa	8	1	4	0	2
	0.1, 10, 20 Vs 45 MPa	1	1	1	2	2

Table 4.24: Total number of proteins up-regulated commonly in all pressure conditions for spore samples

Temperature	**Heat treatment**	**Comparative analysis**	**#A4636 (*A. terreus*)**	**#MTCC479 (*A. terreus*)**	**#NIOCC20 (*A. flavus*)**
4^{o}C	Yes	10, 20, 30 MPa Vs Dormant	6	3	10
		20, 30 Vs 10 MPa	2	4	4
		30 Vs 20 MPa	2	28	15
	No	10, 20, 30 MPa Vs Dormant	0	0	8
		20, 30 Vs 10 MPa	1	2	0
		30 Vs 20 MPa	4	14	5
30^{o}C	Yes	10, 20, 30 MPa Vs Dormant	2	6	4
		20, 30 Vs 10 MPa	0	5	2
		30 Vs 20 MPa	6	10	6
	No	10, 20, 30 MPa Vs Dormant	0	5	8
		20, 30 Vs 10 MPa	4	10	18
		30 Vs 20 MPa	20	14	14

4.7 Checking for the placement of commonly regulated proteins in the KEGG pathway database

Commonly regulated proteins (in all conditions of pressure studied, i.e., 0.1, 10, 20 and 45 MPa for mycelial protein studies; 10, 20, 30 MPa for spore and germination protein studies) were checked for their function in UniProt; after the function of the protein was noted down, the placement of the protein in metabolic pathways was checked in the KEGG pathway database, based on the function. This analysis was performed to know the pathways that were expressed when fungi were grown under simulated deep-sea condition. Figures 4.9 to 4.16 are a graphical representation of protein analysis results for placement in the KEGG pathway

Figure 4.9: Pathways and their total percentage in *A. terreus* (#A4636, deep-sea isolate, mycelial studies) cell proteome

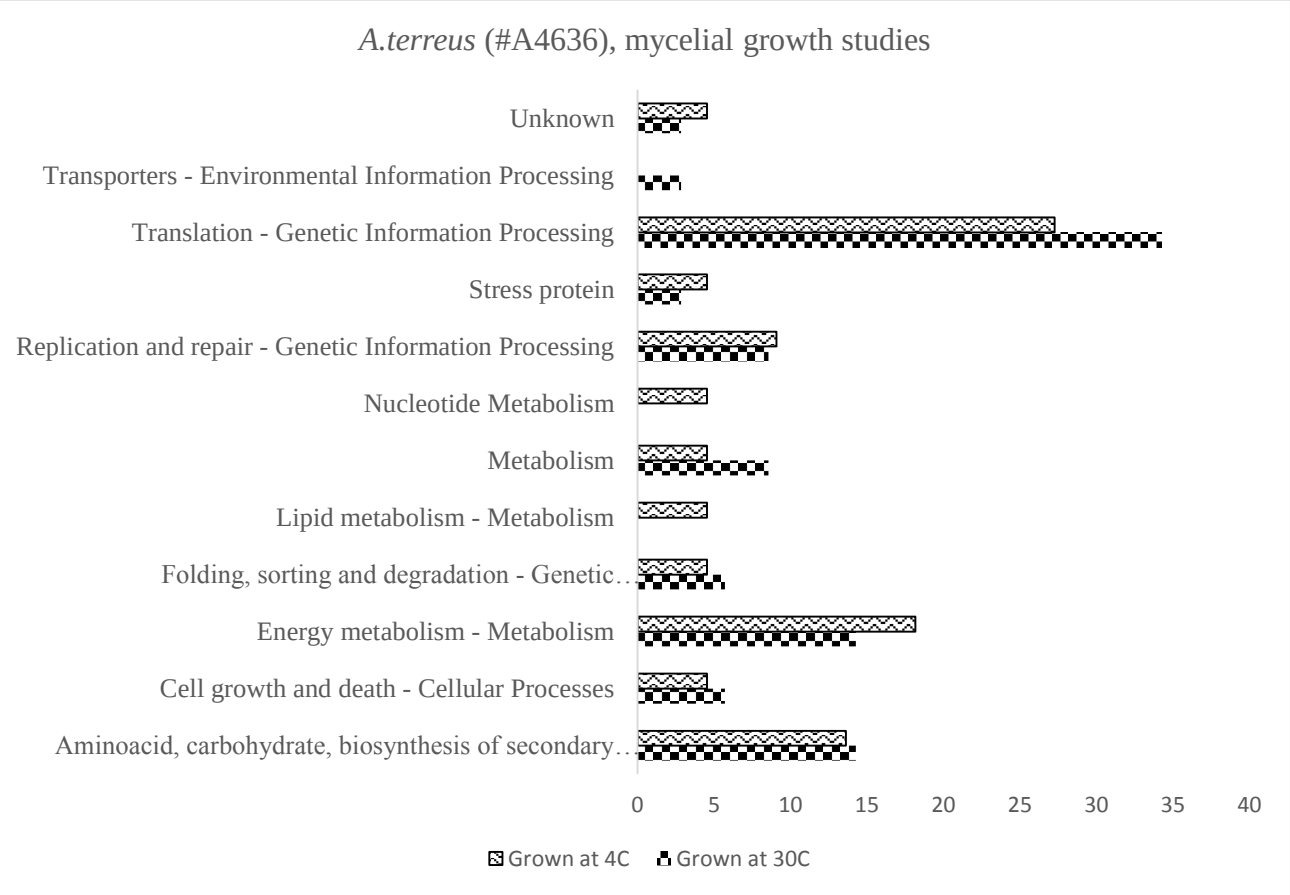


Figure 4.10: Pathways and their total percentage in *A. terreus* (#MTCC479, terrestrial isolate, mycelial studies) cell proteome

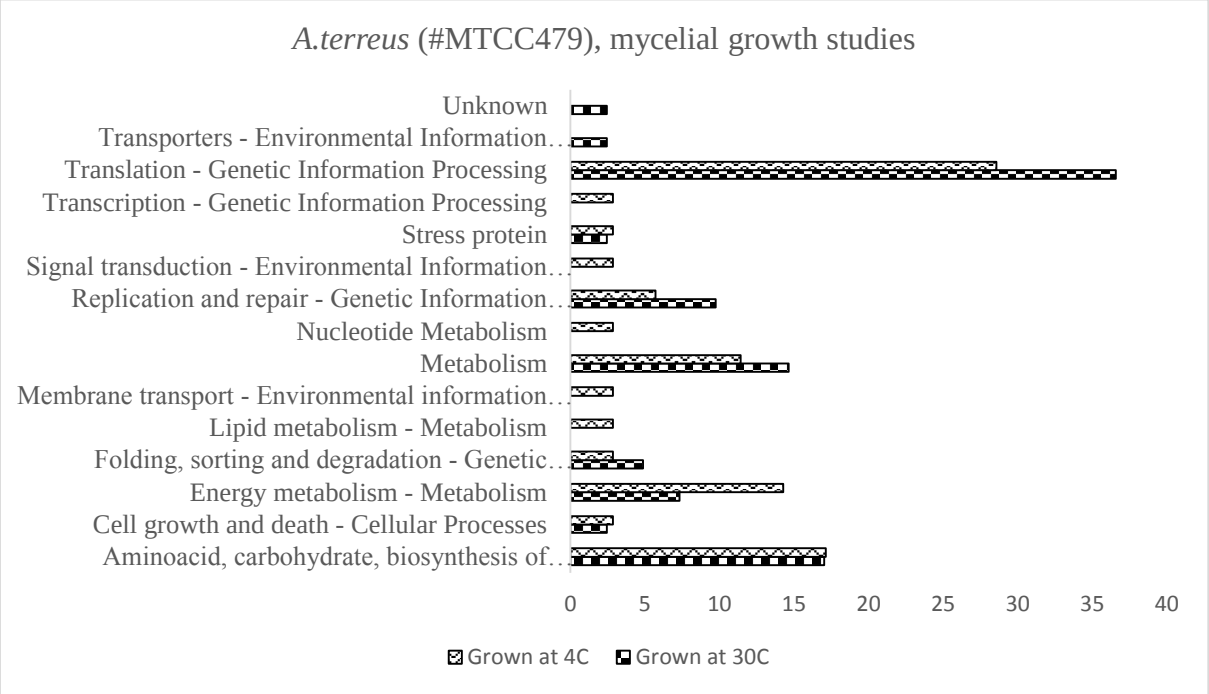


Figure 4.11: Pathways and their total percentage in *A. flavus* (#NIOCC20, deep-sea isolate, mycelial studies) cell proteome

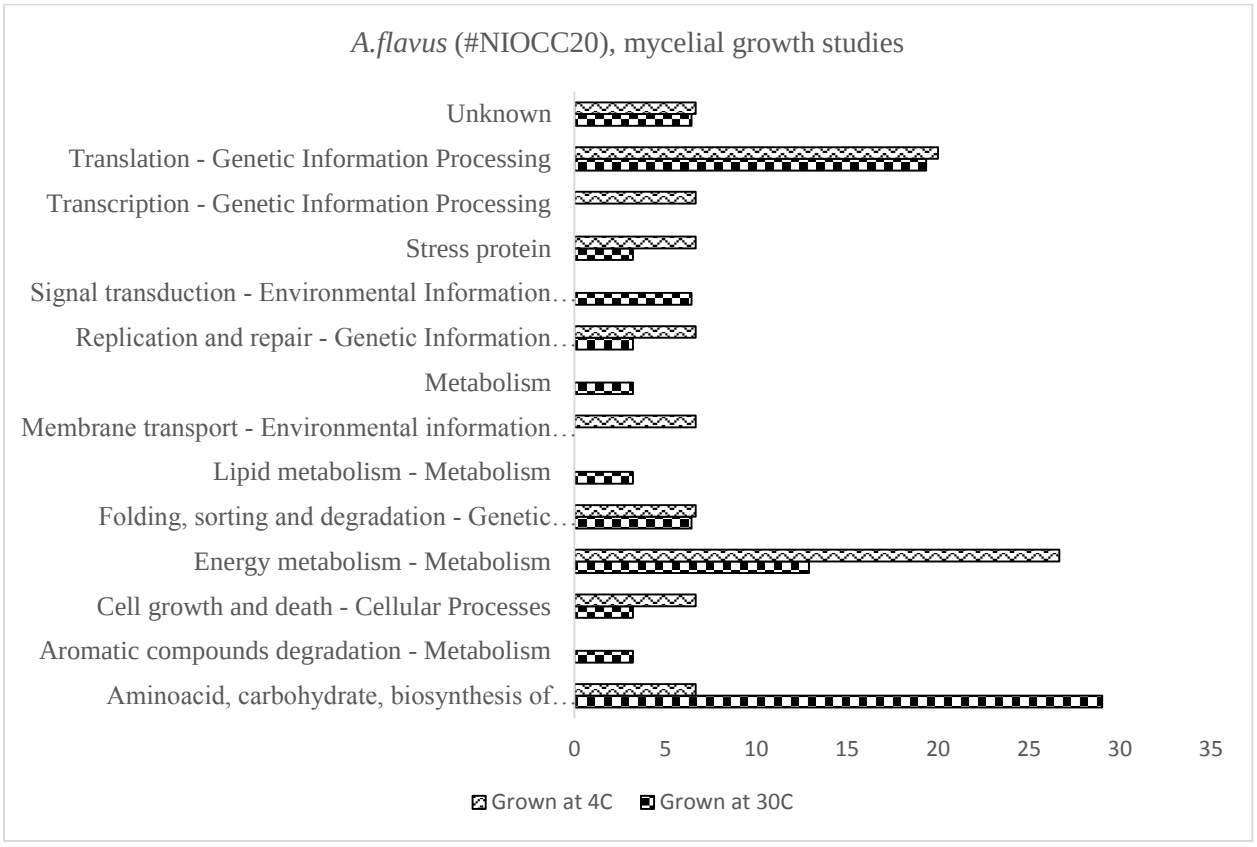


Figure 4.12: Pathways and their total percentage in *Penicillium* sp. (#A4636, deep-sea isolate, mycelial studies) cell proteome

