# Physiological response of fungi from marine habitats to heavy metals

Thesis submitted for the degree of

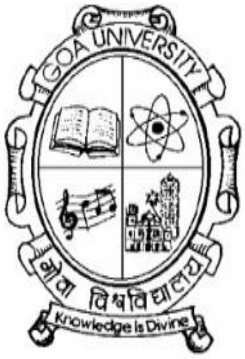
**Doctor of Philosophy**

in

**Microbiology**

to the

**Goa University**




by

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April 2019

# Statement

As required under the University Ordinance OB-9A.10, I state that the present thesis entitled "**Physiological response of fungi from marine habitats to heavy metals**" is my original contribution 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 have been made whenever facilities and suggestions have been availed of.

There were no corrections/modifications suggested by the examiners in the thesis.

**Nikita P. Lotlikar**

**Chairman, Board of Examination** **External Examiner**

# Certificate

This is to certify that the thesis entitled "**Physiological response of fungi from marine habitats to heavy metals**" submitted by Nikita P. Lotlikar 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.

Place:

Date:

Dr. Samir R. Damare
Research Guide
Senior Scientist
National Institute of Oceanography
Dona Paula, Goa, India.

Dedicated to my Grandparents and my beloved brother

# Acknowledgements

My journey of pursuing Ph.D. was always an aided effort. This thesis has been seen through to completion with encouragement and support of numerous people. Before I thank all the people who made this possible, I thank God for giving me the courage, wisdom and patience to successfully complete my work in good health.

I would like to express my sincere gratitude to Dr. Samir R. Damare, my research guide, who has always provided his valuable inputs during my work. His unparalleled patience is the most admirable quality and is worth learning something from. I thank him for correcting my reports and thesis, time and again, without complaints. I respect him for encouraging independent thinking in me and also allowing me to make my mistakes and learn from them.

I want to thank the Directors (past and present) of CSIR- National Institute of Oceanography for rendering me the opportunity to work in the institute. I also thank the Vice Chancellor of Goa University for allowing my work to be affiliated through University.

I am thankful to all my FRC members, Prof. Sandeep Garg, HOD, Microbiology, Prof. M.K. Janarthanam, Dean, Life Sciences, Prof. Sarita Nazareth, Department of Microbiology for their critical comments and scientific inputs that helped my research extensively.

I thank the HODs (past and present) of BOD, NIO, especially Dr. N. Ramaiah for his scientific inputs in my research articles. I also extend my thanks to Dr. Brenda Mascarenhas (GOD), Sir Areef Sardar (GOD), Dr. Parthasarthi Chakraborty (GOD), Dr. B. Nagender

Nath (GOD), Mr. Ram Meena Murti (BOD), Dr. Rakhee Khandeparkar (BOD) and Dr. Siby Kurian (COD) for their help in processing my samples and helping with data analysis.

I am deeply grateful to Dr. Belle Damodara Shenoy (BOD), Dr. Damodar Shenoy (COD), Dr. Shyam Prasad (GOD) and Dr. G.N. Nayak, Department of Marine Sciences, for their words of motivation during the tough phase of my Ph.D. I also thank Dr. Varada S. Damare for her suggestions during my presentations.

I express my sincere thanks to Head Librarian, for providing all the help that was needed in terms of research articles, books and other reference materials.

I am thankful to the Council of Scientific and Industrial Research, India, for the Research Fellowships.

I am most grateful to all my friends and colleagues at N.I.O., specially Anup, Akhila, Genevieve, Larissa, Elroy, Natasha, Priya, Shruti, Bliss, Amara, Mahendra and all the others for their valuable suggestions and timely help and support given for my work.

I wish to thank my parents, my beloved sister and my entire family for being the pillars of strength, encouragement and motivation throughout my life. I believe I am lucky to be surrounded by people who have always tried to keep me positive and happy. By saying this, I wish and pray to God that may he always shower his blessing upon us and help us in all aspects of our lives.

**Nikita P. Lotlikar**

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# Chapter ONE

# Current Scenario of Studies Pertaining to Heavy Metals Tolerance

## 1.1 Introduction

Biogeochemical cycling of metals entering into the environment either from natural or anthropogenic sources, is a well-researched topic to study the dynamics involved in metal homeostasis. But with increasing urbanization and industrialization, the burden faced by the communities involved in these cycles is tremendous. Microbes playing a significant role in recycling of toxic metals released by the industries (Spain, 2003), are subjected to physiological and morphological changes due to the overload of toxic metals causing a selective pressure on their very existence (Ezzhouri et al. 2009). Heavy metals by definition are those metals whose density are greater than 5 $g/cm^3$ (Beveridge et al. 1997) and have proven to be a real threat to natural as well as man-made ecosystems. Metals on entering these systems get transformed into mobile or immobile forms in the environment (White et al. 1995). A variety of fundamental roles are played by these metal ions and their complexes, either being useful in metabolism or proving toxic to the organism. Previous studies have indicated that enhanced metal contamination in soils affects microbial communities in terms of species richness by causing the extinction of sensitive species and also enrichment of particular tolerant species that can have an impact on the organic matter mineralization (Giller, 1998; Kelly et al. 2003). Wastewaters discharged by industries often contain a high dosage of heavy metals that are non-degradable, hence bio-accumulate and magnify in the aquatic fauna leading to pronounced toxicity at higher trophic levels (including humans) in the food chain (Kumar and Krishnan, 2011; Mudhoo et al. 2012).

### 1.1.1 What are heavy metals and their roles?

Metals have been classified time and again based on various aspects. Depending on the position occupied in the periodic table, metals have been classified as Alkali, Alkaline Earth, Rare Earth, Poor and Transition metals. Based on their use in industries, cost, and reactivity, they have been grouped as Toxic, Precious and Radionuclides (Macek and Mackova, 2011). But so far classification of metals based on their biological function put forth by Roane and Pepper (2015), has been the most useful in understanding the role and effect of metals in/on a cell. Here, metals have been broadly classified as the Essential metals (Na, K, Mg, Ca, V, Mn, Fe, Co, Ni, Cu, Zn, Cr, Mo, and W) with known biological functions; the Toxic metals (Ag, Cd, Sn, Au, Hg, Ti, Pb, Al, Ge, As, Sb, Se) which aid in none of the biological processes; and the Non-essential/Non-toxic/adventious metals (Rb, Cs, Sr, and T) whose presence/absence have no effect on cellular functioning (Beveridge et al. 1997; Rajendran et al. 2003). Essential metal ions are important as they form part of vital enzymes and are used for metabolic processes, such as copper which is an essential cofactor in various redox enzyme systems like cytochrome c oxidase, lysyl oxidase and more (Agranoff and Krishna, 1998). Similarly, chromium in the oxidation state of $Cr^{+3}$ binds to a small polypeptide specifically activating the insulin receptor tyrosine kinase, whose deficiency can cause a physiological condition similar to diabetes (Neis, 1999). Therefore the presence of heavy metals in minute concentrations is often beneficial than toxic. In fact, an average adult human body normally contains a copper concentration from 1.4 to 2.1 mg per kg of body weight (Borkow and Gabbay, 2005). The transition elements function by binding to macromolecules such as the proteins, in the transportation of storage molecules and are also involved in enzyme catalyses reactions such as oxidation, reduction,

and hydrolysis. Cations help in maintaining membrane stability, nucleic acid structure and metabolism (Lopez and Vazquez, 2003) among which the monovalent cations because of their ability to weakly bind to ligands, are of great importance in charge neutralization and control of osmotic pressure (Beveridge et al. 2007). Metals which do not have any essential functions are classified under toxic metals (Beveridge et al. 1997; Bellion et al. 2006; White et al. 1995). An important aspect of toxicity of a substance is its availability to target the living cells. As extensively discussed by Shanker (2008), a substance needs to possess a set of properties to qualify as a toxicant. It needs to react with the target and adversely affect its functioning and further alter the target in such a way that the toxicity is traceable. On encountering these effects, the target may undergo improper cell division due to changes in gene expression, impaired ATP synthesis and also impaired membrane functioning. An occurrence of such a cascade indicates towards effects of toxicity. Therefore, although some metals may have essential roles in functioning, all heavy metals at higher concentrations, have deleterious effects on life and are toxic. Leaf chlorosis, reduced stomatal closure in plants are some of the effects caused by high Ni concentrations in soils (Appenroth, 2010). Cadmium causes enzyme deficiency by inhibiting protein synthesis (Levinskaite, 2001). Redox cycling between hexavalent Cr and trivalent Cr produces Reactive Oxygen Species (ROS) that causes oxidative damage to DNA and other cellular components (Borut et al. 2010). The last category of classification includes metals such as rubidium and cesium which do not interfere much with the organisms and therefore their presence/absence does not cause much of a hindrance to the cellular processes. The more pressing concern with respect to the ill effects of metals is their bioavailability, as

high concentrations of heavy metals in a complexed form will not affect any organism, as opposed to minute concentrations of highly toxic metal, in a bioavailable form.

### 1.1.2 Factors affecting the bioavailability of metals

A major factor complicating the study of toxicity is the poor understanding of the complex chemistry that lies between metals, soil, and water (Blust et al. 1987). Bioavailability can be referred to as that fraction of metal available for bioaccumulation by microbiota (Chakraborty et al. 2015). Heavy metals as such are not always found as free metal ions. They can be present sequestered to ligands in the form of soluble metal complexes, as organically bound metals, may also be found embedded into structures of silicate materials or even in insoluble or precipitated forms of carbonates, oxides, and hydroxides (Davies et al. 2001). Therefore as categorized by Sposito, metals can be classified as bioavailable (mobile, non-sorbed, precipitated) and non- bioavailable (non-mobile, sorbed, precipitated). The bioavailability and toxicity of a metal depend on the physicochemical parameters of the environment (Lugauskas et al. 2005), and its fate depends on its speciation apart from edaphic factors such as soil organic matter, redox status and pH (Rajendran et al. 2003; Bellion et al. 2006). In terms of speciation, Cr (VI) under aqueous conditions can exist in three forms which is solely dependent on the pH of the solution. At extreme low pH, Cr (VI) exists as dichromate species ($Cr_2O_7^{2-}$); at low pH of about 6.0 to 6.2, it exists as hydrochromate species ($HCrO_4^-$), while at neutral or basic pH, chromate form ($CrO_4^{2-}$) predominates (Das and Mishra, 2008). Similarly, Cr (III) in its free ionic state easily associates with hydroxide ions to form $Cr(OH)_2^+$, $Cr(OH)_3$ and $Cr(OH)_4^-$ (Hawley, 2004). Cr (VI) being water soluble can easily enter the cell and cause damage to the cellular

components, while Cr (III) hydroxyl forms being water insoluble have low bioavailability and is easily adsorbed onto organic carbon, therefore is less toxic (Fendorf, 1995; Borut et al. 2010; Thatoi et al. 2014). Another example of speciation would be of copper that exists as free soluble cupric ions ($Cu^{+2}$) and copper hydroxo complexes at low pH, but precipitates as copper hydroxide at intermediate pH (6.5 to 12). The cupric ions and mono-hydroxo copper complexes are highly toxic to aquatic organisms as compared to other anionic complexes (Cuppett et al. 2006).

Presence of high amounts of organic matter in soils also hinders in bioavailability of heavy metals. Organic materials such as humic acids, fulvic acids and extracellular proteinaceous exudates containing $COO^-$ groups act as good metal binders via forming complexes and reduce the toxicity (Gadd and Griffiths, 1978; Reichman, 2002). Apart from organic matter, hydrous oxides and clays also determine the availability of metals. This knowledge is essential when microbial metal tolerance studies are concerned as the composition of media and the bioavailability of the embedded metal plays a significant role in determining the tolerance. Complex media often contain organic ingredients such as yeast extracts, peptone, amino acids which have an affinity for metals thereby making a potentially toxic metal unavailable for uptake while the same would be bioavailable in case minimal media or inorganic media is used (Beveridge et al. 1997). Further addition of chelating substances such as EDTA can also render the metal non-available (Gadd and Griffiths, 1978). As discussed earlier, pH also plays an important role in bioavailability of metals. At alkaline pH, cations precipitate out as oxides or hydroxides rendering the heavy metals insoluble thus decreasing the bioavailability (Gadd and Griffiths, 1978). Sometimes complexes may not always leave metal non-available. Though microbial cells can get directly affected by

free metal ions, inorganic and organo-metallic compounds are found to be more toxic and have pronounced effects on microbes (Lugauskas et al. 2005) thus having negative impacts on microbial populations.

Petr in 2010 had stated that the saprophytic fungi that rely solely on the extracellular enzymes such as lignolytic, chitinases, cellulases produced to acquire nutrients, turn target and are most affected by the presence of heavy metals in soils (Al Abboud and Alawlaqi, 2011). Heavy metals affect organisms at different cellular levels and hence have high industrial applications as well.