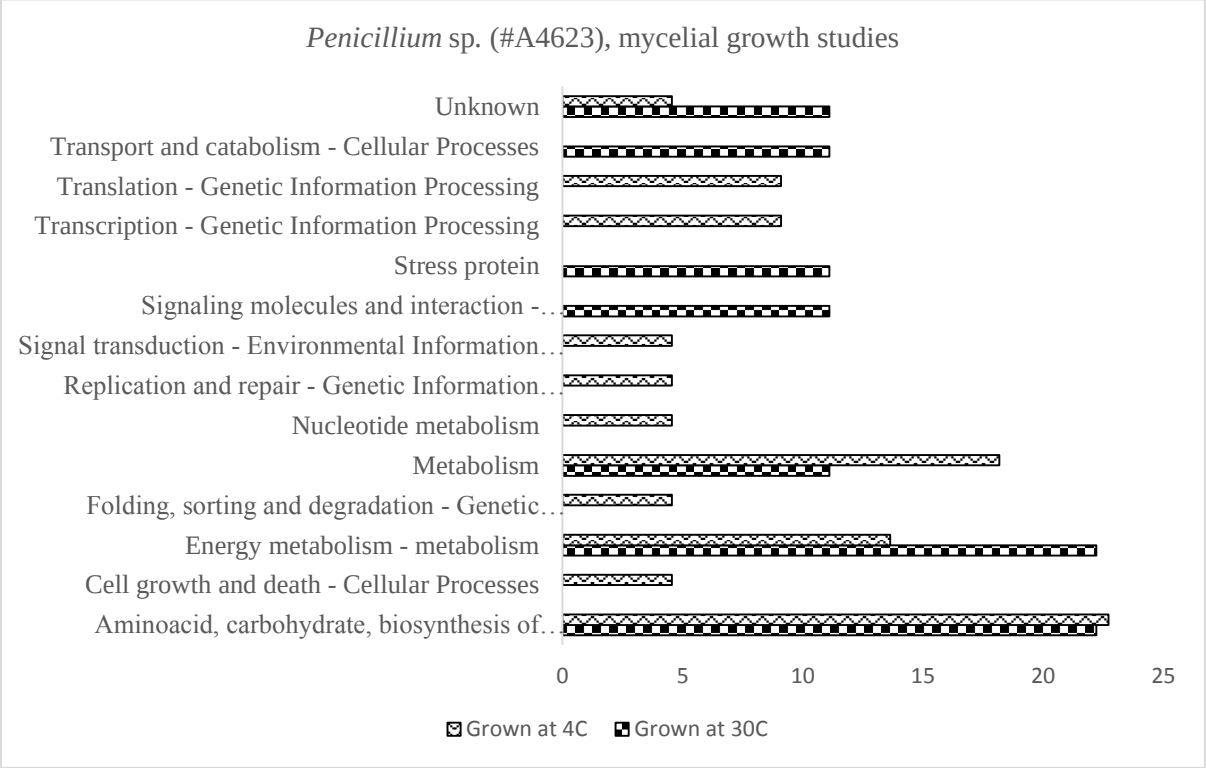


Figure 4.13: Pathways and their total percentage in *A. sydowii* (#CH2, deep-sea isolate, mycelial studies) cell proteome

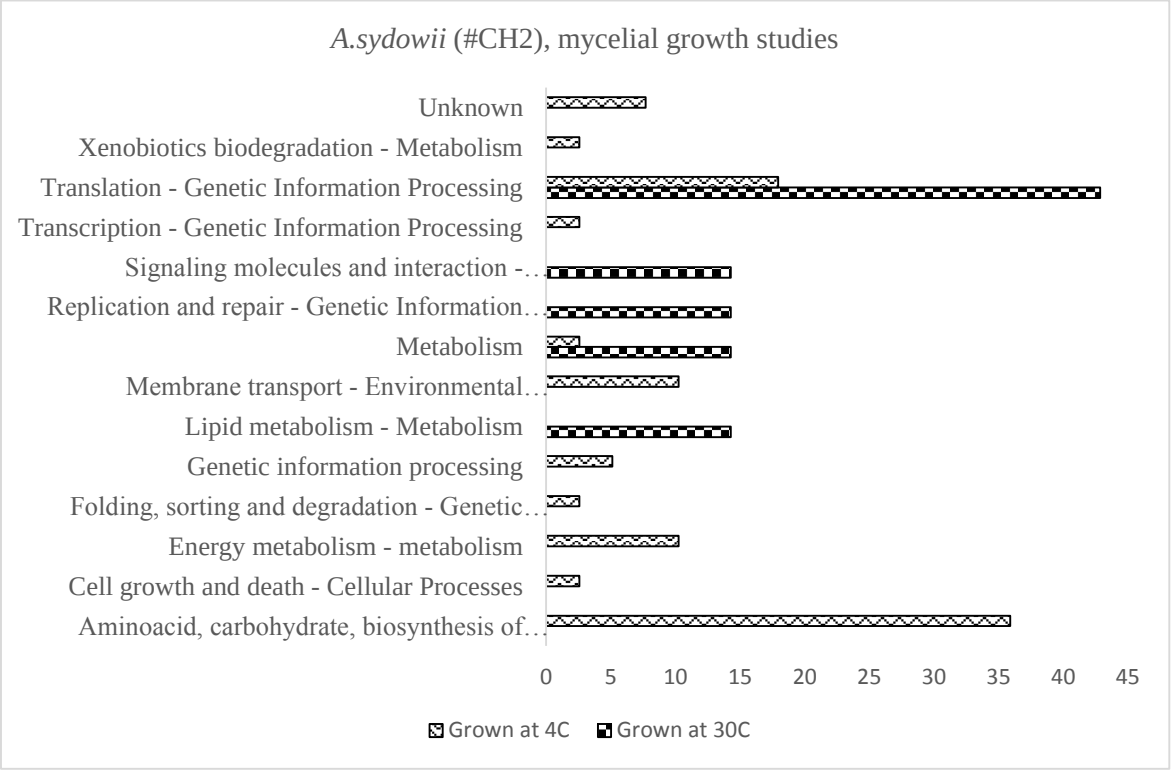


Figure 4.14: Pathways and their total percentage in germinating spore proteome of *A. terreus* (#A4636, deep-sea isolate, conidial germination studies)

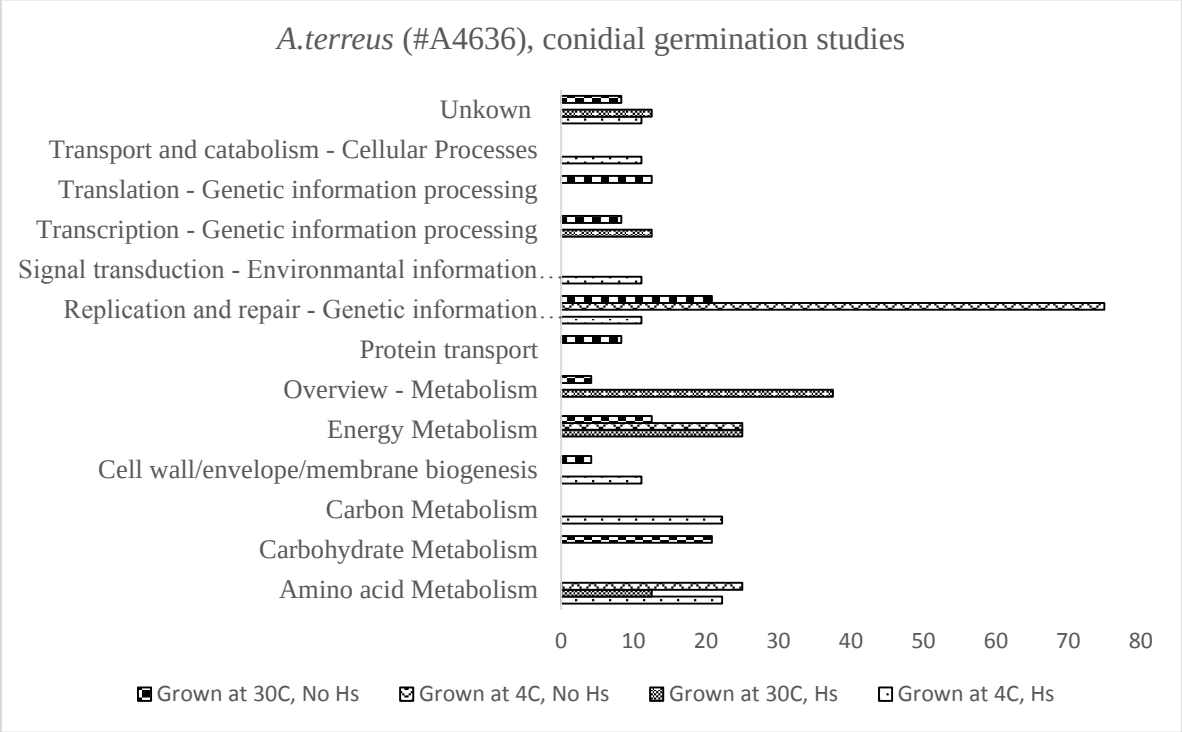


Figure 4.15: Pathways and their total percentage in germinating spore proteome of *A. terreus* (#MTCC479, terrestrial isolate, spore germination studies)

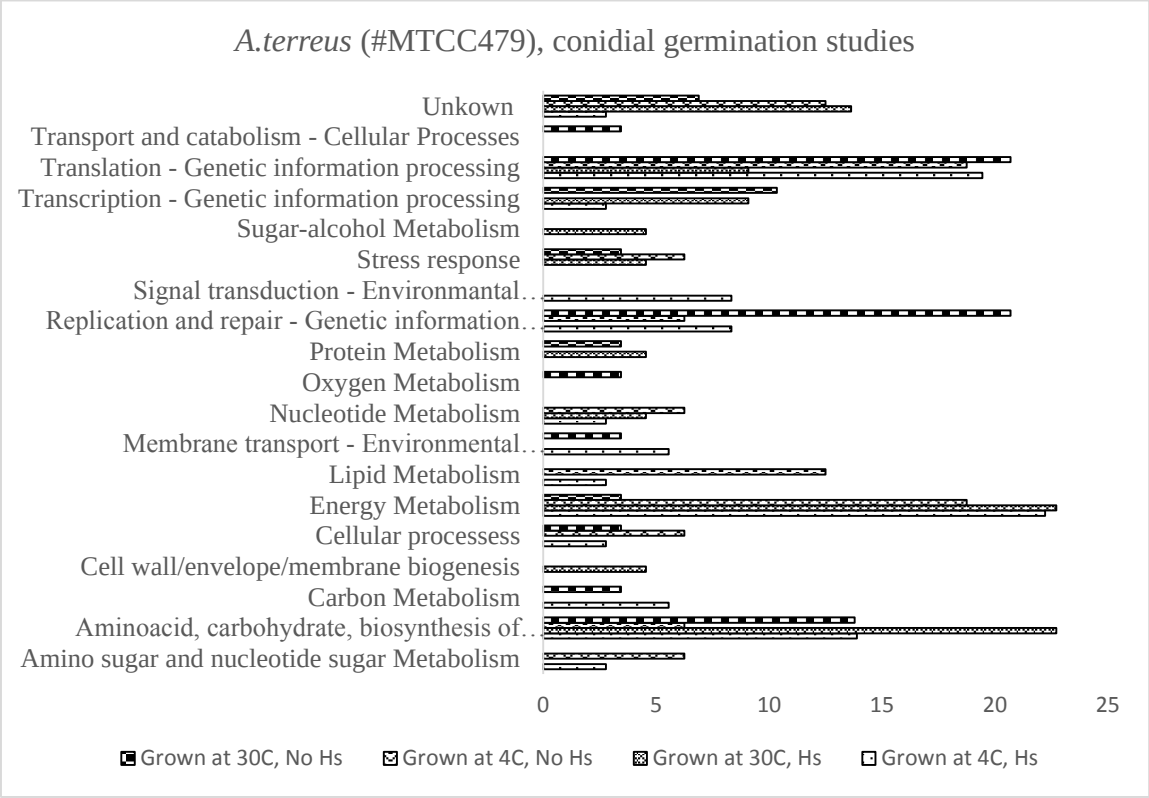
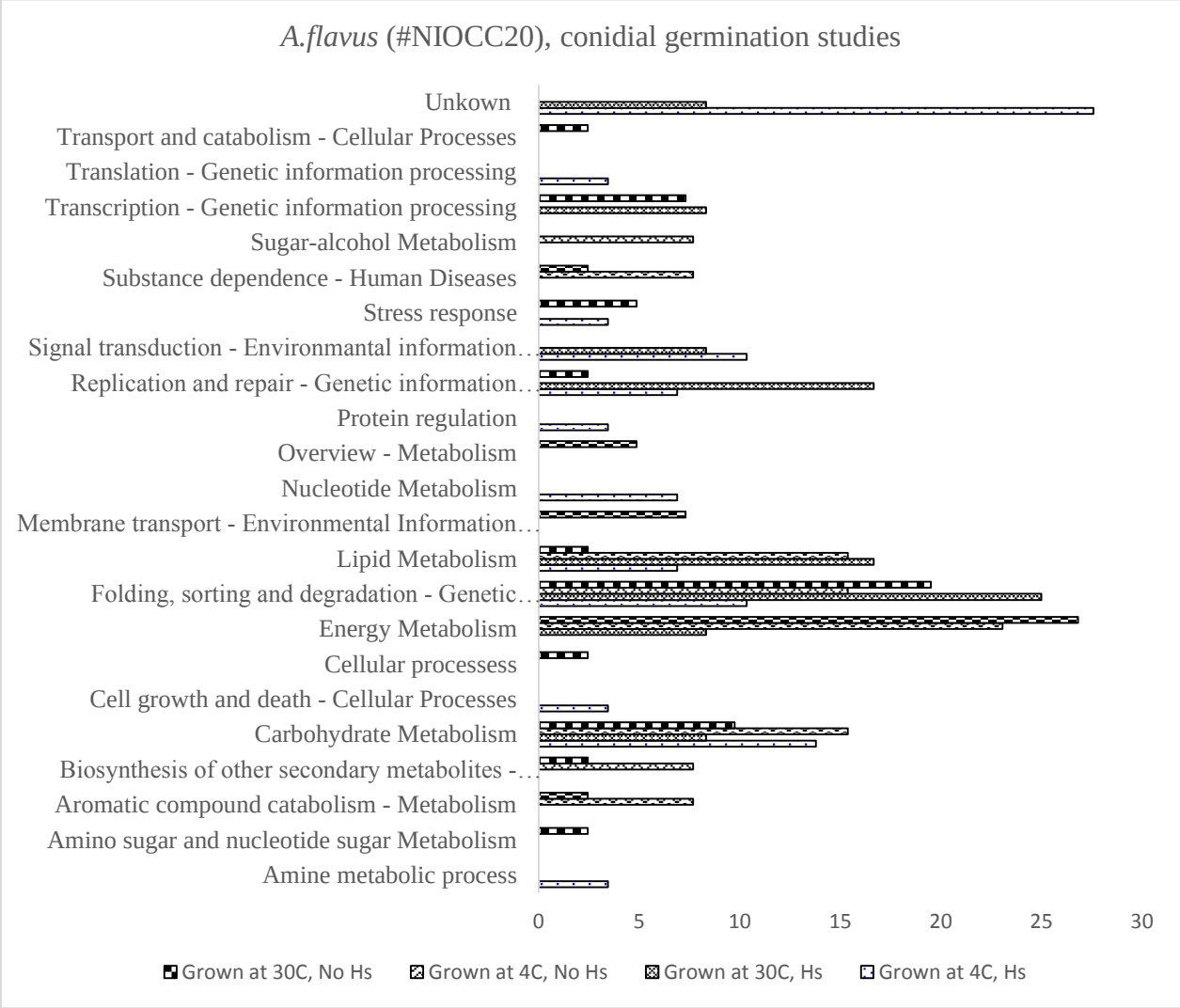


Figure 4.16: Pathways and their total percentage in germinating spore proteome of *A. flavus* (#NIOCC20, deep-sea isolate, spore germination studies)



In the case of down-regulation, proteins involved in house-keeping, cell-division, enzyme production, transport and signalling, DNA repair were mostly down-regulated. As most of these proteins were involved in biomass buildup, it was evident that biomass buildup was in few milligrams under the simulated deep-sea condition

Summary of chapter 4

From the results of statistical analysis of the proteins detected from the mycelia of *A. terreus* (#A4636, deep-sea isolate), 12 pathways were up-regulated across 0.1, 10, 20 and 45 MPa pressure conditions. Mycelial biomass cultivated at 30^{o}C lacked commonly up-regulated proteins in lipid metabolism and nucleotide metabolism while mycelial biomass cultivated at 4^{o}C lacked commonly up-regulated protein transporters.

A. terreus (#MTCC479, terrestrial isolate) showed proteins involved in 15 pathways up-regulated across 0.1, 10, 20 and 45 MPa pressure conditions. There were no commonly up-regulated proteins in lipid metabolism, membrane transport, nucleotide metabolism, signal transduction and transcription for mycelial biomass cultivated at 30^{o}C. There were no commonly up-regulated unknown proteins, and protein transporters for mycelial biomass cultivated at 4^{o}C.

A. flavus (#NIOCC20, deep-sea isolate) showed proteins involved in 14 pathways up-regulated across 0.1, 10, 20 and 45 MPa pressure conditions. The mycelial biomass cultivated at 30^{o}C lacked commonly up-regulated proteins in transcription and membrane transport, while the mycelial biomass cultivated at 4^{o}C lacked commonly up-regulated proteins involved of aromatic compound degradation, lipid metabolism, an overview of metabolism and signal transduction.

Penicillium sp. (#A4623, deep-sea isolate) showed proteins involved in 14 pathways up-regulated across 0.1, 10, 20 and 45 MPa pressure conditions. The mycelial biomass cultivated at 30^{o}C lacked commonly up-regulated proteins involved in cell growth, and death cycle, folding-sorting-and-degradation, nucleotide metabolism, replication and repair, signal transduction, transcription, and translation, while the mycelial biomass cultivated at 4^{o}C lacked commonly up-regulated proteins involved in signalling, stress tolerance, transport, and catabolism.

A. sydowii (#CH2, deep-sea isolate) showed proteins involved in 14 pathways up-regulated across 0.1, 10, 20 and 45 MPa pressure conditions. The mycelial biomass cultivated at 30^{o}C lacked commonly up-regulated unknown proteins as well as proteins involved in the metabolism of aminoacids-carbohydrates-production of secondary metabolites, cell growth and death, energy metabolism, folding-sorting-and-degradation, an overview of genetic information processing, membrane transport, transcription and xenobiotic degradation. The

mycelial biomass cultivated at 4^{o}C lacked commonly up-regulated proteins involved in lipid metabolism, replication, and repair, signalling molecules and interaction.

Statistical analyses of proteins detected in germinating conidia (#A4636) obtained from simulated deep-sea condition revealed that the proteins were from different pathways for all the four conditions of cultivation (30^{o}C, 4^{o}C; with heat shock, the absence of heat shock).

Germinating conidia of *A. terreus* (#MTCC479) in simulated deep-sea condition had commonly up-regulated unknown proteins in addition to proteins involved in pathways of aminoacid-carbohydrate-synthesis of secondary metabolites metabolism, energy metabolism and translation in all the four conditions studied.

Germinating conidia of *A. flavus* (#NIOCC20) in simulated deep-sea condition had commonly up-regulated proteins involved in carbohydrate metabolism, lipid metabolism and folding-sorting-and-degradation in all the four conditions studied.

As the maximum depth of sampling for obtaining deep-sea isolates was 5500 m, conidial germination studies for #A4636, #MTCC479 and #NIOCC20 was also performed at 40, 50, 60 MPa, for 30-day incubation, in all the four conditions (30^{o}C, 4^{o}C; with heat shock, the absence of heat shock). Protein mass fingerprinting (PMF) analysis showed that for each replicate, there were < ten protein hits per sample for germinating conidia at 40 MPa; < six protein hits per sample for germinating conidia at 50 MPa; and ≤ three protein hits per sample for germinating conidia at 60 MPa. As this number of proteins were too less to perform statistical analysis, statistical studies on conidial germination in simulated deep-sea conditions were performed only till 30 MPa.

As a negative control, in-solution digestion was carried out for the three-buffer sample that lacked protein extract. The medium in which the isolates were cultivated in simulated deep-sea condition was preserved in –80^{o}C until methanol precipitation and was in-solution digested to check if there were novel as well as known extracellular metabolites produced by these fungi under simulated deep-sea condition. However, there were no such biomolecules detected. This lack of detection might be because, as biomass buildup was miniscule, the quantity of metabolite produced would be negligible; also, this negligible quantity was beyond detection when it diffused in growth medium leading to further dilution.

Except for details of commonly up-regulated proteins in transcription, translation, and replication and repair, details of other commonly up-regulated proteins are presented as tables (Table 4.25 and Table 4.26) in the forthcoming section. The details of pathways have been presented graphically for individual conditions and the tables below present the details for detected proteins in their respective pathway placements.

Table 4.25: Commonly up-regulated proteins in mycelia biomass (grown in simulated deep-sea conditions) and their metabolic placement as verified in the KEGG pathway database

Pathway	**Proteins detected that were up-regulated**
Amino acid metabolism	Adenosylhomocysteinase; Aspartate aminotransferase; Glutamate dehydrogenase; Ketol-acid reductoisomerase, mitochondrial; Probable dipeptidyl-peptidase 5
The amino acid, carbohydrate, biosynthesis of secondary metabolites metabolism	Catalase, Mycelial catalase Cat1
Aromatic compounds degradation	Alcohol dehydrogenase 1
Carbohydrate metabolism	6-phosphogluconate dehydrogenase, decarboxylating; ATP citrate lyase subunit (Acl), putative; Citrate synthase, mitochondrial; Enolase/allergen Asp F 22; Fructose-bisphosphate aldolase; Fucose-specific lectin FleA; Glucose-6-phosphate isomerase; Glyceraldehyde-3-phosphate dehydrogenase; L-xylulose reductase; Malate dehydrogenase; Phosphoglycerate kinase; Probable endo-1,4-beta-xylanase C; Pyruvate dehydrogenase E1 component subunit alpha; Transaldolase; Triosephosphate isomerase; Uncharacterized protein
Cell growth and death	Actin; Actin act1; Actin gamma; Fimbrin

Pathway	Proteins detected that were up-regulated
Energy metabolism	AAA+ ATPase domain; Aldo/keto reductase; ATP synthase subunit alpha; ATP synthase subunit alpha, mitochondrial; ATP synthase subunit beta; ATP-citrate synthase subunit 1; Cytochrome c; Cytochrome c oxidase subunit 2; Cytochrome P450, putative; Delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial; Dihydrolipoyl dehydrogenase; Glutamate dehydrogenase; Mitochondrial processing peptidase beta subunit; Oxidoreductase, putative; Protoplast secreted protein 2; Pyoverdine/dityrosine biosynthesis protein, putative; Pyruvate dehydrogenase E1 component subunit alpha; Vacuolar ATP synthase catalytic subunit A
Folding, sorting, and degradation	C2H2 finger domain protein, putative; Enolase; Peptidase C19, ubiquitin carboxyl-terminal hydrolase 2; Peptidyl-prolyl cis-trans isomerase; Prefoldin subunit 1, putative
Lipid metabolism	Enoyl reductase domain of yeast-type FAS1; Fatty acid synthase subunit beta, putative; Predicted protein
Membrane transport	ABC multidrug transporter, putative; Ankyrin; Major Facilitator Superfamily protein; Mitochondrial phosphate carrier protein; Outer mitochondrial membrane protein porin; Uncharacterized protein (Fragment)
Metabolism	ADP, ATP carrier protein; Alternative oxidase; Nucleoside diphosphate kinase; Nucleoside phosphorylase domain; Phosphoglycerate kinase; Polyketide synthase; Probable Xaa-Pro aminopeptidase ATEG_00858; Pyruvate dehydrogenase E1 component subunit alpha; Short-chain dehydrogenase reductase SDR; Sister chromatid cohesion factor (Chl12), putative; Tubulin beta chain