### 1.1.3 Where do metals target?

Toxicity caused by exposure to heavy metals can be caused through several processes. Transition metals, for instance, have incompletely filled d orbitals which provide them the opportunity to readily form complexes which may/may not be redox active in nature. Besides, the various divalent cations have ionic diameters and structures resembling each other, which makes their free passage into the cell inevitable allowing them to further displace essential ions or blocking functional groups of biologically important enzymes (Neis, 1999; Lugauskas et al. 2005; Ramasamy et al. 2007; Hefnawy et al. 2009; Ahmad et al. 2011). Reports suggests that heavy metals have serious implications at cellular levels such as generation of ROS instigating oxidative stresses, cause DNA damage and further impair DNA repair mechanisms, loss of membrane integrity via lipid peroxidation, depletion of sulfhydryls, conformational changes in proteins and nucleic acids, many more (Borkow and Gabbay, 2005; Tamas et al. 2014; Shanker, 2008). Redox cycling between Cu (I) and Cu (II) states leads to the production of free hydroxyl radicals that causes oxidative

damage to DNA, plasma membrane and proteins. Cu seems to show a specific affinity for DNA double strands as it binds and crosslinks between the strands thereby destroying the helical structure (Borkow and Gabbay, 2005). Cu has two binding sites to attach to DNA; one is present at every four nucleotides while the second is present at every basepair. Most of the modifications caused in the double helix is the formation of 8- hydroxy-2'-deoxyguanosin that occurs at Guanine and Thymine residues (Borkow and Gabbay, 2005). In case of Chromium, water-soluble Cr (VI) can easily enter the cell through non-specific anion transport being in tetrahedral symmetry which resembles that of sulphate, as compared to the octahedral symmetry of Cr (III) which can enter only via diffusion (Neis, 1999; Borut et al. 2010). Cr (VI) has a redox potential of 1.33-1.38 eV, making it a strong oxidant that rapidly generates high amounts of ROS (Oyetibo et al. 2013). On entering the cell, this Cr (VI) undergoes series of reduction (one electron or two electron reduction) to finally give Cr (III), in the process releasing ROS. Slower the reduction, more is ROS production, more is the DNA damage. Under one electron reduction, Cr (VI) on entering the cell, gets rapidly reduced by intracellular compounds present in cell such as glutathione, ascorbic acid, cysteine, NADH, NADPH, etc. to produce highly reactive, cytotoxic and carcinogenic intermediate Cr (V) complexes (Chourey et al. 2006; Shanker, 2008, Kanmani et al. 2011). Flavin-dependent enzymes, lipoyl dehydrogenase, cytochrome c and glutathione reductase have also been reported to reduce Cr (VI) using one electron reduction mechanism (Thatoi et al. 2014). The redox active and highly unstable Cr (V) intermediates then react with hydrogen peroxide to form free radicals and hydroxyl ions via Fenton like reactions that cause damage (Cervantes et al. 2001; Borut et al. 2010). In the two-electron reduction mechanism, Cr (VI) is directly converted to Cr (IV) without the

unstable Cr (V) intermediate which leads to lesser amounts of ROS generated (Thatoi et al. 2014). As a matter of fact, due to the rapid reduction of Cr (VI) inside the cell, the concentration of Cr in its hexavalent state never reaches equilibrium on either side of the plasma membrane. This is the primary reason why hexavalent chromium is constantly taken up by the cell from its surroundings, and even a small concentration of this anion can kill cell populations (Borut et al. 2010). Not just chromium and copper, both, redox active metals (Fe, V) and redox in-active metals (Cd, Ni, Hg, Zn) catalyze the Fenton or Haber–Weiss reactions releasing free hydroxyl radicals from hydrogen peroxide and superoxide substrates or produce ROS by depleting glutathione and protein-bound sulphydryl groups (Hall, 2002; Bellion et al. 2006). The end products and intermediate products of Cr (VI) reduction display strong affinity towards DNA forming Cr-DNA monoadducts via causing oxidation of DNA bases and sugar thus leading to DNA strand breaks (Shanker, 2008). Metal ions such as Cd (II) bind to the sulphydryl groups of proteins and also break DNA strands thereby exhibiting its toxicity (Beveridge et al. 1997) and may also hamper cellular thiol redox balance in cell causing oxidative stress.

The free radicals released during redox cycling of Cu (I) and (II) causes damage to the plasma membrane as well. Lipid peroxidation occurs due to which the membrane loses its integrity, pores are formed leading to either leakage of intracellular ions along with other components or enhanced accumulation of extracellular ions from the surrounding (Borut et al. 2010). The Cu (II) ions attack the SH- moieties in the membrane giving rise to thiol compounds and Cu (I) ions. Therefore on an average three SH groups get inactivated per Cu ion interaction (Borkow and Gabbay, 2005). Peroxidation of polyunsaturated lipids and oxidation of membrane components are other side effects of heavy metals. Similar toxicity

was reported by Madrid and his colleagues (2011), with regards to nickel that caused lipid peroxidation, DNA strand breaks and DNA-protein crosslinks. The ROS (single oxygen, superoxide, hydroxyl and hydrogen peroxide radicals) generated during Cr (VI) reduction, causes irreversible plasma membrane enzyme inhibition, lipid peroxidation and combine with DNA-protein complexes (Cervantes and Gutierrez-corona, 1994; Borut et al. 2010; Joutlet et al. 2015). Heavy metals not only disrupt the fluidity of plasma membrane, but also cause crosslinking between protein thiol groups and inhibition of proteins such as ATPases (Hall, 2002). Metals can have diverse modes of interaction with proteins and can affect its biological activity. They can bind to free thiols and available functional groups, displace essential ions from metalloproteins, aid in the oxidation of sidechains of amino acids and target non-folded proteins (Tamas et al. 2014). Interfering with protein folding or inhibiting the refolding of damaged proteins leads to accumulation of cytotoxic misfolded proteins that may cause more harm on interaction with other cellular components (Tamas et al. 2014). Despite all these toxic effects and harm caused by heavy metals to organisms, we still have organisms living in heavy metal contaminated sites. Such robust organisms belong to both prokaryotic as well as eukaryotic group and can serve as models to study metal tolerance.

### 1.1.4 Models to study toxicity and tolerance of metals

Tolerance may be defined as "the relative ability of an organism to grow or thrive when subjected to an unfavorable environmental factor or toxin" (Green and Clausen, 2003). The damage caused as a result of metal exposure to cellular components is similar in nearly all types of organisms which makes microbes good models to study its relative effects at

molecular as well as cellular levels (Borut et al. 2010). Microbes such as bacteria, yeast, algae and fungi are known to tolerant heavy metals and have been attempted to study in detail. Compared to bacteria, yeast or fungi prove to be better models to study heavy metal tolerance as its eukaryotic make up show more similarities at mammalian cell and organelle levels (Borut et al. 2010) besides being tolerant to high concentrations of pollutants (Gadd, 2001). Fungi have a selective advantage of being favored over the rest as models due to their ability to survive under extreme conditions of temperatures, nutrient availability, pH and high metal concentration (Iskander et al. 2001; Ezzhouri et al. 2009) besides having a potential for its use in a non-sterile environment (Malik, 2014). Fungi have dominated these studies as they have been found to efficiently solubilize metals better than bacteria (Thangavelu et al. 2006), probably because of the high surface-to-cell ratio of its mycelial network that maximizes enzymatic contact with the substrate (Malik, 2014). Different strategies are used by microbes to combat such stressed conditions. They may use one or more mechanisms to tolerate or detoxify the metal depending on metal concerned, cell age and other environmental parameters. Apart from metal tolerance, microbes may also develop properties such as antibiotic resistance and in turn, get benefitted (Chaterjee et al. 2012).

## 1.2 Literature Review

### 1.2.1 Survival strategies in metal tolerant microbes

Broadly metal tolerance mechanisms are of two types: Extracellular and Intracellular sequestering (Lopez and Vazquez, 2003; Bishnoi and Garima, 2005; Bellion et al. 2006; Hasan 2007; Borut et al. 2010; Anahid et al. 2011). Extracellular mechanism is widely

studied as biosorption and extracellular chelation, while intracellular mechanisms can be further classified as efflux pumps, enzymatic transformation, production of metallothioneins and phytochelatins, complexation with intracellular glutathione and vacuole compartmentalization (White et al. 1995; Beveridge et al. 1997; Spain, 2003; Borkow and Gabbay, 2005; Bellion et al. 2006; Appenroth, 2010)

"Biosorption, is a metabolism and metabolic energy-independent process that requires only a few minutes, but depends considerably on parameters such as pH, temperature, the ratio of the initial metal ion and the initial biomass concentrations, the culturing conditions, and the presence of various ligands and competitive metal ions in solution" (Borut et al. 2010). The process of biosorption can occur in three ways: (1) surface adsorption mechanism where metal ions are rapidly bound to the cell surface, (2) Intracellular accumulation where metal ions cross the cell wall and, (3) extracellular complexation where metal ions bind to excreted metabolic products (Choi et al. 2009; Ahmad et al. 2011).

Cell walls of microbes which are the first sites to be exposure to heavy metals are involved in biosorption process. In bacteria, the Gram-positive bacterial cell walls whose carboxyl groups form peptidoglycan while in Gram-negative bacteria the phosphate groups serve as metal binding sites (White et al. 1995). The survivability of *Klebsiella aerogenes* had increased in Cd (II) solution due to the presence of exopolysaccharides (Borkow and Gabbay, 2005). Fungal cell walls house huge amounts of exceedingly cross-linked polysaccharides (chitin, chitosan, glucan), glucuronic acids, galactosamine, small amounts of glycoproteins, along with melanins and phenolic polymers that contain phenolic units, peptides, fatty acids, which provide ample amounts of oxygen-containing groups such as carboxyl, carbonyl, amino, hydroxyl, phosphate, methoxy and mercapto that are potential

metal binding sites (White et al. 1995; Sag and Kutsal, 2001; Bellion et al. 2006; Yadav et al. 2015). The negative charge on fungal cell surface is due to the presence of phosphate and carboxyl groups (Say et al. 2004), while the amine groups present in chitosan provide a positive charge, both of which may be involved in electrostatic attraction of opposite charged ions or complexation with N or O donors (Naja and Volesky, 2011). In addition to this, biopolymers such as chitin and glucan are predicted to cause metal entrapment in their inter-and intrafibrillar capillarities (Volesky and Holan, 1995). An interesting report suggested the composition of cultivation media plays a crucial role in the metal binding capacity of organisms simply by affecting its cell composition (Baldrian and Gabriel, 2003). Surface adsorption is found in both live and dead cells, but intracellular accumulation and extracellular complexation are found in metabolically active cells. Metabolically mediated transport occurring in the latter processes is difficult to assess and study quantitatively as chemical transport works both ways, in/out of the cell, which includes sequestering metal ions into storage sites as well as the production of extracellular material (Naja and Volesky, 2011).

Extracellular complexation was seen in *S. cerevisiae* that released huge quantities of glutathione in the presence of Ni (II) stress which led to the formation of Ni-glutathione complex that could not penetrate the cell and harm it (Borkow and Gabbay, 2005). The tolerance of brown rot fungus to copper was hypothesized by Tabuko in 1952 due to the production of extracellular oxalic acid (Green and Clausen, 2003). He had also concluded that apart from the formation of insoluble Cu- oxalate complex, the lowering of pH by oxalic acid produced more tolerance. When organic acids are released by organisms, they often provide an abundant source of protons that solubilizes metals from minerals thereby

lowering pH and causing soil acidification (Bellion et al. 2006). A protein named glomalin when released by arbuscular mycorrhiza also sequestered metals ions like Cu, Pb, and Cd (Bellion et al. 2006). Plants also have similar strategies to reduce stress. They maybe single metal or multi-metal resistant and release exudates such as Ni chelators that contain histidine and citrate residues which bind to Ni and reduce uptake (Hall, 2002).

Intracellular mechanisms are far more complicated as compared to the simpler extracellular binding. Most of the heavy metals are taken inside the cell by the nutrient uptake pathways since divalent cations are very similar in size and resemble each other and have the tendency to replace essential cations (Ramasamy et al. 2007). Uptake systems are of two types: one which is unspecific and quick with no utilization of ATP and is more responsible for enhanced uptake and accumulation of heavy metals in cell and two which is substrate specific, slow and requires ATP hydrolysis (Spain, 2003). Different efflux pumps have been studied in various microbes. ATP- driven efflux pumps containing the ATP-binding cassette (ABC) super-families and P-type ATPases, and metal ion/proton antiporters (RND-driven transporter system where proton gradients provide energy for metal transport, have been reported in bacteria (Agranoff and Krishna, 1998; Spain, 2003). In bacteria, one of the most commonly found active transport system is metal exclusion via *czc*CBA system where this structural gene encodes for the outer membrane factors CzcC, a membrane fusion protein CzcB and resistance-nodulation-cell division protein CzcA (Liu et al. 2015). This efflux pump is specific to zinc, cadmium and cobalt export. In Gram-positive bacteria, *Enterococcus hira,* has *cop* operon that codes for four genes. *copA* encodes for Cu(II) uptake ATPase while *copB* encodes for P-type efflux ATPase. *copY* is a repressor protein that binds to the operon in the absence of Cu. When Cu is present, repressor gets detached

from the operon, and the genes *copA* and *copB* can be transcribed (Borkow and Gabbay, 2005). *ChrA* protein belonging to chromate ion transporter CHR superfamily in *P. aeruginosa* is known to export chromate from cytoplasm/periplasm to out of the cell via proton motive force (Thatoi et al. 2014).

Enzymatic transformation is a mechanism by which the toxic metal gets detoxified to a nontoxic form by a series of reactions using different enzymes. Some of the non-enzymatic reductants have already been discussed before. The different enzymatic reductants that are especially known to reduce chromium (in bacteria), are nitroreductases, hydrogenases, iron reductases, flavin reductases, quinone reductases (Thatoi et al. 2014); mitogen-activated protein kinases in yeasts (Pocsi, 2011); and aldehyde oxidases, cytochrome p450, Dt-diaphorases in mammalian cells (Thatoi et al. 2014, Joutlet et al. 2015). Apart from direct reduction of toxicants, bacteria are also involved with indirect reduction of toxicants. A coupling mechanism seems to occur between oxidation of organics and reduction of ferric iron. These reduced ferrous ions then bind to toxic contaminants like U (VI) and Cr (VI) to form insoluble species (Ramasamy et al. 2007).

When a toxic metal ion enters the cell, it is necessary for the organism to come up with a mechanism that will either detoxify it or complex it so that it remains non available to hamper other cellular components. **"**Metallothioneins are a class of ubiquitously occurring low-molecular-weight cysteine- and metal-rich proteins containing sulphur-based metal clusters" (Bellion et al. 2006). Production of these metal-binding proteins has been reported to confer metal resistance to both prokaryotic and eukaryotic life forms (Cervantes and Guitierrez-corona, 1994; Beveridge et al. 1997). Phytochelatins are glutathione derived peptides which also play a role in metal binding. Metal y-glutamy1 peptides

(Phytochelatins) have been reported to be involved in heavy metal detoxification in fungi algae and even plants (White et al. 1995). Production of Intracellular glutathione also plays a very important role as it chelates and sequesters metal ions which would otherwise cause metal initiated cell injuries (Bellion et al. 2006). It also prevents DNA strand breakage by impeding free radical formation by stabilizing Cu (I) state in the presence of hydrogen peroxide and ascorbate (Borkow and Gabbay, 2005). It is also known to act as an intramolecular stabilizer of Cr (VI) as it forms thiolate esters (Borut et al. 2010). These glutathione-metal complexes are either exported out of the cell or sequestered into intracellular compartments such as vacuoles, by membrane-bound transporters to reduce metal concentrations in the cytosol (Rehman and Anjum, 2011; Tamas et al. 2014). It was suggested by Davies (2001), that chromium which alters membrane systems in hyper-accumulating plants, gets eventually retained in cell walls and vacuoles of root cells. Similarly, a Nickel resistant strain of *S. cerevisiae*, showed increased sequestration of $Ni^{+2}$ into the vacuoles accompanied by enhanced production of histidine which has an affinity for $Ni^{+2}$ (Joho, 1995).

Organisms may also produce small heat-stable oxidoreductases called thioredoxins in metal stress. These proteins have various functions such as protein folding and regulation, repair of oxidatively damaged proteins and are also known to maintain redox homeostasis in stressed conditions. A gene *PiTrx1* coding for thioredoxin was produced under Cu and Cd stress by *Paxillus involutus* (Bellion et al. 2006). Other proteins produced to combat oxidative stresses are superoxide dismutase, catalases and peroxidases all of which are active ROS scavengers. Cells are also known to adjust their gene expression to produce more of heat shock proteins/ chaperones and proteasome-encoding genes (Tamas et al.

2014). Damages caused to DNA are promptly dealt with using the DNA repair mechanism comprising of the SOS response enzymes such as RecA, RecG, RuvB (Thatoi et al. 2014). All the above mechanisms whether extracellular or intracellular help the organism to survive such stressed conditions and also reduce the quantities of heavy metals in the surroundings.

### 1.2.2 Physical and chemical treatment methods, no more a boon to industries

Industries releasing effluents that contain oxidized forms of metals ions which are highly soluble, cause more harm to the environment as compared to reduced forms which precipitate out. This affects not only the marine biota where most effluents are released but also affect human lives as these effluents seep into groundwater bodies causing contamination (Ramasamy et al. 2007). Different conventional methods are used by the industries to reduce the amounts of toxic heavy metals in their effluent discharge. Some of these methods include chemical precipitation and sludge separation to remove dissolved heavy metals, chemical oxidation or reduction or other chemical processes like ion exchange, reverse osmosis, electrochemical treatment and evaporative recovery, filtration and adsorption using activated charcoal (Lopez and Vazquez, 2003; Malik, 2004; Sun et al. 2012). All these methods are very expensive, have high energy requirements, results in incomplete metal removal, or generate toxic byproduct containing complexing organic matter that requires further treatment (Malik, 2004; Hasan, 2007; Abbas et al. 2008; Joshi et al. 2011). E.g., methods like precipitation and coagulation are not effective when the metal concentrations lie in between 1-100 mg/L (Bishnoi and Garima, 2005). Under such

conditions, it is mandatory to find techniques that will prove effective, efficient and cheap to remove heavy metal waste from effluents.

Microbial Fuel Cells (MFCs) has been one such promising methods for treatment for wastewaters using proteobacteria (Wang et al. 2008). The proteobacteria act as biocatalysts that utilize the substrates (waste) and generate electrons which move towards the cathode under electric current and reduce the acceptor (hexavalent chromium). Thus the use of biological agents to reduce toxic waste from the environment can solve the current issue of metal contamination. Bioremediation is defined as "the utilization of microorganisms to reduce or eliminate environmental hazards by mediating desired chemical reactions or physical processes" (Skladany and Metting, 1993). Fungi as a potential biological agent in bioremediation of heavy metal waste are rapidly getting recognized (Bishnoi and Garmi, 2005) a part from others such as molds, bacteria, seaweeds which also can be used (Chaterjee et al. 2012). The strategy used by bioremediation is to locate and identify a strain (bacterial/fungal) that can modify the contaminant into a product which is no longer harmful (Chaterjee et al. 2012). Microbes are now being genetically engineered for remediation purposes. *NixA* gene from *Helicobacter pylori* that coded for Ni transport was inserted in *E.coli* by Krishnasamy and Wilson. On inserting the gene, it expressed glutathione S-transferase metallothionein protein which helped the strain to accumulate four times more Ni as compared to the wild-type (Rajandran et al. 2003). Processes such as bioleaching that utilizes heterotrophic fungi and the metabolic products released by it to extract metals from solid waste and reports have been made where fungal species such as *Penicillium* and *Aspergillus* have a potential for metal bioleaching (Anahid et al. 2011). Due to mycelial nature of fungal biomass and its high biosorptive properties, these can be

readily used to treat metal wastes. Dead biomass is always more preferred as compared to live fungus because it does not have any limitations such as the toxic effect on growth, need of feed or nutrients, maintenance of physiological parameters like pH or temperature, etc. Besides metals sorbed on dead biomass can be desorbed by chemical treatment and biomass can be reused.

### 1.3 Why this Research?

Microbes isolated from marine habitats such as mangroves have demonstrated the immense potential for the production of secondary metabolites which can have many scientific applications. Fungi found in such regions have adapted to the osmotic pressures caused due to high salts levels by having excellent efflux systems to exclude the cations (Gunde-Cimerman et al. 2009). These eukaryotes can, therefore, be expected to have similar mechanisms to exclude heavy metals if subjected to the stress and needs to be explored. Though terrestrial fungi have been extensively studied for their metal sorption properties (Rulcker and Schnurer, 1993; Subbaiah and Yun, 2013), studies pertaining to marine fungi still remain sparse. Further, more than a fair share of the existing literature is based on biosorption and extracellular accumulation which extensively describes the functional groups available on cell walls to scavenge heavy metals. This further limits the current methods of bioremediation to permanently solve the issue of metal contamination. E.g. hexavalent chromium after its reduction gives the trivalent form which in the presence of soluble organic matter forms chromium trivalent complex. Unfortunately when this complex comes in contact with manganese oxides in soil or water, reverts back to hexavalent form (Fendorf, 1995). This makes it mandatory to understand the mechanism of

detoxification and which is not restricted to extracellular chelation. Lastly, the current literature still lacks the study on protein profiling of marine-derived fungi showing tolerance to metals. Understanding the mechanisms involved in metal tolerance in marine-derived organisms will help in devising methods of practical applications of such organisms in salt laded effluents containing metals, which even today face a challenge to detoxify.

### 1.4 Research Aims and Objectives

Based on the few of the many lacunae found in this field of metal tolerance research, an attempt was made to add a fraction of knowledge to the existing literature, and the following objectives were framed for this work.

Objective ONE: To isolate and identify fungi from marine habitats.

Objective TWO: To check selected isolates for tolerance to metals such as Cr, Pb, Cd, Ni and Cu (one or two metals to be studied in detail).

Objective THREE: To study protein profiles of selected fungal isolates with respect to salinity, temperature and heavy metal stresses.

# Chapter TWO

# Fungal Diversity and its Importance

## 2.1 Marine Fungi and their Significance

Fungi are one of the most important decomposers involved in recycling of the minerals. They function in almost all the habitats with sediments being their main domain of activity which provides them with a substrate to grow upon. Mangroves are one such habitat with interesting adaptations such as prop roots, pneumatophores, viviparous seedlings (Sarma and Hyde, 2001) with rich biodiversity and biologically active secondary metabolites (Devi et al. 2012) owning to the second most important marine ecosystem in terms of productivity (Manoharachary et al. 2005; Alsheikh-Hussain et al. 2014). Energy transfer to higher trophic levels in this bio system begins from the detritus-based food chains where the fungi along with other microbes play a crucial part by decomposing an enormous load of raw organic carbon and releasing surplus amounts of nutrients in the mangrove sediments. Woody substrata prove as an eminent source for carbon and energy (Tsui and Hyde, 2004) on which fungi rather than bacteria, thrive on and act as principal sources of increasing nitrogen and protein enriched fragments in the decomposed matter (Sarma and Vittal, 2000). Fungi have also been found in deep-sea regions where unfavorable conditions occur such as high pressure, low temperatures and nutrient deficiencies (Raghukumar et al. 2010). Direct detection techniques have revealed fungal hyphae existing in anoxic sediments of Arabian Sea questioning roles of aerobic organisms in such anoxic environments (Raghukumar et al. 2014). Damare and Raghukumar (2008), hypothesized that marine fungi could be responsible for macro-aggregation of sediments and carbon sequestration. Along with being the pioneers for any decomposition process, fungi stand out as efficient biomass degraders (Alsheikh-Hussain et al. 2014) and rich sources of bioactive compounds and secondary metabolites (Devi et al. 2012) which have wide application in medicine, food and other industries. The overall fungal diversity in the world is estimated to be more than 1.5

million species (Hawksworth, 2001) of which just 7% have been described (Branco, 2011). About 1500 species is the latest estimate of marine fungi (Alsheikh-Hussain et al. 2014) of which 150 species of true mangrove fungi have been reported from Indian mangroves despite of one third of global fungal diversity existing in India (Manoharachary et al. 2005).

**2.2 Materials & Methods (Objective ONE: To isolate and identify fungi from marine habitats)**

**2.2.1 Isolation of marine-derived fungi**

Sediment samplings were carried out in mangrove region of Divar (15°29'N, 74°12' E) during the year 2012- 2014 at low tides, Figure 1a, and Arabian Sea sediments (sediment sampling was carried across the cruise track of different sampling stations using box grab at 15°30' N, 73°40' E to 12°47' N, 74°01'E, Figure 1b) in the year 2013. The salinity of the sediment samples varied from 32 to 35 PSU while temperature varied between 29°C to 40°C (mangrove sediment) depending on the season, with pH close to 6-6.5.

Sediment sample (0.5 g) from mangroves was suspended in 10 ml of sterile seawater and vortexed briefly for direct spread-plate technique (Chesters, 1940). Aliquots (100 µL) of the suspension were spread-plated on various sea salt amended (10%, 20% and 30%) growth media (Table 1) such as potato dextrose agar (PDA), malt extract agar (MEA), Czapek's Dox agar (CDA), Zobell marine agar (ZMA), corn meal agar (CMA), Sabouraud's dextrose agar (SDA) and 50% sediment extract agar (50 g sediment suspended in 100 mL of distilled water amended with 0.5M EDTA and antibiotic and subjected to shaker conditions overnight. The suspension was then filtered through 0.22 µm filter paper to give sediment extract).