Pathway	Proteins detected that were up-regulated
Nucleotide metabolism	Pc22g08770 protein; Pyrimidine biosynthesis enzyme THI11
Signal transduction	Aspartic endopeptidase Pep2; G-protein complex beta subunit CpcB; Pc12g05080 protein; Phosphoinositide phospholipase C; Predicted protein; Putative uncharacterized protein
Stress proteins	Hsp70 chaperone (HscA), putative; Heat shock 70 kDa protein; Molecular chaperone Hsp70; Molecular chaperone (ABC1), putative
Transport and catabolism	AP-1 adaptor complex subunit mu, putative; Pc12g05080 protein
Transporters	GTP-binding nuclear protein GSP1/Ran
Xenobiotics degradation	Gamma-glutamyltranspeptidase
Unknown proteins (details of accession number is indicated within the braces)	BYS1 domain protein, putative (B8N2Y9); Predicted protein (Q0CL64); Putative uncharacterized protein (Q0CIJ2, Q0CUP4, B8N2F5, B8NHR1, B8MRZ8, B6QHP4); SET domain-containing protein (A0A017SQ59)

Table 4.26: Commonly up-regulated proteins in conidial germination (grown in simulated deep-sea conditions) and their metabolic placement as verified in KEGG pathway database

Pathway	**Proteins detected that were up-regulated**
Amine metabolism	Amine oxidase
Amino acid metabolism	Adenosylhomocysteinase; Cystathionine gamma-synthase; Dihydroxy-acid dehydratase; Glutamate synthase; Glycylpeptide N-tetradecanoyl transferase; Major allergen Asp f 2; Probable Xaa-Pro aminopeptidase ATEG_00858; Vacuolar protease A
The amino acid, carbohydrates, and biosynthesis of secondary metabolites	Catalase; Spermidine synthase
Amino-sugar metabolism	Chitin synthase 6
Aromatic compounds catabolism/degradation	2-oxo-hepta-3-ene-1,7-dioic acid hydratase, putative; Alcohol dehydrogenase 1
Biosynthesis of other secondary metabolites	Benzoate 4-monooxygenase cytochrome P450, putative; NRPS-like enzyme, putative
Carbohydrate metabolism	6-phosphogluconate dehydrogenase, decarboxylating; Alpha-1,2-Mannosidase; Enolase; Glutamate decarboxylase; Glyceraldehyde-3-phosphate dehydrogenase; Malate dehydrogenase, mitochondrial; Malate dehydrogenase, NAD-dependent; NAD(+)-isocitrate dehydrogenase subunit I; Phosphatidylinositol 4-kinase (STT4), putative; Phosphoenolpyruvate carboxykinase; Possible apospory-associated protein c; Pyruvate carboxylase; Pyruvate decarboxylase; Pyruvate decarboxylase PdcA, putative; Pyruvate dehydrogenase E1 component subunit alpha; Succinate dehydrogenase assembly factor 2, mitochondrial; Transketolase

Pathway	Proteins detected that were up-regulated
Carbohydrate, amino-sugar and nucleotide-sugar metabolism	UDP-glucose 4-epimerase
Carbon metabolism	Alpha-1,2-Mannosidase; Extracellular endoglucanase, putative; L-xylulose reductase; Probable beta-glucosidase I; Probable feruloyl esterase B-2; Probable glucan endo-1,3-beta-glucosidase eglC; Succinyl-CoA ligase alpha-chain, mitochondrial
Cell growth and death	C6 transcription factor, putative; Cell cycle control protein (Cwf4), putative; Cupin domain protein
Cell wall/envelop/membrane biogenesis	Putative metallocarboxypeptidase ecm14; Putative uncharacterized protein
Cellular processes	Actin; Actin Act1; F-actin capping protein alpha subunit, putative
Energy metabolism	3-ketoacyl-acyl carrier protein reductase; Acetoacetyl-CoA reductase; ATP synthase subunit alpha; ATP synthase subunit beta; ATP synthase subunit d, mitochondrial; ATPase family AAA domain-containing protein 1; Calcium-dependent mitochondrial carrier protein, putative; Citrate synthase; Cytochrome c oxidase assembly protein COX11, mitochondrial; Cytochrome c oxidase polypeptide VIb; Cytochrome c peroxidase Ccp1, putative; Cytochrome P450 51; Cytochrome P450, putative; Fatty acid oxygenase PpoC, putative; Glutathione reductase; Glyoxylate/hydroxypyruvate reductase, putative; Heat shock 70 kDa protein; NAD(+)-isocitrate dehydrogenase subunit I; NADH-ubiquinone oxidoreductase 49 kDa subunit, mitochondrial; NAD-specific glutamate dehydrogenase; Non-heme chloroperoxidase; Oxidoreductase, zinc-binding dehydrogenase family, putative; Vacuolar ATP synthase catalytic subunit A

Pathway	Proteins detected that were up-regulated
Folding, sorting, and degradation	ATP dependent RNA helicase, putative; C2H2 finger domain protein, putative; C6 finger domain protein, putative; HECT domain protein; Mitochondrial processing peptidase beta subunit, putative; Peptidyl-prolyl cis-trans isomerase; Proteasome regulatory particle subunit Rpt4, putative; Pyoverdine/tyrosine biosynthesis protein, putative; RNA exonuclease Rex3, putative
Lipid metabolism	Fatty acid synthase subunit beta, putative; Patatin-like phospholipase domain-containing protein ATEG_02594; Predicted protein
Lipid and energy metabolism	Mitochondrial carnitine: acylcarnitine carrier, putative
Membrane transport	ABC multidrug transporter; ADP, ATP carrier protein; Mitochondrial ADP, ATP carrier protein (Ant), putative; Mitochondrial phosphate carrier protein, putative
Metabolism	Acetyltransferase component of pyruvate dehydrogenase complex; Nonribosomal peptide synthase, putative; Pyruvate dehydrogenase E1 component subunit alpha
Nucleotide metabolism	IdgA domain protein; Nucleoside diphosphate kinase; Ribose-phosphate pyrophosphokinase 2; Uricase
Oxygen metabolism	Superoxide dismutase [Cu-Zn]
Protein and RNA transport	Ran GTPase-activating protein 1
Protein metabolism Protein regulation	Mitochondrial processing peptidase beta subunit 2-heptaprenyl-1,4-naphthoquinone methyltransferase, putative

Pathway	Proteins detected that were up-regulated
Protein transport	AP-1 complex subunit beta-1
Signal transduction	Autophagy regulatory protein Atg2, putative; Dynamin GTPase, putative; Protein kinase family protein; Rho GTPase activator (Bem2), putative; Two-component osmosensing histidine kinase (Bos1), putative
Stress response	30 kDa heat shock protein; Glutathione reductase; Heat shock protein Hsp30/Hsp42, putative; Heat shock protein 60, mitochondrial; Heat shock 70 kDa protein; Heat shock protein SSC1, mitochondrial; Prefoldin subunit 1, putative
Substance dependence - Human diseases	Histone H2B
Sugar-alcohol metabolism	Inositol-3-phosphate synthase
Transport proteins	Acetylglutamate kinase, putative; dsDNA-dependent ATPase Rad54, putative; GTP-binding nuclear protein GSP1/Ran; Nuclear pore complex subunit Nup159, putative; Plasma membrane ATPase; Vacuolar protein sorting protein DigA

Unknown proteins (details of accession number is indicated within the braces)	3-ketoacyl-CoA thiolase (Q0CXZ0); Annexin (Q0CY55); cAMP-regulated D2 esterase, putative (B8NAK6); Cullin binding protein CanA, putative (B8N863); DUF185 domain protein (B8NBB3); DUF89 domain protein (B8NDA9); Ferulic acid esterase (FaeA), putative (B8MYQ9); Glutathione-S-transferase theta, GST, putative (B8NW05); HEAT repeat protein (DRIM), putative (B8N4K1); HIT finger domain protein, putative (B8N498); Integral membrane protein (B8N2A5); Kinesin family protein (B8NLX8); Predicted protein (Q0CL64); Predicted protein (Q0CID3); Putative uncharacterized protein (Q0CIJ2); Putative uncharacterized protein (Q0CXB0); Sphingolipid long chain base-responsive proteinPIL1(Q0CRL1)

- ***Generation of phylogenetic trees to finalise the protID for species-specific database search***

The taxonomy ID of the organisms used for MS/MS search was - Swiss-Prot: *A. terreus* NIH2624, *A. flavus* NRRL 3357; NCBI: *A. terreus* txid_33178. *A. flavus* txid_5059. *Penicillium* sp. and *A. sydowii* were checked across the complete genus-specific database in both the databases

- ***Processing the spectral data with data extractor***

The total spectra generated after MS analysis for the 71-minute protocol ranged approximately between 3300–5488. Spectra processed for MS/MS analysis was between 63–2653 and mzXML files were generated for all technical replicates.

- ***Species-specific MS/MS search in NCBI and Swiss-Prot databases***

Swiss-Prot database based species-specific search was finalised. *A. terreus* NIH2624, *A. flavus* NRRL 3357 databases were used; *Penicillium* sp. and *A. sydowii* were checked across the complete genus-specific database in Swiss-Prot.

- ***Autovalidation of the generated spectra***

The false discovery rate was within 1% for all the samples, when analysed with their respective technical replicate.

- ***Generation of peptide and protein lists***

Table 4.27: Total number of protein hits obtained after MS analysis in organism-specific UniProtKB (Swiss-Prot) database (mycelial samples). * Genus database

Total number of Proteins – Mycelial samples								
Pressure (MPa)	**0.1 MPa**		**10 MPa**		**20 MPa**		**45 MPa**	
Organisms Temperature	**4ºC**	**30ºC**	**4ºC**	**30ºC**	**4ºC**	**30ºC**	**4ºC**	**30ºC**
Aspergillus terreus (#A4636)	32	211	209	133	50	84	404	14
Aspergillus terreus (#MTCC479)	244	169	48	273	198	155	116	20
Aspergillus flavus (#NIOCC20)	61	236	91	64	45	72	285	44
Penicillium sp.* (#A4623)	41	147	147	116	411	22	284	23
*Aspergillus sydowii** (#CH2)	355	126	68	198	503	62	363	26

Table 4.28: Total number of protein hits obtained after MS analysis in organism-specific UniProtKB (Swiss-Prot) database (dormant and germinating conidial samples).

Ht – Heat treated conidia (70ºC, 1 hour); No Ht – absence of heat treatment

Total number of proteins in													
		Ungerminated and germinating conidia at											
Pressure (MPa)		**10 MPa**				**20 MPa**				**30 MPa**			
Heat treatment	**Dormant conidia/spore**	**Yes**		**No**		**Yes**		**No**		**Yes**		**No**	
		4ºC	**30ºC**	**4ºC**	**30ºC**	**4ºC**	**30ºC**	**4ºC**	**30ºC**	**4ºC**	**30ºC**	**4ºC**	**30ºC**
A. terreus (#A4636)	107	94	72	8	38	61	39	10	45	41	36	19	57
A. terreus (#MTCC479)	268	62	125	10	69	74	158	6	73	84	102	32	60
A. flavus (#NIOCC20)	234	74	144	20	84	61	111	7	151	38	17	5	47

- ***Statistical analysis to check for the regulation of proteins***

The total number of commonly regulated proteins, across different pressure conditions, were identified and noted down for mycelial growth as well as conidial germination studies.

- ***Checking for the placement of commonly regulated proteins in the KEGG pathway database***

Proteins that were up-regulated across pressure conditions were checked for their function and based on function, was looking for placement in metabolic pathways in the KEGG pathway database. Based on these details, pathways and their percentage contribute in cell cycles were calculated.