**Figure 1a. The sampling site for mangroves sediment collection.**

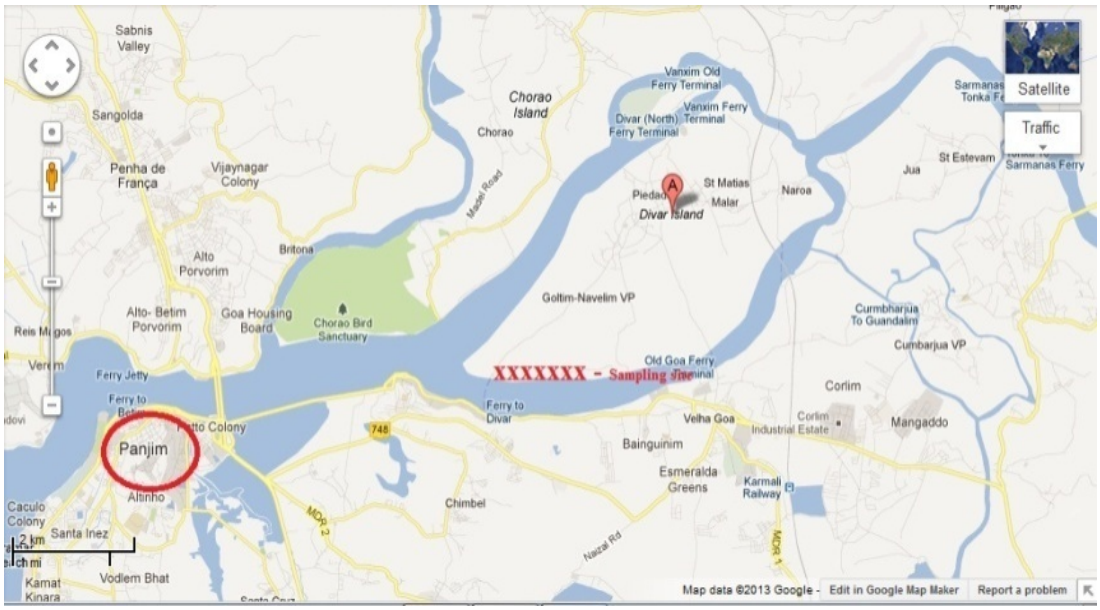



**Figure 1b. Cruise track for the Arabian Sea sediment collection.**

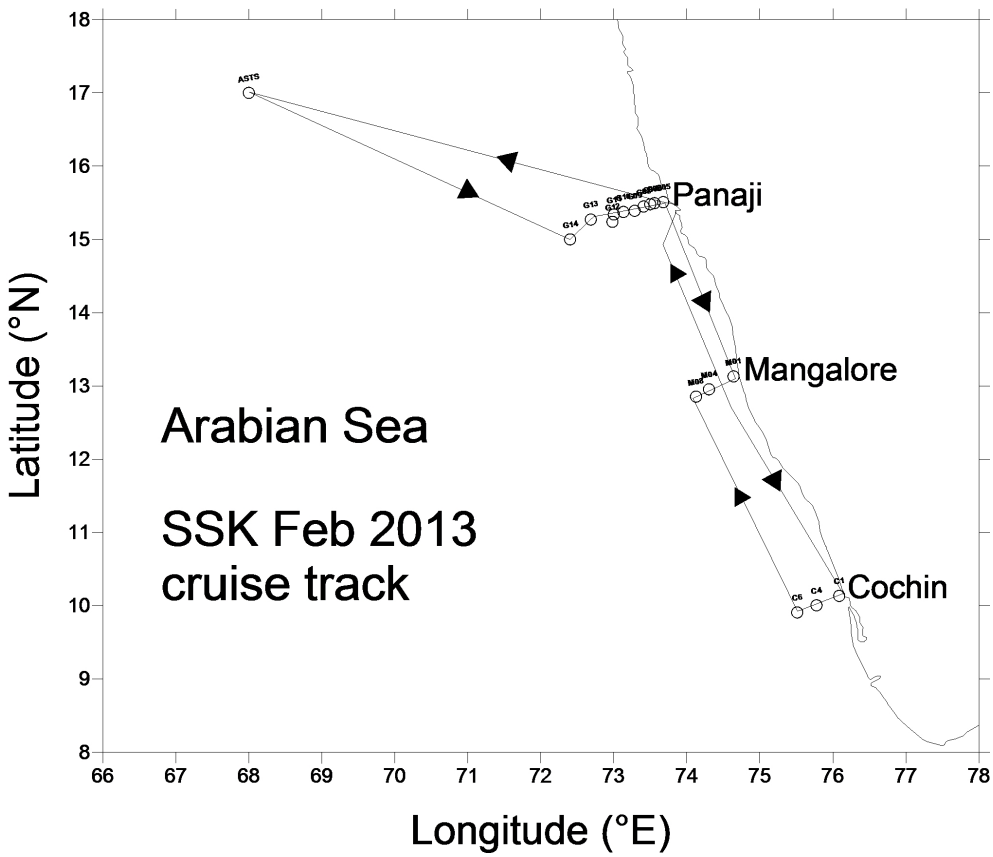


**Table 1. Samplings carried out in Mangroves and salt pan regions**

| Date | Place | Time | Height (m) | No of Samples | No of isolates | Media used |
|---|---|---|---|---|---|---|
| 26112012 | Divar | 15:27 | 0.38 | 1 | 25 | PDA, MEA, CDA, ZMA, CMA, SDA, sediment agar in 10%, 20% and 30% sea-salt water |
| 12122012 | Divar | 15:36 | -0.02 | 5 | 96 | PDA, MEA , CDA, ZMA, CMA, SDA, sediment agar in 10%, 20% and 30% sea-salt water + cycloheximide amended CDA, MEA in 10% sea-salt water |
| 19092013 | Divar | 16:42 | 0.27 | 4 | 45 | Mangrove twigs and leaves placed on PDA, MEA , CDA, ZMA, CMA, SDA in 10%, 20% and 30% sea-salt water |
| 21032014 | Saltpan (Ribandar) | 12:00 | 0.33 | 5 | 4 | PDA, MEA, CDA, SDA in 10%, 20% and 30% sea-salt water + cycloheximide , Basal Mineral Agar, seawater agar |
| 26032014 | Divar | 12:39 | 0.59 | 5 | 3 | PDA, MEA, CDA, SDA in 10% and 20% sea-salt water + cycloheximide, Seawater agar |
| 3052014 | Divar | 07:02 | 0.3 | 5 | 17 | PDA, MEA, CDA, SDA in seawater and in 10% and 20% sea-salt water + cycloheximide, seawater agar |

**Table 2. Samplings carried out in Arabian Sea**

| Cruise | Place | No of samples | No of isolates | Media used |
|---|---|---|---|---|
| SSK046 | Arabian Sea | 10 | 21 | PDA,MEA,CDA,ZMA,CMA,SDA |
| SSK056 | Arabian Sea | 44 | 172 | full strength and 1:5 strength PDA,MEA,CDA,ZMA,CMA,SDA |

All the above media were amended with antibiotic (750 µg/mL).Further for subsequent samplings, all the above media were also amended with cycloheximide (100 µg/mL). Cycloheximide which is a protein synthesis inhibitor in eukaryotic cells and is used as a fungicide to prevent the growth of rapidly growing molds (Pedersen, 1992). Another isolation technique used, where 50 % sediment extract agar (100 µL) was poured in sterile 96 well plates and left to solidify. Suspensions of sediments samples made in sterile seawater were serially diluted,and10 µL aliquots of each dilution were pipetted in one row each of the 96 well plate. In order to isolate fungi associated with mangroves, dead decaying leaves and twigs of mangroves were collected in sterile polythene bags, and rinsed with sterile seawater and placed on above growth media. Apart from this, accumulated froth due to turbulence caused by tides were also collected in sterile 50mL tubes and spread-plated on growth media. Froth acts as a spore trapper and can be used to isolate fungi. The plates were incubated at room temperature for 3 days to one month, and the fungal isolates were sub-cultured to get pure colonies. The cruise sediments were spread-plated in a similar manner on full strength and 1:5 strength of MEA, CDA, SDA, CMA, PDA, and ZMA prepared in seawater (Table 2) and amended with antibiotic as above.

### 2.2.2 Molecular identification of marine-derived fungi

DNA extraction of all the procured isolates was carried out using ZR Fungal/Bacterial DNA MicroPrep Kit (Zymo Research Corp., USA). Further using ITS primers (ITS1 - TCCGTAGGTGAACCTGCGG and ITS4 - TCCTCCGCTTATTGATATGC)the entire ITS region was amplified by PCR (White et al. 1990) using the following protocol.Initial denaturation step at 95ºC for 5 minutes; denaturation step at 95ºC for 1 minute; annealing

temperature of 52ºC for 45 seconds; elongation step at 72ºC for 1 minute and a final elongation step at 72ºC for 10 minutes and steps 2, 3 and 4were repeated for 35 cycles. Some isolates were also sequenced using 18S rDNA gene whose ITS sequencing did not give GA results. The primers used were NS1- GTAGTCATATGCTTGTCTC and NS8- TCCGCAGGTTCACCTACG where the 18S region was amplified by PCR using the following protocol.Initial denaturation step at 95ºC for 5 minutes; denaturation step at 95ºC for 1 minute; annealing temperature of 54ºC for 45 seconds; elongation step at 72ºC for 45 seconds and a final elongation step at 72ºC for 10 minutes and steps 2, 3 and 4were repeated for 35 cycles. The amplicons were purified using AxyPrep PCR Cleanup Kit (Axygen Biosciences, USA) and were then uni-directionally sequenced using Applied Biosystems (ABI) 3730 DNA stretch sequencer, with the XL upgrade and the ABI prism big dye terminator version 3.1 Cycle sequencing ready reaction kit. The raw sequences were analysed using the Blast tool (Altschul et al. 1990) to get the closest reference sequences. The partial ITS and 18S sequences were then submitted to GenBank database with accession numbers KX685942; KT956257; KX685943; KX845384- KX845387; KT956258; KT956259; KY788339; MG584873- MG584983; MG589523- MG589634; MG580928- MG580938. The phylogenetic trees for all submitted sequences were constructed using Mega software ver 7 using Neighbour Joining method (Figure 2a, 2b, 3 and 4). On identifying all the isolates, diversity indices (Species richness, Species evenness and Species diversity) were calculated using Primer 6 software ver 6.1.10.

**Figure 2a. Phylogenetic analysis of fungal isolates (ITS sequences) isolated from mangrove sediments, inferred from NJ analysis using MEGA software. (part one)**

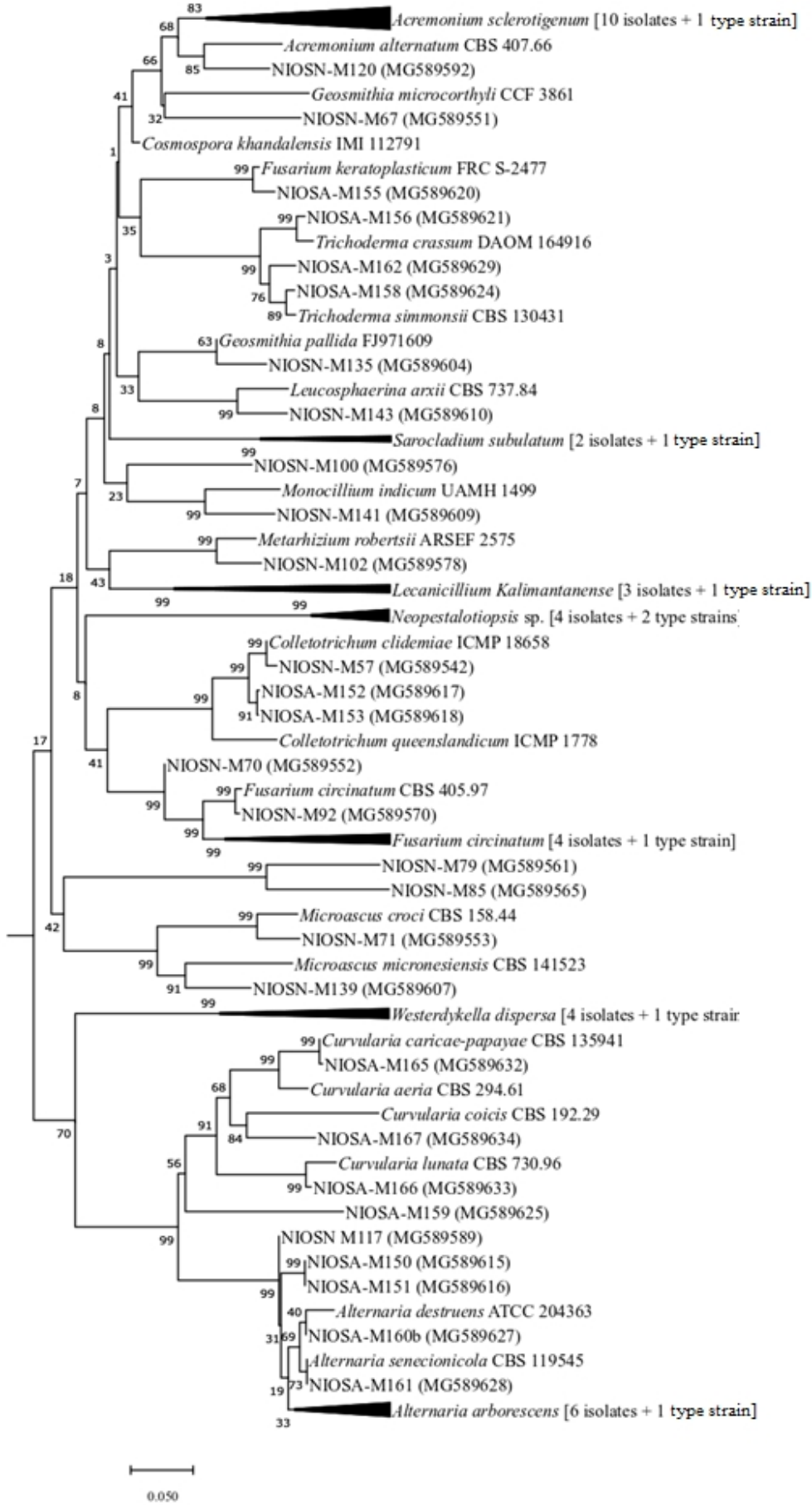
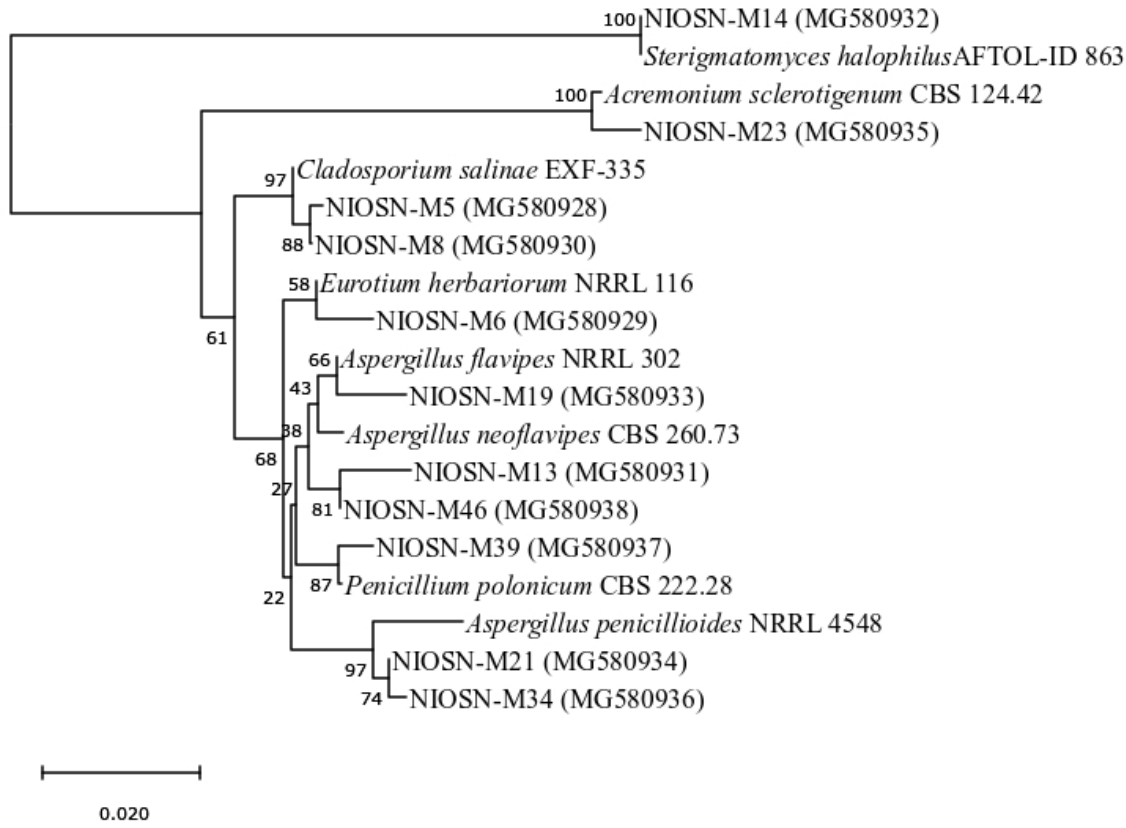


**Figure 2b. (part two)**

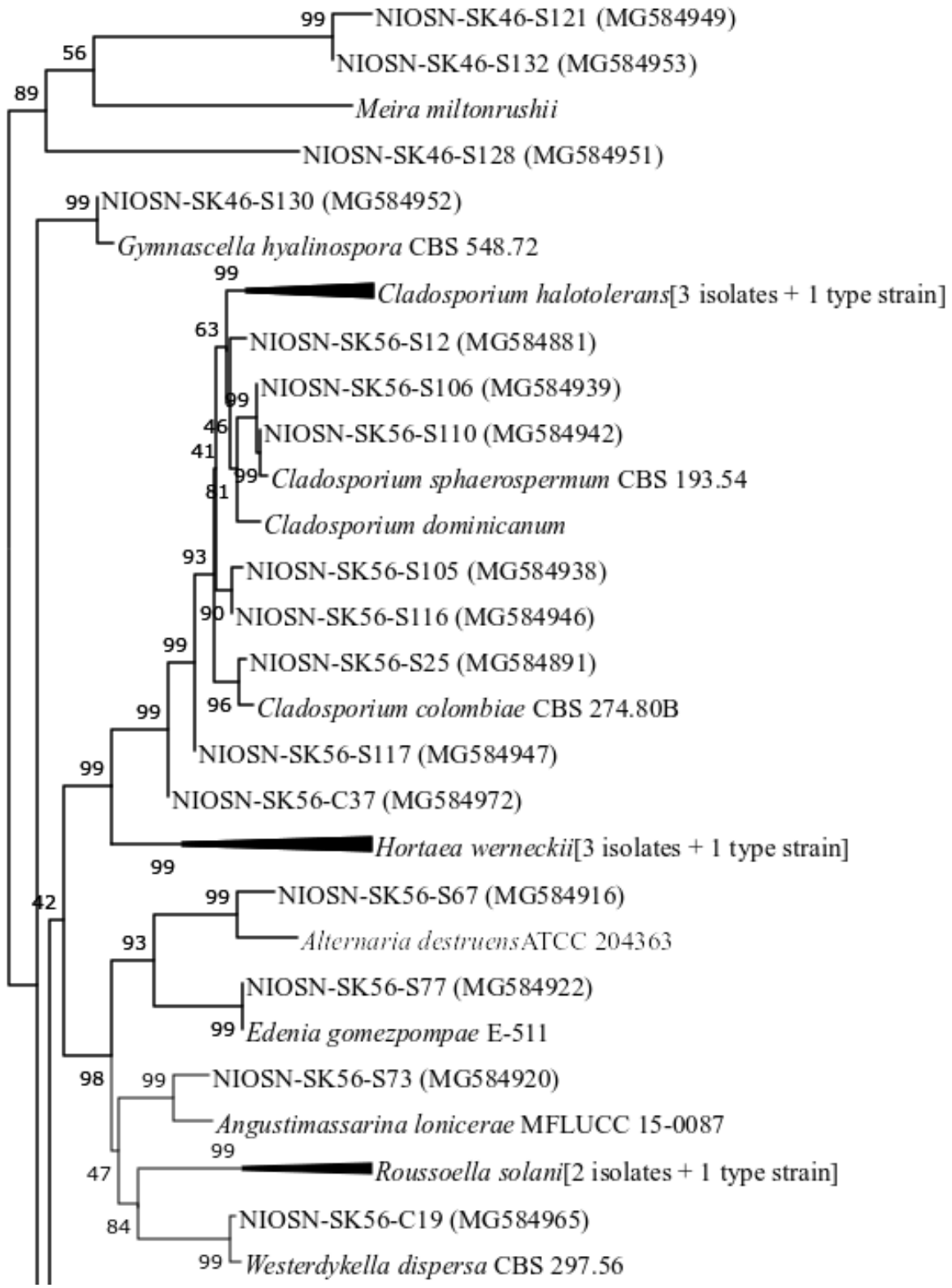
The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 6.79747622 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is 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 (Tamura et al. 2004) and are in the units of the number of base substitutions per site. This analysis involved 179 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 926 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al. 2018).

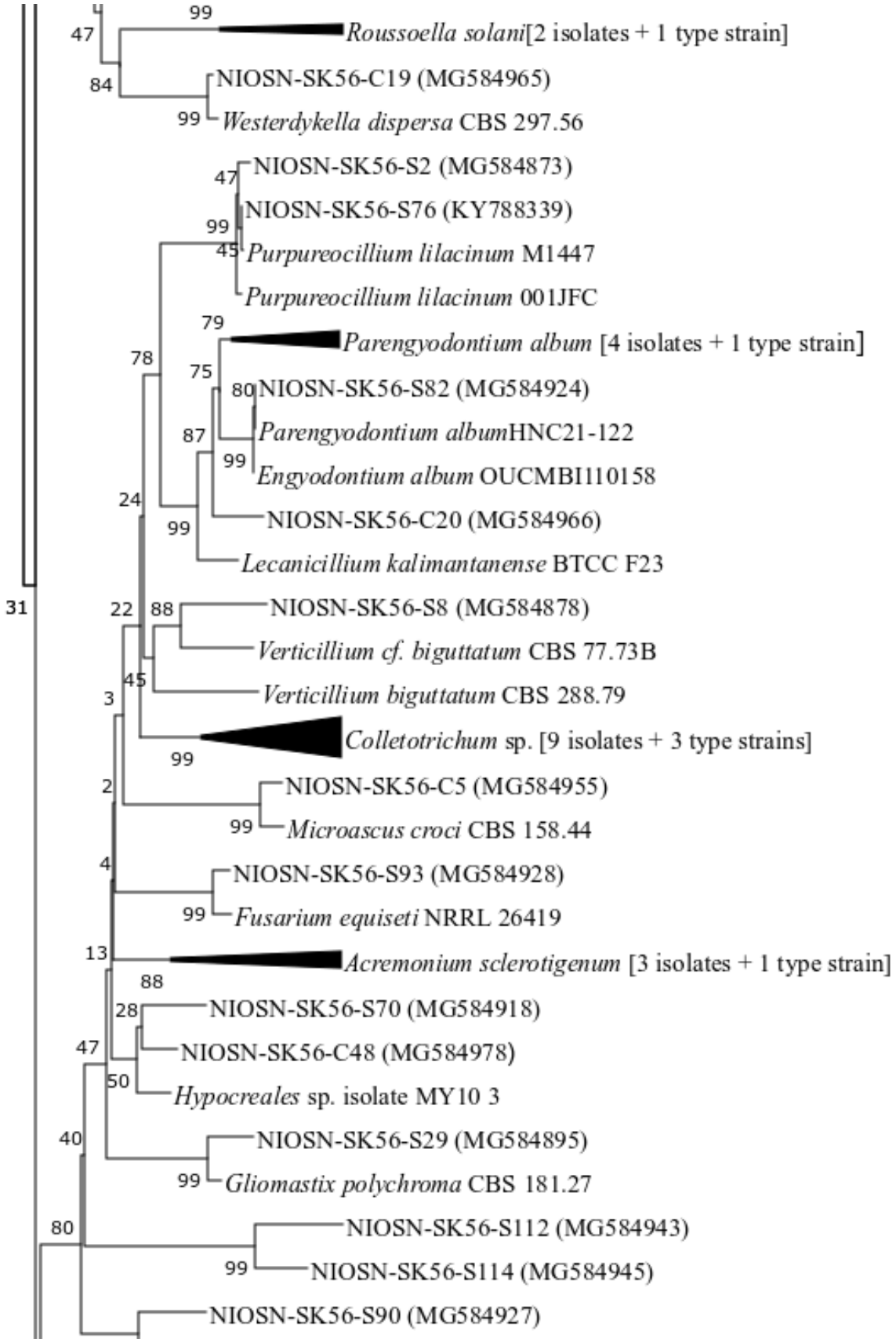
**Figure 3. Phylogenetic analysis of fungal isolates (18S sequences) isolated from mangrove sediments, inferred from NJ analysis using MEGA software.**