Prelude to chapter 5

This chapter discusses the details and reasoning behind each procedure that was finalised for standardising protocol of all the three objectives studied.

Chapter FIVE

DISCUSSION

Chapter 5 – Discussion

5.1 Fungal isolates used in this study as deep-sea inhabitants

The four fungal species tested in this study have been reported as inhabitants of sub-seafloor sediments (Jones et al. 2015). Conidia/spores of all isolates studied here displayed contact inhibition. Tween 80 was used for suspending conidia as it was a surfactant that reduced contact inhibition by de-clumping the conidia. A count of 30-50 x 10^6 conidia ml^{-1} of sample ensured that the total number of germinating conidia were not reduced by contact inhibition.

5.2 Effect of heat-treatment in conidial germination studies

The results of providing heat treatment as an additive for conidial germination suggest that heat was not necessarily a triggering factor for conidial germination, a result also obtained in a previous study (Jesenska et al. 1993), although it had a positive effect on germination of the conidia of *A. terreus* after a 15-day incubation in this study and a 20-day incubation in Damare et al. (2008).

5.3 Role of chemical inducers in conidial germination studies

The chemical inducers evaluated in conidial germination studies were known to play a role in the start of cell life cycle amongst other organisms. Thiamine was a vital player in carbohydrate breakdown in humans (Lonsdale 2006) and triggered conidial germination by initiating cellular processes (Mkrtchyan et al. 2016). The absence of trigger for the start of the pyruvate cycle was probably the only rate limiting factor (Laere et al. 1982) for dormant spore to initiate development. Acids softened fungal cell wall (Rao 2011) and allowed the diffusion of nutrients between conidia and external environment. Sucrose helped in initial swelling (Wooley and Thoene 2004) of conidia for uptake of nutrients to initiate germination.

5.4 Conflicts of in vitro and in vivo studies

In all the above studies as well as in the current study, the inducers were provided freely, as a one-component additive for the experimental setup; whereas in deep-sea, these inducers always occur as minerals and in combination with other compounds and or elements. While none of the one-component additives employed in this study was a trigger for germination of conidia, a combination of different compounds/elements, as present in

the open ocean may play a role in triggering conidial germination in deep-sea. Also, varying the concentrations of additives may trigger germination.

5.5 Upper-limit of piezotolerance for conidial germination

There was a possibility that high hydrostatic pressure improved hydration of biomolecules and could be the reason for conidial germination even at 40 MPa (Mozhaev et al. 1996, Heremans and Smeller 1998, Chen et al. 2006) as observed from our experiments conducted over a 30-day incubation period. No mycelial growth was observed at that pressure. This observation indicates that increasing pressure is detrimental to rapid growth, thereby reducing the rate of mycelial development. Therefore, protein studies on conidial germination were done on samples grown at 10, 20 and 30 MPa. Interestingly, for a 30-day incubation at 50 and 60 MPa, less than ten conidia were germinating for all the isolates, This proved that an upper limit for initiating germination might exist in case of increased pressure.

5.6 Inducers and conidial germination

If not for the necessity of inducers, all wet surfaces might be covered with fungi (Florian 1992). The exogenous dormancy of conidia was broken with the advent of favourable conditions; endogenous dormancy of conidia broke only by ageing or by a physiological shock that permitted nutrient entry or by endogenous inhibitors that leached out of the spore (Moore et al. 2011). Spore activation was done by either acid or alkaline treatment, protein denaturation or by heat treatment in amoeba (Cotter 1973). Some studies had also documented the enzymes or pathway-intermediates, which occurred and ensured full germination (Laere 1986). Dijksterhuis (2007) has studied the correlation between heat-treatment, the medium of growth, external pressure and pH of the medium that affects conidial activation and dormancy. While the swelling of spore during the start of germination was mainly due to osmosis, the development of germ tube happened only after the initiation of cellular processes such as transcription and translation, which required energy (Feofilova et al. 2012). Numerous studies have documented heat-induced inactivation of conidia in the food industry and heat-induced activation of conidia for germination studies. However, none of these studies gave a convincing explanation for the role played by heat in either inducing or inhibition of germination. The exact role of the effect of any inducer in spore germination was hard to document as spore germination involves extremely rapid physiological changes (Moir and Smith, 1990).

5.7 Effectiveness of the size and charge of beads in protein extraction

The quantity of protein extracted from dry biomass was higher as compared to the quantity of proteins extracted from wet biomass, a fact also observed by Lakshman et al. 2008. For protein extractions, the type of beads used along with its size play a crucial role in the total content of the extracted analyte/compound (Gibbons et al. 2014). Zirconium beads yielded maximum protein due to their spiky-surface that aids in cell wall lysis, in addition to being charge-neutral. Zirconium beads were also suitable for lysing robust samples due to the higher density, almost double as compared with the glass beads of the same size, and so gave a good yield of intra-cellular molecules from half the quantity of biomass as compared with glass beads, a fact also reported by Fujimoto et al. 2004.

5.8 Designing protein extraction buffer

Buffers are essential components in analytical chemistry as they maintain biomolecules under study in a stable form (Urbansky and Schock 2000). The total quantity of protein extracted from any biomass varied as per the extraction buffer used (Shimizu and Wariishi 2005). The buffer composition for protein extraction was based on the type of action of each buffer component. Tris-HCl solution buffered the system to maintain the ionic strength of the medium (El-Harakany et al. 1984). EDTA acted by chelating essential divalent cations preventing unnecessary enzyme action and thereby reducing oxidative damage. Detergents such as SDS and CHAPS manipulated hydrophobic-hydrophilic interactions easing solubilisation. CHAPS was a zwitterionic detergent while SDS was anionic. Therefore SDS was used for the extraction of cell membrane proteins as it solubilised the plasma membrane (Tukmachev et al. 1979). Urea and thiourea increased the solubility of hydrophobic peptides by permanently altering their structure (Rabilloud et al. 1997). It was observed that the combined strength of these salts in the buffer should not exceed 9 M. At higher concentrations, urea and thiourea crystallise. The optimum ratio between urea and thiourea was reported as 9: 0 (nil) or 8:1 or 7:2 or 6:3 (Table 3.2) and the choice of the ratio between the two salts may correspond to the type of proteins being extracted (Berkelman et al. 2004).

DTT was preferred as it is a di-thiol as compared to β-mercaptoethanol, which is a mono-thiol. The quantity of reducing agent in the buffer should suffice the complete breaking of the disulphide bonds which could occur even in 50 mM suspension, depending on the type of cell under study. IPG buffer and ampholytes were zwitterions that can either

donate or accept an H^+ ion during the extraction process (Westermeier 2001). Glycine, being the only non-chiral amino acid, could fit into both the hydrophilic and hydrophobic environment thereby increasing the number and types of proteins extracted. Magnesium chloride in the extraction buffer aided in the stabilisation of the extracted proteins (Bridge et al. 2003). The total concentration/strength of constituents in a buffer depended on the quantity of bio-molecules expected to be extracted although all buffers may not work with equal efficacy for all types of extractions (Ugwu and Apte 2004).

Depending on the solubility and non-degradability of the protein in a particular buffer, pH of the extraction buffer was designed. It has been reported that the activity of proteases is high in fungi as the mycelium ages. When the mycelium was lysed, proteolysis started dynamically, which needed to be curtailed by the addition of protease inhibitors such as PMSF or inhibitor cocktails. Bridge et al. 2003 reduced protein degradation by working at low temperatures along with the addition of PIC, also followed in the standardised protocol. In the case of intracellular protein study, it was encouraged to use the multiple-buffer system for protein extractions.

5.9 Effect of the medium of cultivation of biomass on protein profile

Medium composition altered the biomass (Cooke 1968) and so the protein profile produced. Hence different media were tested to check for biomass production under simulated deep-sea conditions. One semi-synthetic medium (CDB), two complex media (MEB, PDB) and one undefined medium (SBCD) were used for the study. For synthetic medium CDB, sucrose was replaced with 5% glucose. Deep-sea was saturated with oxygen (Damare 2007) and glucose concentration altered dissolved oxygen levels (Mascarin et al. 2015). None of the media was selective for any fungi studied here. As oceans were known to harbour oligotrophic conditions, one complex medium (MEB) was checked in $1/5^{th}$ strength. Forty-five MPa was set for checking growth as the isolates were isolated from depths of 4500 m and above. When proteins were extracted from *A. terreus* mycelia grown in modified CDB, the residual background on SDS gel (Figure 3.6) was minimal to none. This clarity could be because CDB was an inorganic medium with glucose as the only organic carbon source. Conidia required minimum nutrition to germinate and develop, and so the effect of media on conidial germination studies was not conducted.

5.10 Advantages and drawbacks of various precipitation protocols

Details about alcohol-induced protein precipitation have been reported by Yoshikawa et al. 2012. The addition of 0.2M sodium hydroxide to the protein pellet for dissolution did not alter the pattern of the protein bands (Nandakumar et al. 2003). TCA precipitation, though capable, caused residues on the SDS gels as also reported by (Kim et al. 2007). Fic et al. (2010) have described in detail the time-taken and protein recovery for various protein precipitation methods, where methanol/chloroform precipitation was fastest at 30-60 minutes, followed by acetone-TCA precipitation, acetone precipitation and TCA precipitation. The percentage recovery of proteins was highest with methanol-chloroform precipitation (88.52 ±11.62%). Therefore, modified methanol precipitation was used for all in-solution analytes as it was rapid and most efficient, next only to BioRad micro spin columns precipitation. Probably with consecutive steps in the salt removal protocol, there could be a loss in the total quantity of protein resulting in fainter bands in SDS gels as indicated by Charmont et al. 2005.

5.11 Action/function of chemicals used in in-solution and in-gel digestions

Destaining of gels was vital for in-gel digestion as dyes interfered with MS analysis (Gundry et al. 2009). Potassium ferricyanide was an oxidising agent that helped in destaining (Shevchenko et al. 1996) when combined with sodium thiosulphate for silver stains. As coomassie stain was milder than silver stain (Candiano et al. 2004), potassium ferricyanide- sodium thiosulphate mixture was used for detaining. DTT and IAA solutions were prepared in ammonium bicarbonate, a salt that was volatile, which made it compatible with MS analysis. Also, 0.01-0.1 M ammonium bicarbonate had a pH around eight, making it an ideal solution for trypsin to act. DTT was a reducing agent, converting the disulphide bonds in cysteine to free sulfhydryl groups; IAA was an alkylating agent that assured lack of re-oxidation to form disulphide bonds; enzymatic digestion diffused into protein samples and cleaved the proteins to peptides, which was an essential step for generating spectra (Gundry et al. 2009). IAA reacted with remaining cysteine to enable carbamidomethylation, an essential post-translational modification step used for the identification of proteins in tandem mass spectrometry (Rombouts et al. 2013). Heat-assisted denaturation and reduction. Proteases were used for digestion. The most widely used protease for digestion was trypsin due to its exact specificity, availability, ease and cost of material (Hustoft et al. 2012, Giansanti et al. 2016). Acetonitrile had a high

dielectric constant value that made it miscible with a wide range of ionic and non-polar solvents. Therefore, acetonitrile was used in the mobile phase of LC-MS. Formic acid, an organic acid, altered pH at little volume thereby not diluting/altering the digest.

5.12 Advantages of gel-free proteomics

The sensitivity of gel-based proteomics was limited depending on the dye used for staining. While coomassie blue had a sensitivity of 50 ng, silver staining had a sensitivity of 0.5 ng (Schuchard et al. 2003). Although fluorescent dyes had sharper sensitivity, it was not recommended when the gel had to be processed for MS analysis. When proteins were present in tiny concentrations, they cannot be separated based on their physiochemical properties as the concentration of proteins will be too low to be detected by staining (Baggerman et al. 2005). Given these constraints, only in-solution digestion for protein analysis was retained.