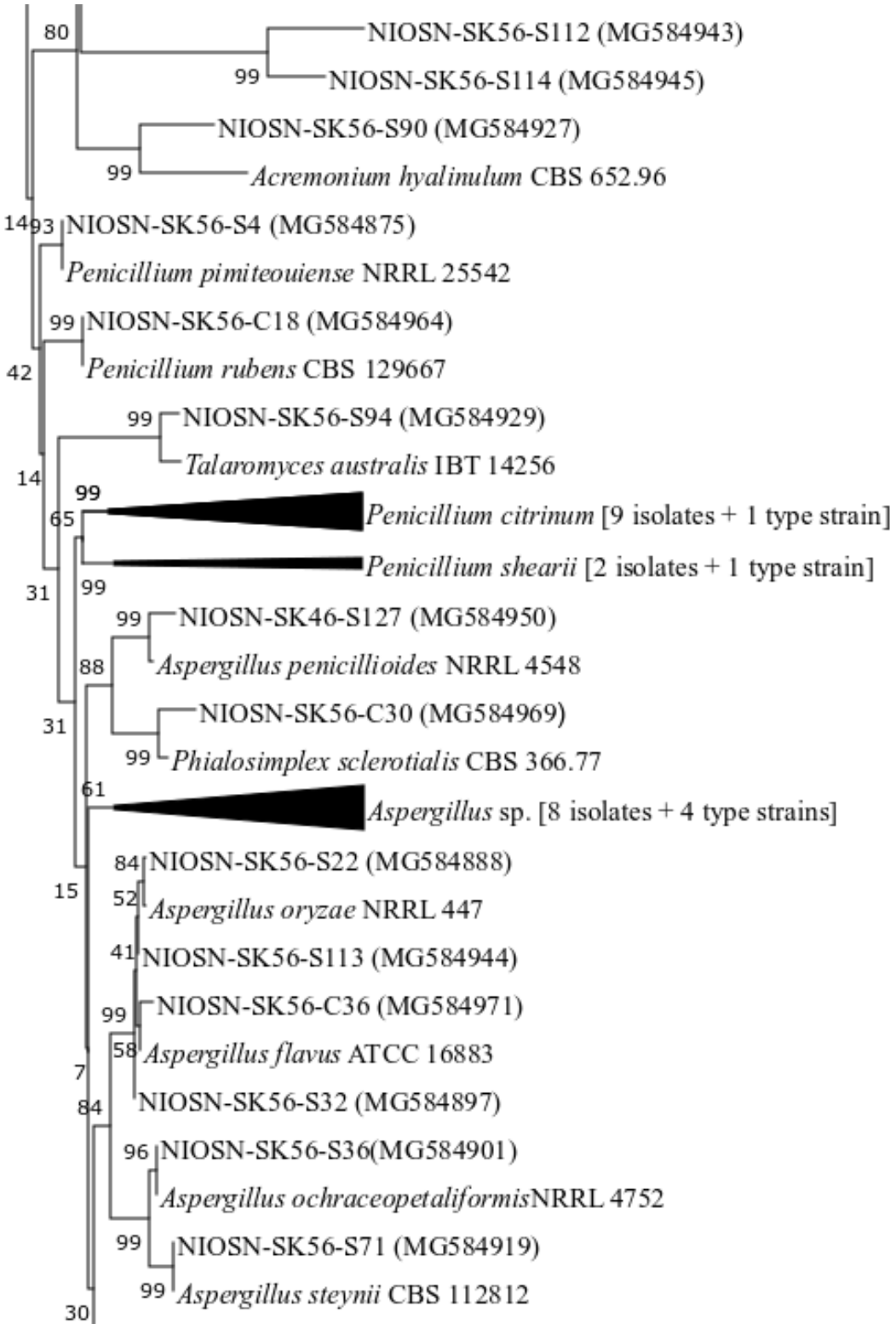


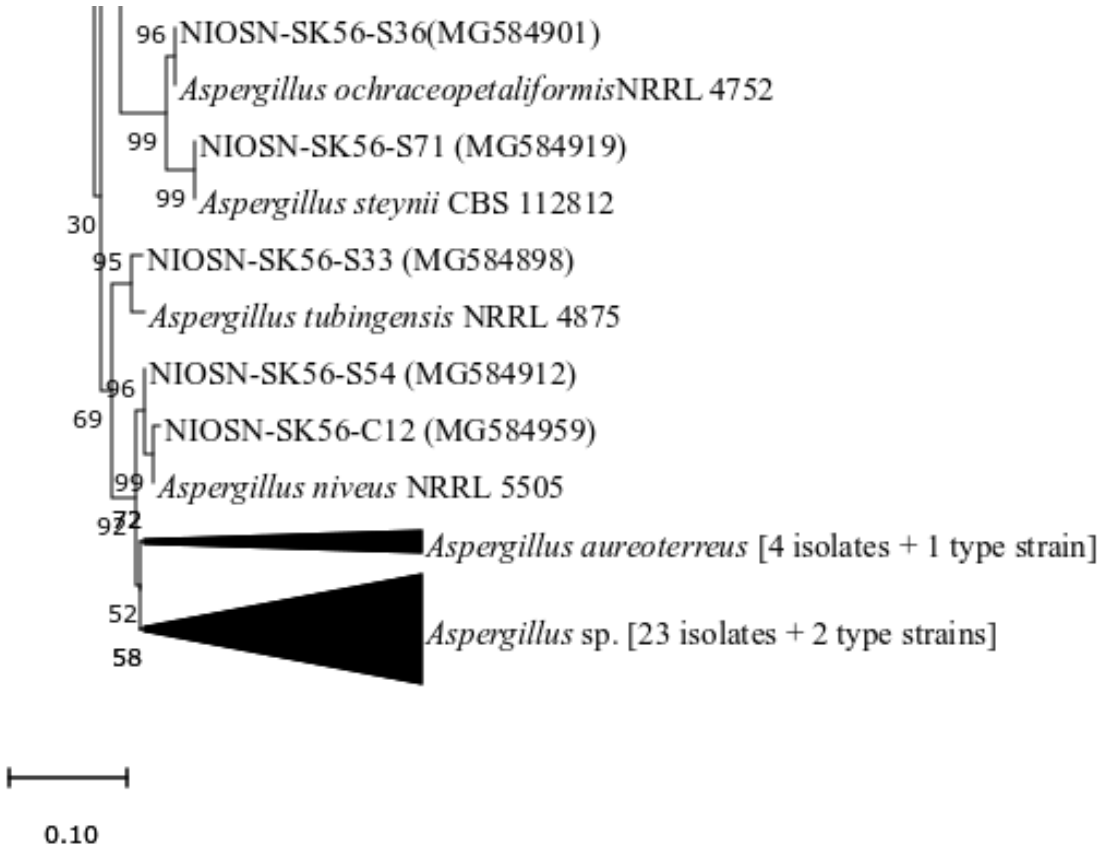

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.26315840 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is 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 (Tamura et al. 2004) and are in the units of the number of base substitutions per site. This analysis involved 19 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 636 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al. 2018).

**Figure 4. Phylogenetic analysis of fungal isolates (ITS sequences) isolated from Arabian Sea sediments, inferred from NJ analysis using MEGA software.**




Roussoella solani[2 isolates + 1 type strain]
NIOSN-SK56-C19 (MG584965)
Westerdykella dispersa CBS 297.56
NIOSN-SK56-S2 (MG584873)
NIOSN-SK56-S76 (KY788339)
Purpureocillium lilacinum M1447
Purpureocillium lilacinum 001JFC
Parengyodontium album [4 isolates + 1 type strain]
NIOSN-SK56-S82 (MG584924)
Parengyodontium albumHNC21-122
Engyodontium album OUCMBI110158
NIOSN-SK56-C20 (MG584966)
Lecanicillium kalimantanense BTCC F23
NIOSN-SK56-S8 (MG584878)
Verticillium cf. biguttatum CBS 77.73B
Verticillium biguttatum CBS 288.79
Colletotrichum sp. [9 isolates + 3 type strains]
NIOSN-SK56-C5 (MG584955)
Microascus croci CBS 158.44
NIOSN-SK56-S93 (MG584928)
Fusarium equiseti NRRL 26419
Acremonium sclerotigenum [3 isolates + 1 type strain]
NIOSN-SK56-S70 (MG584918)
NIOSN-SK56-C48 (MG584978)
Hypocreales sp. isolate MY10 3
NIOSN-SK56-S29 (MG584895)
Gliomastix polychroma CBS 181.27
NIOSN-SK56-S112 (MG584943)
NIOSN-SK56-S114 (MG584945)
NIOSN-SK56-S90 (MG584927)


80
NIOSN-SK56-S112 (MG584943)
99
NIOSN-SK56-S114 (MG584945)
NIOSN-SK56-S90 (MG584927)
99
Acremonium hyalinulum CBS 652.96
14
93
NIOSN-SK56-S4 (MG584875)
Penicillium pimiteouiense NRRL 25542
99
NIOSN-SK56-C18 (MG584964)
42
Penicillium rubens CBS 129667
99
NIOSN-SK56-S94 (MG584929)
Talaromyces australis IBT 14256
14
99
65
Penicillium citrinum [9 isolates + 1 type strain]
Penicillium shearii [2 isolates + 1 type strain]
31
99
99
NIOSN-SK46-S127 (MG584950)
88
Aspergillus penicillioides NRRL 4548
31
NIOSN-SK56-C30 (MG584969)
99
Phialosimplex sclerotialis CBS 366.77
61
Aspergillus sp. [8 isolates + 4 type strains]
84
NIOSN-SK56-S22 (MG584888)
15
52
Aspergillus oryzae NRRL 447
41
NIOSN-SK56-S113 (MG584944)
99
NIOSN-SK56-C36 (MG584971)
58
Aspergillus flavus ATCC 16883
7
NIOSN-SK56-S32 (MG584897)
84
96
NIOSN-SK56-S36(MG584901)
Aspergillus ochraceopetaliformisNRRL 4752
99
NIOSN-SK56-S71 (MG584919)
99
Aspergillus steynii CBS 112812
30




The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 5.74124748 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The tree is 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 (Tamura et al. 2004) and are in the units of the number of base substitutions per site. This analysis involved 163 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 883 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al. 2018).

### 2.2.3 Estimating fungal diversity using culture independent method from marine habitats

Fungal diversity was identified from mangrove sediments (with/without tidal influence) and Arabian Sea sediments (coastal/open sea). Total genomic DNA from the sediments were extracted using PowerSoil® DNA Isolation kit (MO BIO laboratories, Carlsbag) as per manufacturer's protocol. The purity of the extracted DNA was estimated using Nanodrop spectrophotometer (Thermo, US) and a total of 2000 ng was vacuum dried in the vacuum concentrator (Eppendorf, Germany) and outsourced to Genotypic technology, Bangalore, to obtain sequenced amplicon of ITS regions using Illumina MiSeq platform. The Illumina paired raw reads were quality checked using Fast QC tool, QIIME pipeline was used to ensure quality and clean sequences clustered into Operational Taxonomic Units (OTUs) at 97% similarity using uclust. The sequences were annotated using Unite database. The raw Illumina pair reads were deposited to NCBI.

## 2.3 Results and Discussion

Divar mangroves being located along the Mandovi estuary, face a direct tidal effect along with the constant influence of disturbances caused by barge movement carrying metal ore, across the estuary.While the off-shore sediment samples taken from Arabian Sea seabed remained unaffected by anthropogenic activities.Isolation and maintenance of fungal cultures from different marine habitats from different studies have been well described (Alsheikh-Hussain et al. 2014),where most frequently used media include potato dextrose agar, corn meal agar, yeast extract and peptone agar, etc. On implementing various media, it was observed that not a single media could isolate all types of cultures. In comparison

between organic media (PDA, SDA, MEA, ZMA, and CMA) and inorganic media (CDA), PDA supplemented with salt showed maximum numbers of isolates. CDA at 20% sea-salt concentration also supported different fungi, both filamentous and non-filamentous types. Least isolates were obtained at 30% sea-salt amended media, where most appeared stressed and crumpled. The environment and substrates in which these organisms are found are quite complex. Fungal preferences and succession varies with the mangrove substrates, litter and detritus involved, as fungi found in mangroves are known for their substrate specificity and niche (Ananda and Sridhar, 2004) therefore, all fungal species do not grow media or substrates currently used for isolation (Raghukumar and Schaumann, 1993). Media such as sediment agar on petri-plates as well as 96 well plates, failed to support the growth of fungal isolates while froth spread-plated on solid media, harboured a few sporulating fungi. Soil is one habitat with the scarcity of nutrients with readily utilizable carbon having a severe limitation (Lockwood and Filonow, 1981), therefore total failure to isolate fungi from sediment agar is not unexpected. Further, the presence of volatile fungi-static factors, ammonia and ethylene act as inhibitory factors in soil (Schippers and Palm, 1973; Pavlica et al. 1977). A total of 380 fungal isolates were obtained, out of which 145 isolates belonged to mangrove sediments and 175 isolates to Arabian Sea sediment. Inability to mimic the exact marine environment of isolation in the laboratory led to the loss of isolates. Since the first and second objectives of the thesis were carried out simultaneously, all procured isolates were not identified at one time. During the subsequent sub-culturing, handling of cultures and conduction of experiments in objective two, few more isolates were lost in the process. The results shown in this objective, are specific to the isolates that grew on subsequent culturing and were identified using

molecular techniques. It is to be noted that not all of these isolates were used for the experimental purpose and not all isolates used in objective two, survived and therefore were not identified.

The isolates that survived subsequent culturing (245) belonged to 23 different families, 39 different genera (Figure 5) and 92 different species. The mangrove sediment isolation led to 131 fungal isolates belonging 13 different families, 26 genera (Figure 6a) and 70 species (Figure 6b) with one isolate that could be identified up to class level of Ascomycetes. The Arabian Sea sediments yielded 114 fungal isolates belonging to 25 different genera (Figure 7a) and 46 (Figure 7b) different species. Most of these isolates belonged to *Penicillium*, *Aspergillus*, *Cladosporium*, *Acremonium*,*Alternaria* and *Curvulari*a genera which have also been widely reported in the literature and belong to class Ascomycota. Ascomycotina is the largest subdivision in the taxonomic classification of fungi and has up to 2700 genera and 28500 species (Manoharachary et al. 2005), therefore being abundant and rapidly proliferating, these fungi would probably be masking the growth of other fungi. Studies carried out previously to study cultivable fungi from mangroves also showed ascomycetes to be abundant fungi in terms of number of isolates (Sarma et al. 2001; Sarma and Vittal, 2000; Sarma and Vittal, 2001; Shini et al. 2009-10; Tsui and hyde, 2004). On calculating the diversity indices for both the marine habitats (Table 3), it was observed that species richness and diversity were more in mangrove sediments as compared to Arabian Sea sediments. Further the distribution of species were more even in mangrove sediments as compared to the latter.