5.13 Overview of results of pathway analysis

From the results of the pathway analysis of commonly up-regulated proteins, there were 20 pathways expressed during mycelial growth; proteins involved in xenobiotic degradation were exclusive only to mycelial biomass. Studies on germinating conidia in simulated deep-sea conditions had proteins from as much as 33 pathways expressed. Proteins involved in amine metabolism, amino sugar and nucleotide sugar metabolism, carbon metabolism, cell-wall/envelope/membrane biogenesis, cellular processes, membrane transport, oxygen metabolism, protein metabolism, protein regulation, protein transport, substance dependence and sugar-alcohol mechanism were exclusive to conidial germination studies.

Amine metabolism involved proteins with organics that are weakly basic. Amines were involved in almost all steps of DNA, RNA and protein synthesis; requirements for metabolism of amines was high during the initial stages of acute growth (Gloria 2003). In humans, alterations in amine metabolism altered hypertension levels (Sjoerdsma 1961). Therefore amine metabolism may play an important role in conidial germination under high-pressure conditions. N-acetyl-D-glucosamine, an essential constituent of chitin, was an amino sugar (Salton 1965). Amino sugar metabolism was necessary for chitin build-up in germinating conidia. Also, amino sugars served as sources of both carbon and nitrogen (Matano et al. 2014). Proteins involved in the metabolism of nucleotide sugars were responsible for building blocks of DNA and RNA, deoxyribose and ribose sugars. Carbon

metabolism involved the conversion of carbon to a monosaccharide (as in Calvin cycle) or any other organic molecule whereas carbohydrate metabolism focussed on the production of energy from sugars (Mulukutla et al. 2015). As a growth medium supplied was sourced only in primary elements, it was not surprising that carbon metabolism was observed only in conidial germination. Initiation of carbon metabolism may be responsible for the development of germ pore and germ tube. In bacteria, proteins involved in cell wall/envelope/membrane biogenesis were responsible for cell wall development (Veeranagouda et al. 2014). These proteins may be occurring during the elongation of the germ tube followed by the development of mycelia. Proteins involved in cellular processes carried out essential functions within a cell for survival (Shoemaker and Panchenko 2007). In conidial germination, these proteins may be involved in triggering activity from the state of dormancy as these proteins were identified even in ungerminated conidia. Oxygen metabolism occurs in all aerobic organisms for the production of energy (Venojarvi 2010). Protein metabolism was pivotal in acclimatisation to low temperatures (Guy 1990), management of injury (Biolo et al. 1997) and many more functions. Regulation of genome leads to regulation of transcriptome and proteome. Protein regulation is the changes that occurred in protein level, localisation, activity, and interaction; therefore, it was intimately connected to signalling molecules (Lee and Yaffe 2016). While protein regulation served as a response to extracellular signals outside cells, within the cell, it served as a response to protein degradation (Cooper 2000). Protein transport played a significant role in moving proteins to its destination for action, within the cell (Yoneda 2006). Sugar alcohols were intermediates of carbohydrate metabolism (Makinen 1984). Proteins involved in these metabolisms proved the regular functioning of a growing cell in simulated deep-sea conditions.

Proteins involved in the metabolism of amino acid-carbohydrate-biosynthesis of secondary metabolites **we**re intermediates that could proceed in any of these three metabolic pathways depending on the necessity arising at that moment of survival and growth (Jensen 1986). Benzene ring formed the basis for aromatics; degradation of aromatics was essential for maintaining the balance of elements in nature (Dagley 1971). Persisting presence of aromatics within cells was stressful to the cell (Jimenez et al. 2010); hence it was crucial that they are degraded. Proteins involved in cell growth and death may be responsible for the proliferation and abrupt arrest of growth (Cooper and Youle 2012).

All processes that ended up generating ATP fell under energy metabolism that sustained life (Martin and Thauer 2017). Post Translational Modifications (PTM) of proteins was essential for the functioning of protein (Lodish et al. 2000). The maintenance of protein in a particular folded state, sorting for translocation within and outside cell and apoptosis of degraded protein was performed by the folding-sorting-and-degradation mechanisms (Hebert and Molinari 2007). Evolution of organisms happened by processing genetic material (Patel 2001). Proteins involved in genetic information processing maybe responsible for adaptations in simulated deep-sea conditions. Lipid metabolism involved the production, utilisation, and oxidation of lipid within the cell; lipids were insoluble in water and were storehouses of high-energy which came of utility during distress (Dashty 2014). Proteins involved in membrane transport took part in selective permeability of molecules within and outside the cell (Pardee 1968, Oxender 1974, Jiang 2017). The function could also include osmoregulation in this study due to the difference in pressure conditions within and outside the cell. Nucleotides were precursors of life as they were the building blocks of nucleic acids; degradation of nucleotides provided products that got into many other pathways such as energy metabolism (Henderson and Paterson 2014). Therefore, proteins involved in nucleotide metabolism played a role in overall metabolism. Preservation of genetic information and the process of transferring this information with precision and accuracy fell under replication and repair (Eggleston 2007). All proteins that carried out genetic information processing should be house-keeping proteins. The ability to convert external stimuli into signals and passing them within the cell to localise the preparation of response for the stimuli was performed by proteins involved in signal transduction, signalling molecules and their interaction; the biomolecules involved either identified the components of the received signal or integrated multiple received signals (Duffy and Perrimon 1996). Mechanisms of signal transduction played an essential role in translation (Morley 1994) and acted as a platform for discovery of new drugs (Persidis 1998). Transcription and translation processes formed the central dogma of life. Transport proteins were also called as carrier proteins. While transport proteins were involved in the transporting biomolecules within and outside the cell, protein transport was the study of how proteins were transported within the cell to the site of action. Transport proteins maybe named as mitochondrial carrier proteins, membrane carrier proteins, etc. depending on the target site for the action of the protein.

Xenobiotics are particles/compounds that are foreign to a biosphere (Fetzner 2002); they may not be toxic and hence need not necessarily be a pollutant. Xenobiotic pathways were usually studied to understand if there were toxic intermediates in mechanisms of degradation (Singleton 1994) and therefore have high potential in bioremediation (Magan et al. 2010).

5.14 Stress proteins or chaperons

Highly conserved proteins were stress proteins; although heat-shock proteins (HSPs) were discovered in response to heat stress, they were induced by other stresses also and therefore were called stress proteins; determinants of stress proteins were expressed on the surface of the cell requiring specific techniques for extraction (Young 1990), which was not possible in this study. The conditions for the synthesis of HSPs in cardiovascular biology was due to the presence of reactive oxygen species, osmosis of ions in ion channels, deactivated proteases, alterations in cell wall surface, the external activity of collagenase, proteases, cytotoxic lysosomal enzymes from other microbes in the same biosphere (Benjamin and Mcmillan 1998). Stress proteins were named HSP n where "n" denoted the molecular weight of the protein (Latchman 2004). Latchman described the functional role of major eukaryotic stress proteins with possible inference and discussion. In general, HSPs were involved in the appropriate folding of proteins so that the folded proteins can proceed with their earmarked function; some minimum number of HSPs were involved in the destruction of damaged proteins. The diverse function of stress proteins included proper protein folding, protein transport, modulation of protein activity, protein complex assembly/dismantling, refolding of misfolded proteins, protection against protein aggregation by solubilising protein aggregates and destruction of damaged proteins (Arslan et al. 2006).

5.15 Dark proteome

At normal growth conditions that lacked external stress, the level of stress proteins in prokaryotes was 1.5%; on the other hand, when the cell was actually under external stress, stress protein expressions went up to a maximum of 15% in bacteria (Neidhardt and Vanbogelen 1987, Sedlackova 2008). However, this level was still unknown for eukaryotes due to a high level of the dark proteome. The dark proteome were proteins that can never be observed by experiments and therefore were inaccessible for homology modelling (Perdigao et al. 2017).

With more than 40% proteins in eukaryotes lacking functional annotation (Horan et al. 2008, Luhua et al. 2008), it was not surprising that this study has also detected almost 70% unknown proteins in dormant conidia and 50% unknown proteins in mycelial expression studies. Although there are non-experimental methods to devise function for an unknown protein using gene prediction from contigs (Dhanyalakshmi et al. 2016), it was far from being foolproof (Whisstock and Lesk 2003). Nevertheless, protein domains of unknown function in fungi may perform essential functions as proven in bacterial studies (Goodacre et al. 2013). Notably, there were no predicted proteins that were commonly up-regulated across any of the conditions studied. Neither was any conserved hypothetical protein detected.

Proteins rarely acted alone and usually acted as protein-protein complexes; there was a massive number of proteins within a proteome; as cells were dynamic, only some proteins were expressed at any point of time (Bhadauria et al. 2007). In eukaryotes, dark proteome accounted for 54% of the total proteome (Perdigao et al. 2015). Hence, the number of proteins detected in experiments conducted can never be 46% as all proteins are not expressed at all points of time. It was critical to relate function to the structure of the protein so that the significance and dynamics of an organism in an environment can be correlated. (Chakravarty et al. 2015). An amino acid sequence can have multiple structures and functions based on PTM. As this study involved treatment of protein to get it to primary structure and enzymatic digestion for PMF analysis, the result of pathway analysis was preliminary. Therefore sub-proteome analysis was not possible at this stage.

5.16 Observations and conclusions

The results of protein summary obtained indicated that each isolate may or may not produce the same set of proteins under identical conditions. For all isolates, proteins that were commonly up-regulated were not retained as "up-regulated proteins" when the baseline for statistical analysis was changed. Similarly, the commonly up-regulated proteins were not conserved/signature proteins. Also, some pathways expressed during conidial germination in simulated deep-sea conditions were completely absent in mycelial growth and survival studies under simulated deep-sea conditions.

Proteins involved in house-keeping such as glycolysis, pentose-phosphate-pathway, kerb's cycle, electron transport, signalling, cell-wall generation, cell division and cell growth were commonly down-regulated. On the one hand, few proteins involved in

one pathway were up-regulated whereas, on the other hand, most proteins from the same pathway were down-regulated, which explained the slow rate of growth under extreme conditions. This study provides the preliminary results by exhibiting the proteins produced by fungi cultivated under simulated deep-sea conditions.

Chapter SIX

SUMMARY

Chapter 6 – Summary

Based on the results observed in this study, the following observations are made and summarised as mentioned below.

6.1 Point-wise summary of the study

- A solution of 0.1% tween 80 was used for harvesting conidia from agar plates as spores being hydrophobic cannot be harvested in water-based solutions.
- Conidia/spores were better as inoculum than broken mycelia as inoculum. Broken mycelia released lysozymes.
- For all experiments involving conidia, a count of 30–50 x 10^6 conidia mL^{-1} was used as inoculum as contact inhibition was minimum at this concentration.
- 30-day was the minimum period of incubation for studying spore germination, mycelial growth and survival at elevated hydrostatic pressures. When conidia were incubated for lesser than 30 days at low temperature (4^{o}C), the results obtained for the total number of germinating spores were lesser than 25% for *A. terreus* at all conditions of elevated pressures.
- After completion of incubation, biomass was harvested by filtering using vacuum pump instead of centrifugation. Pre-weighed, dried, 0.45 µm nitrocellulose membrane filters were used in the filtration unit to harvest biomass. Filtration ensured minimum loss of biomass.
- Lyophilising is the best method to preserve cellular contents and store biomass.
- Modified CDB (sucrose replaced with glucose) was recommended for the cultivation of fungi to study the mycelial protein profile, as long as the biomass build up is not adversely affected.
- High-speed homogenisation with bead-beating using zirconia beads and a multi-buffer system of protein extraction yielded maximum proteins.
- Single or two-step salting-out methods worked well for protein precipitation as there was minimal loss of proteins.
- In-solution digestion that is gel-free proteomics had more advantages than in-gel digestion that is gel-based proteomics. Hence, for the comparison of protein profiles, only in-solution analysis was carried out.
- A 71-minute protocol in LC-MS-QToF for PMF eluted most peptides and gave maximum protein coverage.

- Statistical analyses provided the commonly regulated proteins across pressure conditions.
- Proteins involved in carbohydrate metabolism and the central dogma of life were mostly up-regulated.
- Presence or absence of specific pathways within an organism exposed to different temperature conditions proved that the organism's way of adaptability is unique to environmental changes.
- The presence or absence of specific pathways amongst the isolates studied point that each isolate adapts itself to the identically simulated deep-sea conditions in different ways.
- No universal survival and growth strategy could be applied to all fungal isolates studied here.
- House-keeping proteins had higher fold-change regulation than stress- and unknown-proteins showing that adaptability for survival and acclimatisation are prioritised before growth.
- The slow growth in deep-sea conditions was due to the down-regulation of intermediate proteins and enzymes involved in house-keeping.
- Germination and growth of spores in deep-sea conditions are triggered by numerically more number of active pathways than mycelial survival and growth under the same conditions. Hence most spores remain ungerminated under the deep-sea conditions.

Prelude to chapter 7

This chapter briefs the importance of this study and hints about the future perspectives.

Chapter SEVEN

FUTURE PERSPECTIVES

Chapter 7 – Future perspectives

This study described for the first time, a set of proteins expressed in a eukaryote under simulated deep-sea conditions. Knowing the biochemistry of an organism under deep-sea conditions can lead to biochemical, medical and other advances (Thurber et al. 2014). The importance of this work, the necessary modifications that could be used in the future for improving the results obtained from this study are discussed in this chapter.

7.1 Importance of this work

Some studies on spore germination have mentioned the use of an inducer or additive as a spore-germination trigger. This study documented that fungal conidia needed only water and minimum nutrients to germinate, not only at normal conditions of room temperature and atmospheric pressure but also under elevated hydrostatic pressures and low temperature. This is an important finding as it is the first step in explaining the ubiquitous presence of fungi in the atmosphere as well as in hydrosphere.

There is no greater advantage for the conidia to germinate to into hypha/mycelia in the nutrient impoverished deep-sea sediments. More sampling and analyses from high organic carbon accumulating trenches could provide clues as to designate the exact ecological roles of fungi. The effect of inducers for breaking either exogenous or endogenous dormancy cannot be entirely extrapolated from this study as conidia/spores were harvested from freshly cultivated isolates. Also, the period that conidia get buried within the sea bed before initiating germination is not known. Tracking the fate of each spore in the ocean is also not feasible.

From this study, it is ascertained that any specific entity or set of proteins cannot be held responsible for growth under simulated deep-sea conditions. When there was more than one organism growing in the identical/same environment, the response pattern was different for each organism as detected in this study and also in a previous study (Tesei et al. 2012). With this finding, it is proposed that instead of studying only stress proteins for survival, adaptation, and growth of an organism in stressful conditions, studying the function, structure, dynamics, and sequence of all the proteins expressed in a particular condition may hold the key for breakthroughs.

7.2 Path Ahead

The total number of proteins obtained may change when the proteins would be digested with different enzymes; when the column used for carrying out PMF analysis and

solvents used for LC would be altered. Studying the post-translation modifications (PTM) of the expressed proteins would make more relevance than studying chaperones for extremophilic life as there were few HSPs, and no cold shock protein was produced, as observed in this study. However, studying PTM is challenging as the NMR for this structural analysis is required to be carried out under high-pressure, which involves processes from extracting the protein sample till digestion to be carried out under elevated pressure. A sub-proteome analysis could shed more light on protein structure and function in stress conditions.

Alternatively, whole genome sequencing of the deep-sea isolates and working on transcriptomics can provide clues of proteins that were unidentified or missed in this study.

BUFFER COMPOSITIONS

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SEMINARS

Composition of buffers

10mM TE buffer (pH 8.0) 10 mM Tris-HCl @ pH 8.0 1 mM Na_2EDTA @ pH 8.0		Acrylamide/Bis-acrylamide 29.2 g Acrylamide 0.8 g Bis-acrylamide Make-up to 100 mL with deionized water
10X TBE buffer 108 g Tris 55 g Boric acid 7.5 g Disodium EDTA Make-up to 1 l with deionized water		10X SDS electrophoresis buffer (pH 8.3) 30.2 g Tris 144 g Glycine 10 g SDS Make-up to 1 l with deionized water
Resolving buffer 1.5 M Tris-HCl @ pH 8.8 pH to be adjusted using 6 N HCl		Stacking buffer 0.5 M Tris-HCl @ pH 6.8 pH to be adjusted using 6 N HCl
3-Buffer system for protein extraction *Buffer 1*: 0.5 M Tris-HCl at pH 8.3, 2 % CHAPS, 20 mM $MgCl_2$, 2 % DTT *Buffer 2*: 9 M urea, 4% CHAPS, 100 mM DTT. This buffer was prepared in 40 mM Tris-HCl (pH 8.0) *Buffer 3*: 6 M urea, 3 M thiourea, 4 % CHAPS, 100 mM DTT. This buffer was prepared in 40 mM Tris-HCl (pH 8.0)		

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Details of conferences attended, papers presented and list of publications

List of publications, conference proceedings, workshops, and seminars attended

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An improved method for protein extraction from miniscule quantities of fungal biomass. 2018. **A Krishnaswamy**, N Barnes, NP Lotlikar, SR Damare. 2018. *Indian Journal of Microbiology*. doi: 10.1007/s12088-018-0752-y

Conference presentations

1. ***Oral presentation*** titled '*Protein profile of fungi grown in simulated deep-sea condition*' at the Asian Mycological Congress (AMC 2015) held at Goa University, Goa, India, Oct. 5-8, 2015.
2. ***Oral presentation*** titled '*Protein expression studies in fungal spores, subjected to various elevated hydrostatic pressure using proteomic techniques*' at the High pressure Bioscience and Biotechnology (HPBB 2014) held at Oniris, Nantes, France, Jul. 15-18, 2014.
3. ***Oral presentation*** titled '*Protein expression studies for the differentiation of germination phases in fungal spores using 2-D electrophoresis technique*' at National conference on Fungal Diversity and Biotechnology for Food and Chemicals (MSI 2014) held at Annamalai University, Parangipetti Campus, India, Feb. 27-28, 2014.

Seminars/Workshop/Training attended

1. Participated in a **National Seminar** 'Advances in Microbiology and Marine Microbiology' held at Goa University, Goa, India, Mar. 13, 2015.
2. Participated in Scientific writing and effective communication **Workshop**, held at Goa University, Goa, India, Jan. 5-6, 2015.
3. Participated in **Workshop-cum-Training** on 'Basic operations of Ultra-high Definition MS and its application for analyzing small and large molecules' held at CSIR-NIO, Goa, India, Nov. 5-7, 2014.

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SHORT COMMUNICATIONS

An Improved Method for Protein Extraction from Minuscule Quantities of Fungal Biomass

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Abstract Filamentous fungi are ubiquitous eukaryotes having chitin as a major constituent of the cell wall. Chitin is tough to lyse due to which the intracellular fungal proteins are not readily accessible. The problem is further enhanced when the biomass to be analyzed for protein studies is too little due to the extreme experimental parameters under consideration such as increased or lowered pH, temperature, hydrostatic pressure, nutrients, etc. The method described here is capable of obtaining proteins from minuscule quantities of biomass (~5 mg lyophilized biomass). In this study, different lysing conditions and varied composition of extraction buffers were tried to obtain maximum protein of high quality. Lysis with zirconium beads in a combination buffer system (Tris–$MgCl_2$ buffer, urea buffer I and urea buffer II) was best for extracting proteins from the fungal isolates used. The protocol described here provides for a simple and quick method for extraction of high-quality proteins from very less biomass that could be extended to other tough to lyse biological material also.

Keywords Fungi · Homogenizer · Biomass · Protein · Zirconium beads

Protein extraction is the most critical step of proteomic studies, which often differs with the type of organism and requires thorough standardization to obtain good quality proteins for analysis. Extraction of proteins from filamentous fungi has always posed challenges due to the robust, chitinous cell wall. Although liquid nitrogen crushing of fungal mycelia is the most commonly used method for extracting proteins [1–3], it has limitations when the amount of biomass is less [4, 5]. The problem of less biomass arises when biomass build-up reduces drastically as a result of the experimental parameters, for example, the extreme conditions of temperature, pressure, pH, etc. Despite the limitation of biomass quantity, an improved method of extraction of intracellular proteins from biomass as less as 5 mg (dry weight), without the need of liquid nitrogen based homogenization is described in this work. Various lysing methods and extraction buffers were evaluated to extract proteins using lyophilized biomass of *Aspergillus terreus* (Strain #A4636). The efficiency of the standardized method is demonstrated in terms of the quantity and quality of the extracted proteins as viewed on the SDS–polyacrylamide gel.

To check the efficiency of the standardized protocol, eight randomly chosen fungal isolates from eight different genera, isolated from different marine habitats, oyster mushroom, and one unidentified filamentous fungus were used. Fungal cultures were grown in modified Czapek Dox broth (CDB—sucrose replaced with 5% glucose and prepared in seawater) for 6–7 days at room temperature (28 °C, static conditions), the biomass harvested, washed to remove residual seawater and media, lyophilized, and stored in airtight container till used for protein extraction. *Pleurotus ostreatus* was grown on Czapek Dox agar for

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12 days at room temperature, the mycelia were harvested, and all the steps described above were followed.

It was reported that lyophilized biomass gave better protein profiles than wet biomass [2], hence lyophilized fungal biomass was used for entire analysis. To ascertain the best lysing condition, different methods of homogenization were tried such as sonication and vortexing with glass beads, mechanical disruption using FastPrep homogenizer (FastPrep®24, MP Biomedicals, USA) with lysing matrix A (MP Biomedicals, USA) and zirconium beads (D6006, Zymo Research, USA) (Table 1). Five hundred µL of each extraction buffer and 5 µL of 100 X Protease Inhibitor Cocktail (PIC, Serva #39104) were added to the lyophilized biomass for protein extraction individually. All the homogenizations using FastPrep homogenizer were performed at 6.5 m/s for 60 s. In case of single buffer extractions (S. no. 1, 2 and 3, Table 1), homogenization was performed twice with a rest time of 5 min, while for multi-buffer extractions (S. no. 4 and 5, Table 1), homogenization was performed twice for the first buffer (rest time of 5 min) followed by single homogenization for the remaining two buffers with the same biomass/pellet. The homogenized lysate tube was centrifuged at 14,000 rpm, 4 °C for 15 min and the supernatant was collected. In case of multi-buffer extraction, supernatants were collected in a single tube.

Protein yield from sonicated biomass was very less (Table 1), as high-intensity sonication breaks the mycelia into smaller filaments but does not rupture the cell wall, thereby not releasing all the proteins [6]. Zirconium beads provided the best lysis of fungal mycelia, which reflected in the protein levels obtained (Table 1). Maximum yield of proteins with zirconium bead lysis could be due to their spiky-surface that aids in cell wall lysis; in addition, they are neutral in charge and higher in density (almost double as compared to glass beads of the same size) [7].

Constituents of extraction buffers in this study were selected based on their function. The Tris–HCl solution used in this method was chosen to maintain the ionic strength of the medium [8]. Detergents such as SDS and CHAPS control hydrophobic-hydrophilic interactions easing solubilization, while urea and thiourea increase the solubility of hydrophobic peptides by altering their structure [9]. The ratio between urea and thiourea varies typically from 9:0, 8:1, 7:2 to 6:3, as the combined strength of urea and thiourea in the buffer, should not exceed 9 M

Table 1 Details of different methods of lysis and the extraction buffers used for protein extraction from lyophilized biomass of *Aspergillus terreus* #A4636

S. no.	Buffer name	Buffer composition	Method of lysis	Type of beads	Protein concentration (µg/mL)
1	TE buffer	10 mM Tris–HCl/EDTA buffer (TE buffer) + 5 µL of 0.7 M β-mercaptoethanol	Sonication (5 min) and vortexing (60 min)	Glass beads (0.125–0.25 mm Ø)	< 2
2	Yeast breaking buffer (YBB)	YBB buffer (MP FastPrep protein isolation kit, Cat 116550600) + 5 µL of 0.7 M β-mercaptoethanol	FastPrep Homogenization (6.5 m/s for 60 s)	Lysing matrix A (MP Biomedicals, USA 6.223 mm Ø)	< 2
3	Urea with SDS	1.5% SDS, 9 M urea, 25 mM Tris–HCl [pH 6.8], 10 mM EDTA and 0.7 M β-mercaptoethanol [modified from [14]]	FastPrep homogenization (6.5 m/s for 60 s)	Zirconium (0.1 mm Ø)	549
4	Tris–glycine buffer	Tris–glycine @ pH 8.3 (3 g Tris, 14.4 g glycine in 1L deionized water, SDS running buffer) [1] + urea buffer I (9 M urea, 4% CHAPS, 100 mM DTT—prepared in 40 mM Tris–HCl pH 8.0) [modified from [3], [9]] + urea buffer II (6 M urea, 3 M thiourea, 4% CHAPS, 100 mM DTT—prepared in 40 mM Tris–HCl pH 8.0) [15]	FastPrep homogenization (6.5 m/s for 60 s)	Zirconium (0.1 mm Ø)	251
5	Tris–$MgCl_2$ buffer	(Buffer 1) Tris–$MgCl_2$ @ pH 8.3 (0.5 M Tris–HCl, 2% CHAPS, 20 mM $MgCl_2$, 2% DTT) [16] + (buffer 2) urea I buffer (9 M urea, 4% CHAPS, 100 mM DTT—prepared in 40 mM Tris–HCl pH 8.0), + (buffer 3) urea II buffer (6 M urea, 3 M thiourea, 4% CHAPS, 100 mM DTT—prepared in 40 mM Tris–HCl pH 8.0) [15]	FastPrep Homogenization (6.5 m/s for 60 s)	Zirconium (0.1 mm Ø)	1752

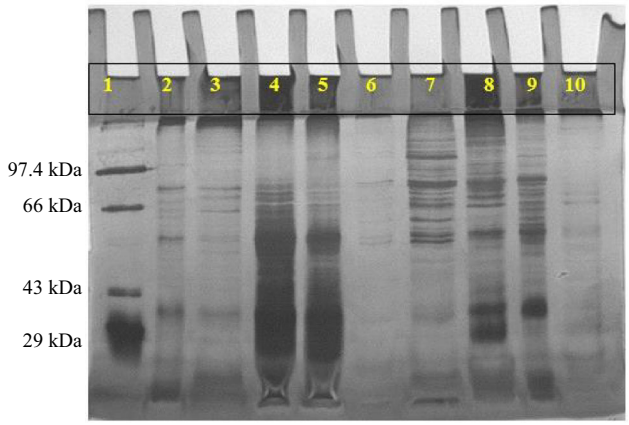

Fig. 1 Mycelial protein profiles of *Aspergillus terreus* #A4636 obtained using FastPrep homogenizer lysing and different buffers. Lane 1: protein molecular weight marker (PMW-Medium, Merck, India); lane 2: YBB buffer, # A4636; lane 3: YBB buffer, # MTCC479; lane 4: urea buffer, #A4636 (with SDS); lane 5: urea buffer, #A4636 (with IPG buffer); lane 6: Tris–glycine combination buffer, #A4636; lane 7: standardized buffer, #A4636; lane 8: standardized buffer, #MTCC 479 (*A. terreus* strain); lane 9: urea buffer, #MTCC 479 (with IPG buffer); lane 10: Tris–glycine combination buffer, #MTCC 479

Table 2 Fungal isolates used in the study and proteins obtained from the lyophilized biomass ($\sim$ 5 mg) by the standardised method[a] as quantified by Qubit assay kit

Fungal isolates used	Protein concentration (mg/mL)
Sarocladium strictum	3.14
Cladosporium cladosporioides	2.78
Hortaea werneckii	3.6
Acremonium alternatum	3.66
Purpureocillium lilacinum	2.48
Penicillium citrinum	1.04
Engyodontium sp.	1.028
Aspergillus flavus	2.42
Unidentified filamentous fungus	2.35
Pleurotus ostreatus	3.84

[a]Lysing with zirconium beads using FastPrep homogenizer and Tris–$MgCl_2$ 3-buffer system

since at higher concentrations, urea and thiourea crystallize [10]. DTT is preferred since it is a di-thiol as compared to mono-thiol β-mercaptoethanol [11]. Magnesium chloride aids in stabilization of the extracted proteins [12].

Proteins obtained from first two methods (S.No. 1 and 2, Table 1) using TE and YBB buffers were too less (> 2 µg/mL). Tris–$MgCl_2$ 3-buffer system (Table 1, S. no. 5) yielded maximum proteins from lyophilized biomass of *A. terreus*, during standardization of the method. Figure 1 is representing the quality of proteins obtained using different methods tried with lyophilized biomass of *A. terreus* #4636, with lane 6 showing the best protein profile. Tris–$MgCl_2$ 3-buffer system and FastPrep homogenization with zirconium beads were evaluated for its efficiency for protein extraction from different fungal isolates (Table 2). The method worked well for all the fungal cultures tested in this study, with the protein concentrations obtained above 1 mg/mL (Table 2).

It has been reported that the activity of proteases is very high in fungi, especially as mycelium ages [12]. When mycelium lyses, proteolysis starts dynamically, which could be stopped by addition of protease inhibitors and by working at low temperatures [1]. In this method, protease inhibitor cocktail (PIC) was added along with lysing buffer and lysed contents were intermittently cooled in ice (5 min) between homogenization pulses to neutralize the effect of proteases released during lysis. The intermittent cooling in ice also helped to nullify the heat generated during homogenization.

It was not possible to extract proteins from 5 mg biomass using conventional liquid nitrogen crushing in pestle and motor. Hence no relative quantity of protein extracted by liquid nitrogen crushing could be mentioned. Instead, comparison with previous studies of protein extractions using liquid nitrogen is reported in Table 3, which shows that recovery of proteins with respect to biomass employed using this method is quite high.

Quantification of the desalted protein extracts (done using BioSpin chromatography columns, BioRad, USA) was performed using Qubit Assay kit (Invitrogen, Q33211 USA) as per manufacturer's protocol. Desalting of proteins helps in removal of salts and other chemicals from the extraction buffers. The desalted protein extracts were electrophoresed using SDS-PAGE to check the quality and the yield of extracted proteins on 12% resolving gel with 6% stacking gel at a constant voltage of 90 V for one h and 45 min. Hundred µg of protein extracts were loaded in each well. The gel was stained using silver staining method [13] (Fig. 2). The quality of the proteins obtained was good as evident from the SDS–polyacrylamide gel with distinct bands seen in almost all the lanes (Fig. 2).

The method of protein extraction described here is a rapid protocol (requiring around 20 min, excluding the time required for lyophilization) to obtain sufficient quantity of high-quality proteins. This method nullifies the disadvantage of biomass availability for protein extractions, thereby opening up avenues to study protein profiles of samples/organisms which are difficult to cultivate or sparsely obtained.

Table 3 Comparison of protein yields obtained from different methods reported in the literature with the method reported in this study

S. no.	Biomass quantity	Wet/dry biomass	Method	Protein concentration/yield	References
1	500 mg	Dry	Pestle and mortar grinding (mycelium stored in − 20 °C, freeze-dried before grinding) French press in case of yeasts *Buffer*: Tris–glycine buffer (3 g trizma, 14.4 g glycine and 1L deionized water, pH 8.3)	15–100 mg/mL	[1]
2	1 g	Dry	Mechanical grinding with liquid nitrogen on the freeze-dried sample, stored in − 20 °C *Buffer 1*: TCA-acetone extraction procedure *Buffer 2*: Phosphate buffer extraction	56.46 mg/g dry weight (buffer 1) 43.19 mg/g dry weight (buffer 2)	[2]
3	100 mg	Dry	Pestle and mortar grinding of mycelia frozen in liquid nitrogen, lyophilized over night *Buffer*: 6 different buffers were used for standardization	Not available	[3]
4	Not mentioned	Wet	Liquid nitrogen freezing followed by mechanical grinding *Buffer*: 7 M urea, 2 M thiourea, 4% CHAPS, 0.8% IPG buffer, 20 mM DTT	Not available	[17]
5	~ 5 mg	Dry (lyophilized)	Bead-beating homogenization *Buffer*: Tris–$MgCl_2$, urea buffer I, urea buffer II	1.04–3.66 mg/mL corresponding to 208–732 mg/g dry weight of biomass	Present study

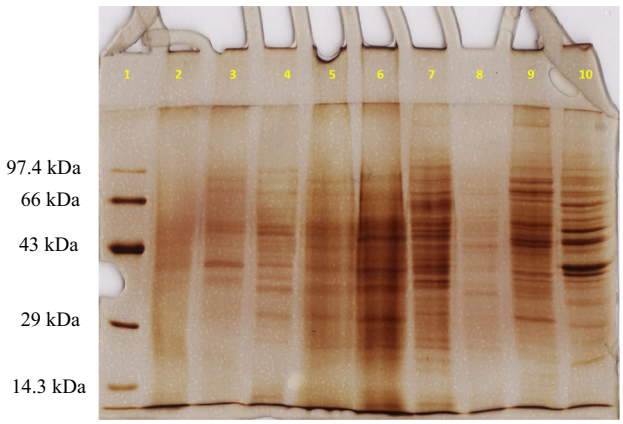

Fig. 2 SDS-PAGE profile of proteins extracted from nine different fungal cultures using the optimized Tris–$MgCl_2$ buffer FastPrep homogenization with zirconia beads. Lane 1: protein molecular weight marker (PMW-Medium, Merck, India), lane 2: *Sarocladium strictum*, lane 3: *Cladosporium cladosporioides*, lane 4: *Hortaea werneckii*, lane 5: *Acremonium alternatum*, lane 6: *Purpureocillium lilacinum*, lane 7: *Penicillium citrinum*, lane 8: *Engyodontium* sp., lane 9: *Aspergillus flavus*, lane 10: unidentified filamentous fungal isolate

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Contribution of authors The idea was conceived by the corresponding author during the work on deep-sea fungal cultures, where obtaining biomass from fungi growing at elevated hydrostatic pressure is a challenge. The first author was involved in standardization of the method and drafting of the manuscript, and the work is part of her doctoral thesis to be submitted to Goa University. The second and third authors have validated the method by evaluating it for extraction from different fungal cultures. This is an original work by the authors and has not been published elsewhere either completely, in part, or in any other form. The manuscript has not been submitted to another journal. The results described here have been approved for publication by the responsible authority of the institute where the work was carried out, and all the persons entitled to authorship have been named.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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CERTIFICATE

This is to certify that ~~Mr.~~ / Ms. / ~~Dr.~~ Akhila Krishnaswamy *has participated in the National Conference on* "Fungal Diversity and Biotechnology for Food and Chemicals" *and* 40th Annual Meeting of the Mycological Society of India, *held at Centre of Advanced Study in Marine Biology, Faculty of Marine Sciences, Annamalai University, India during February 27-28, 2014 as Delegate / ~~Organising committee member~~ / ~~Volunteer~~ and delivered an ~~Invited lecture~~ / ~~Award lecture~~, made Oral / ~~Poster~~ presentation on "*Protein expression studies for the differentiation of germination phases in Fungal spores using 2d electrophoresis technique".

Dr. M. KALAISELVAM
Organising Secretary

Prof. K. KATHIRESAN
Dean & Convener

Prof. N. RAAMAN
Secretary, MSI

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8th International Conference on High Pressure Bioscience & Biotechnology
15-18 July 2014, Nantes, France

Certificate of Attendance

This is to certify that

Akhila Krishnaswamy

CSIR-National Institute of Oceanography

India

has attended the 8th International Conference on High Pressure Bioscience & Biotechnology which took place at Nantes (France) 15-18 July 2014.

Nantes, 15th July 2014

On behalf of the HPBB 2014 Organizing Committee

Marie de Lamballerie

Chairman

Agilent Technologies

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Department of Biotechnology

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Head, Biotechnology	Workshop Co-ordinator	Distinguished Professor	Research Professor
		University of Florida, USA	

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