**Figure 5. Piechart depicting culturable diversity from marine habitats.**

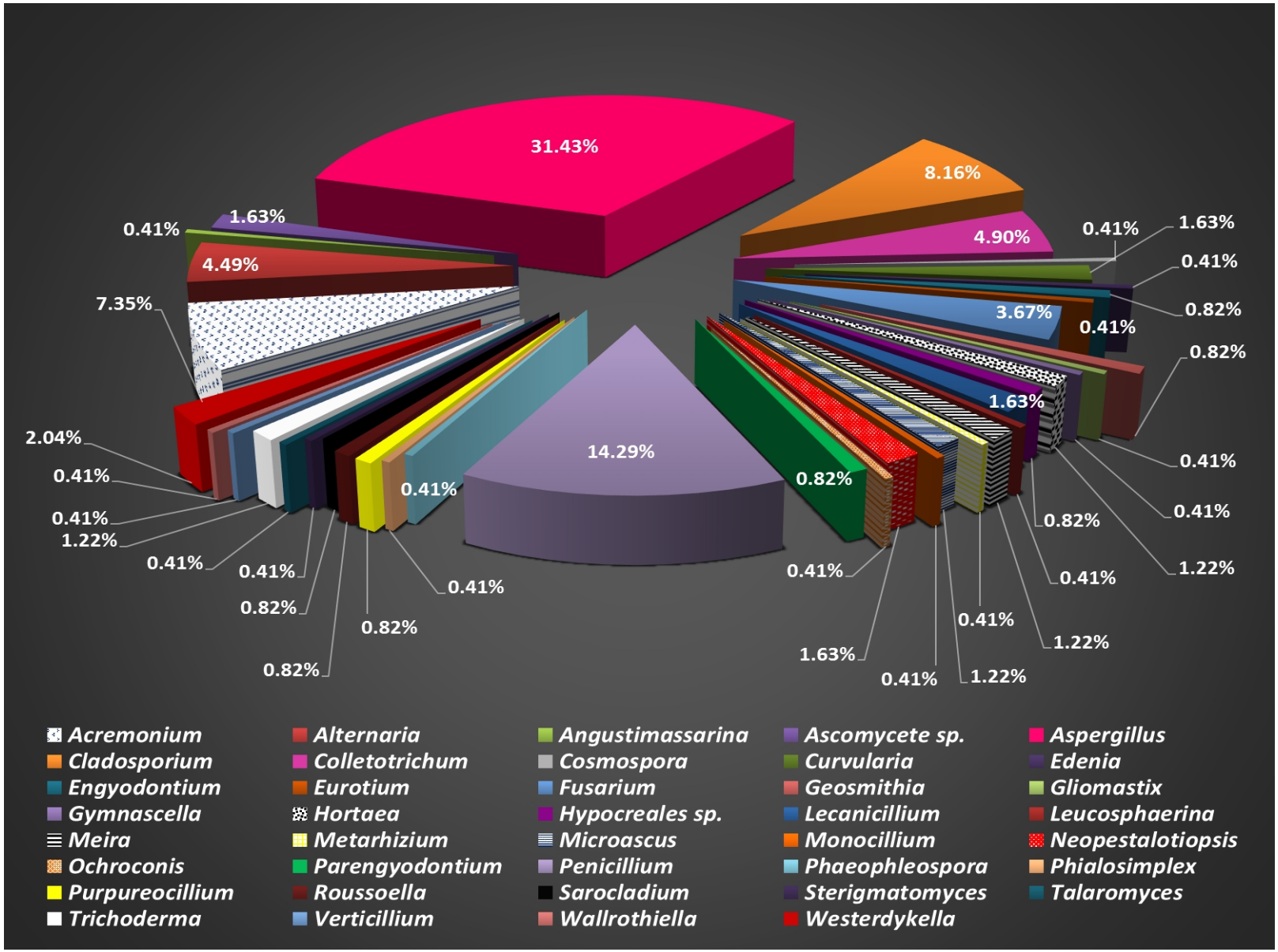


**Figure 6. Bar graph depicting culturable fungal diversity from mangrove sediments.**

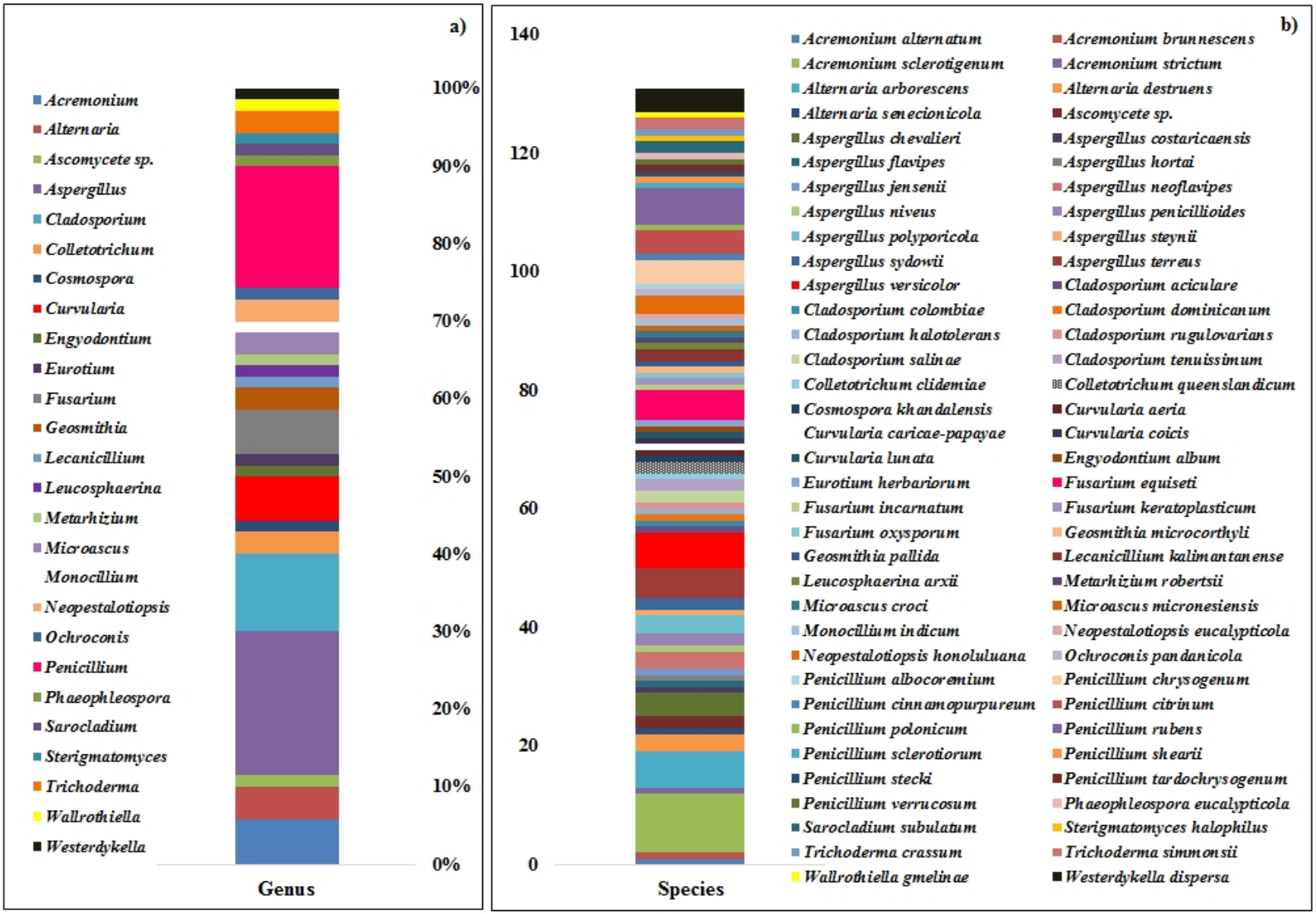


**Figure 7. Bar graph depicting culturable fungal diversity from Arabian Sea sediments.**

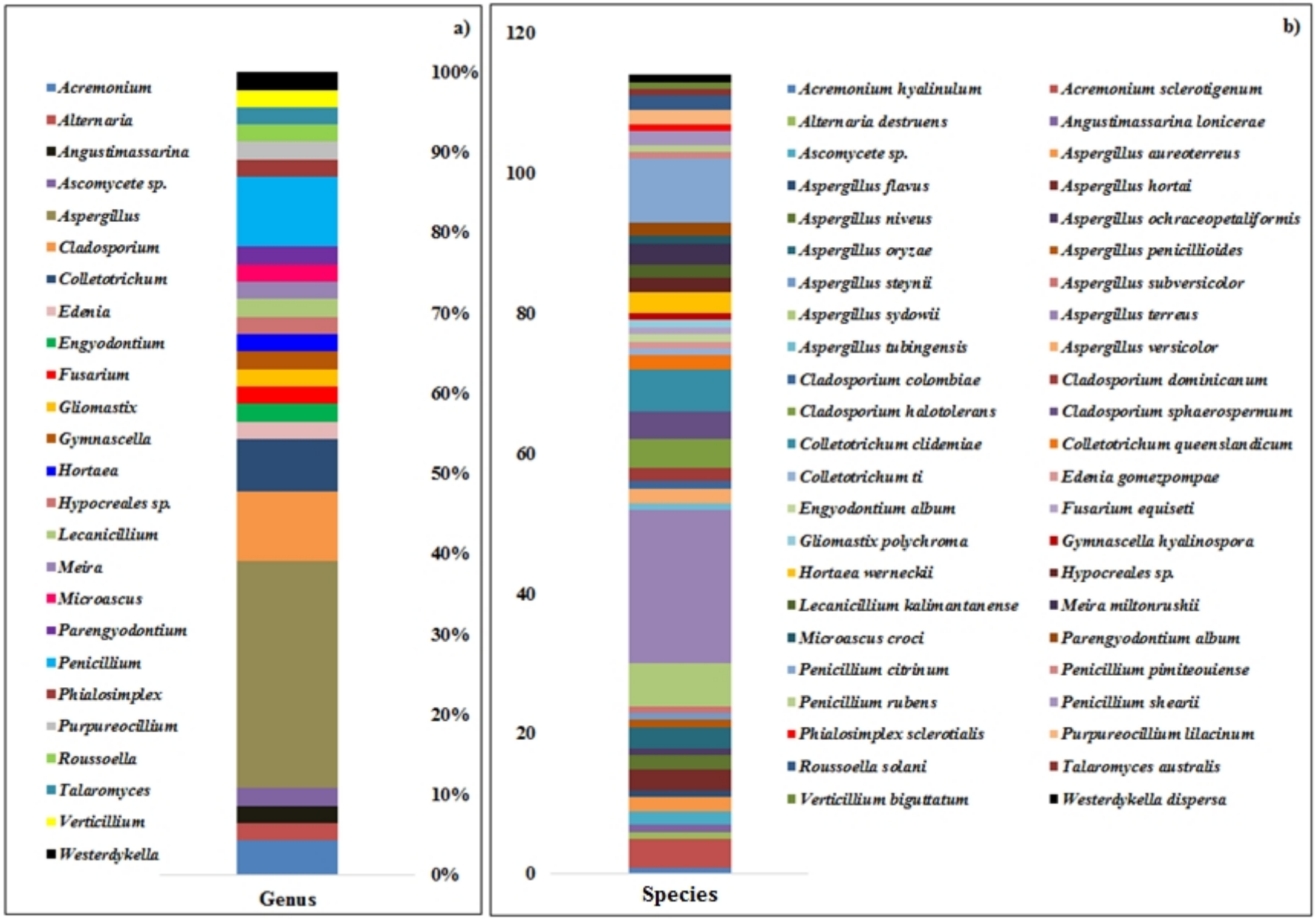
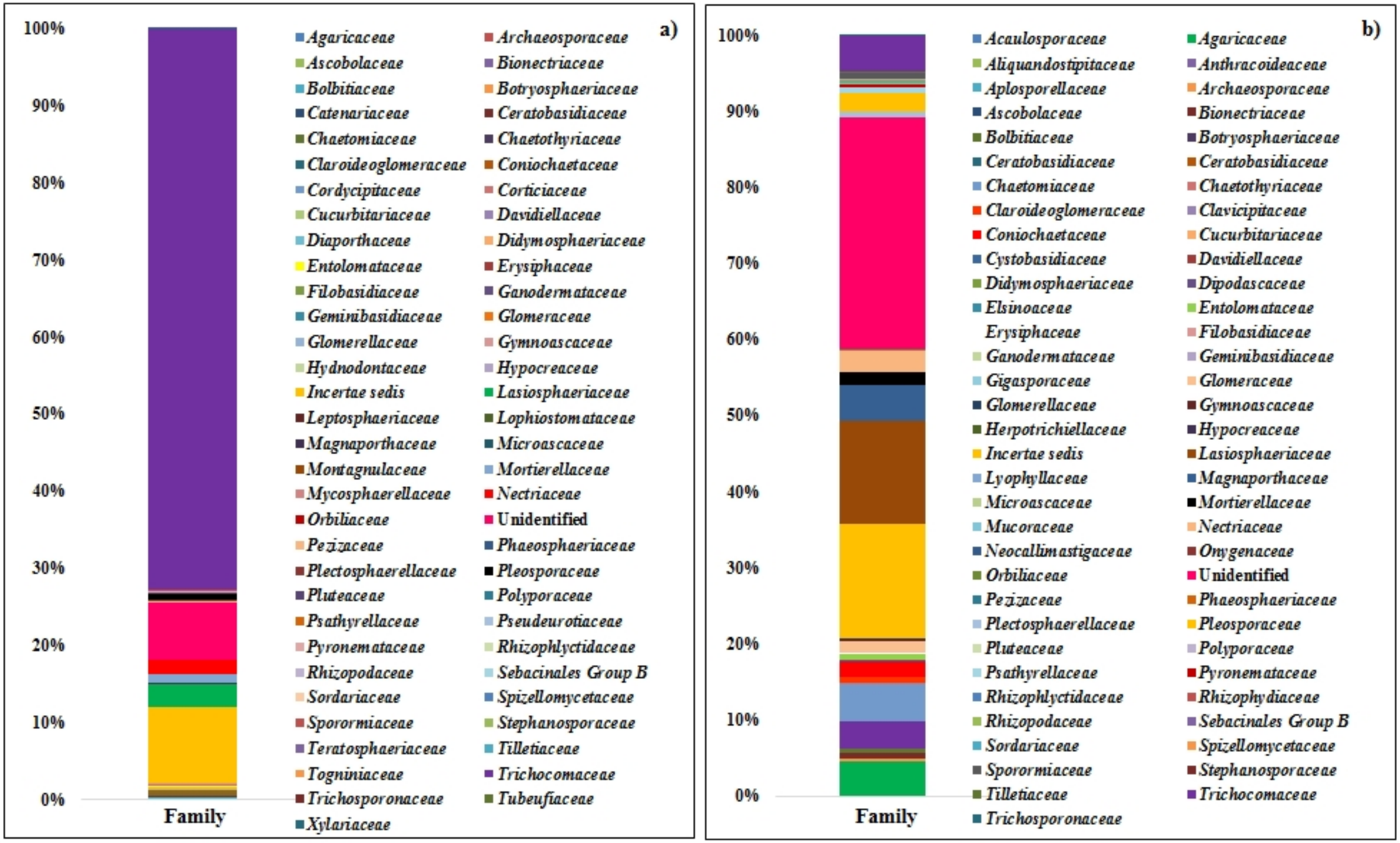


**Table 3. Comparative analysis of diversity indices between isolates procured from Mangrove sediment and Arabian Sea sediment.**

| Sample type | S (Number of species) | N (Number of isolates) | d (Species richness) | J (Species evenness) | H' (Species diversity) |
|---|---|---|---|---|---|
| **Mangrove sediment** | 26 | 131 | 5.127998 | 0.799546 | 2.604999 |
| **Arabian Sea sediment** | 25 | 114 | 5.067355 | 0.712236 | 2.292599 |

Sediment samples of mangrove region, M1 (with the effect of tides) and M2 (without effect of tides) identified 144194 and 196788 rRNA gene sequences respectively. The sequences in M1 clustered to give 2261 OTUIDs that belonged species to 61 families (Figure 8a), 109 Genera and 205 different species while in M2 they clustered to give 1110 OTUIDs that belonged to 62 families (Figure 8b), 117 genera and 206 different species. The rRNA gene sequences in sample M1 belonged to phylum *Ascomycota* (133471- absolute count), *Basidiomycota* (2610), *Zygomycota* (1536), *Glomeromycota* (186), *Chytridiomycota* (52), *Rozellomycota* (6), and Unidentified (6333). Among all the genera, the most abundant that were recorded were *Penicillium* sp. (45.2%), *Aspergillus* sp. (26.9%) and *Candida* sp. (8.8%). Similarly, the rRNA gene sequences in sample M2 belonged to phylum *Ascomycota* (131778), *Basidiomycota* (28104), *Zygomycota* (4058), *Glomeromycota* (5349), *Chytridiomycota* (743), *Rozellomycot*a (434), *Incertae sedis* (64) and Unidentified (26258). The most abundant genera found were that of *Zopfiella* sp. (4.8%), *Coprinus* sp. (4.5%), *Cercophora* sp. (3.5%), *Retroconis* sp. (2.4%) and P*homa* sp. (2.3%). Further, Unidentified fungi accounted for 4.4 % in M1 and 13.3% in M2 samples respectively. NGS data revealed 92.6% and 1.81% of sequences belonged to *Ascomycota* and *Basidiomycota* from sediment subjected to tidal fluctuation (M1) while 66.96% and 14.28% sequences belonged to mangrove sediment not under the influence of tides (M2). Mangrove habitats house huge stores of lignocellulosic substrates, supporting diverse dwelling populations of fungi (Alsheikh-Hussain et al. 2014; Sarma et al. 2001). With all the culturing techniques employed in this study, only 0.1% of total diversity was cultivable when compared with the results obtained from NGS analysis of uncultured forms which holds true for studies carried out previously to obtain culturable diversity (Handelsman, 2004). It was interesting to note that the community differed in both the sediments.

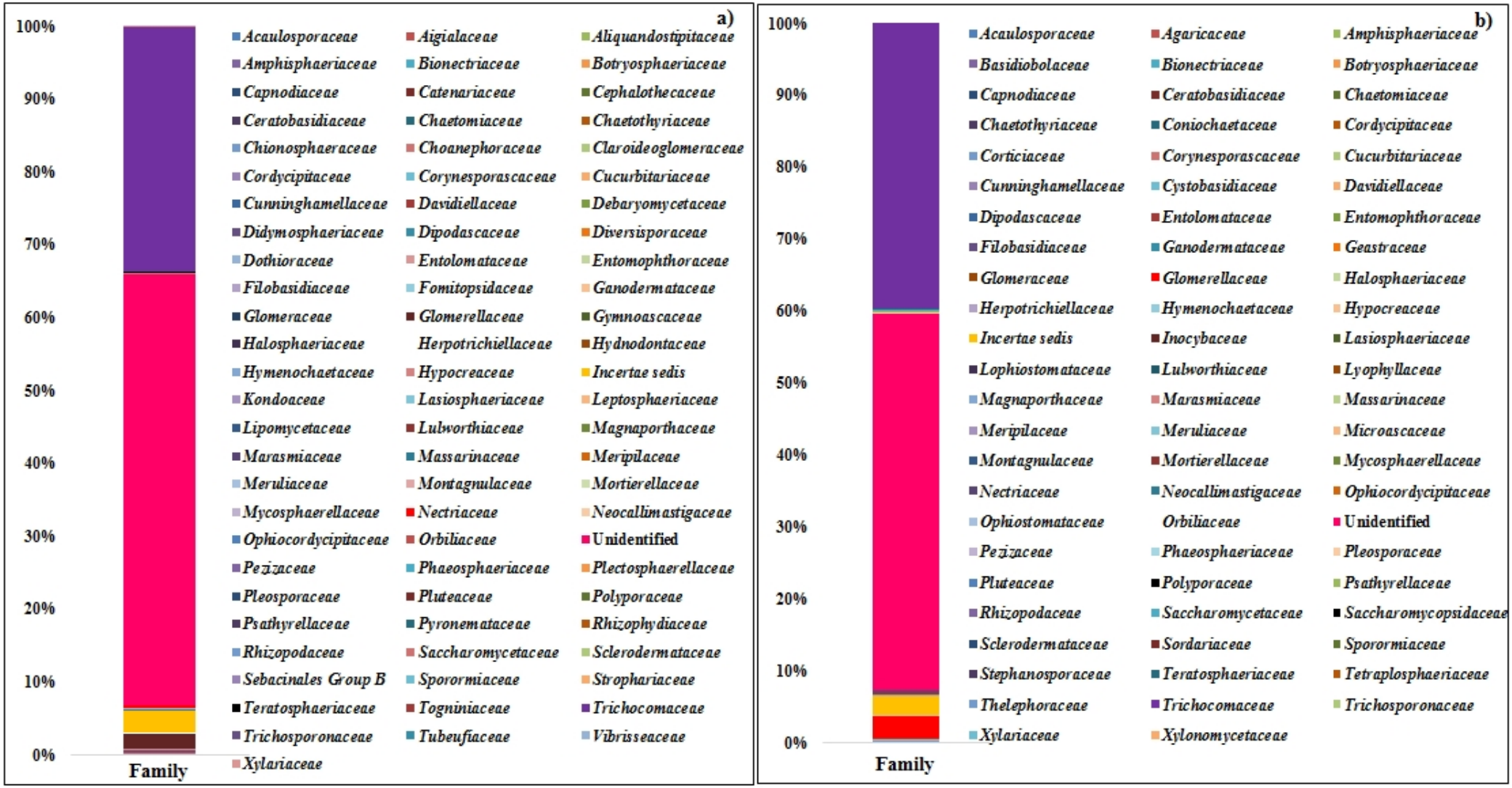
**Figure 8. Bar graph depicting distribution of fungal diversity at family level from mangrove sediments.**



Mangrove habitats generate huge amounts of litter and woody debris providing a variety of substrates for fungi to thrive on (Manoharachary et al. 2005) which can account for the high diversity of fungi in sample M2 which was sediments found in the core of mangrove habitation. Periodic submergence of sediments under estuarine waters due to tides can cause a change in community structure which could be seen from sample M2. All isolates except one, cultured under laboratory conditions belonged to phylum Basidiomycota, while fungi from all different phylum persist in the mangrove habitat. Therefore it is evident that the culturing techniques prove inefficient to isolate different forms of fungi, and preferably isolate only the ones which are robust and outnumber the rest. Techniques involving isolations from soil are specific to sporulating fungi which grow faster (Parkinson, 1965; Choi et al. 1999). Not just the media, but factors such as host specificity (Sarma and Vittal, 2000), succession, time of sample collection and incubation period plays equally an important role in isolation of fungi (Sarma and Hyde, 2001). In order to isolate slow growers which are often masked by rapidly proliferating fungi, amendment of growth inhibitors in growth medium has also been reported. Lesser concentrations of antifungal agents such as cycloheximide, rose bengal (Choi et al. 1999) and Natamycin (Pedersen, 1992) have been used before to retrieve slow growing fungi.

NGS analysis of Arabian Sea sediments, C1 (coastal sediment) and C2 (Deep Sea sediment), also proved that fungi are abundant in these regions. Sample C1 retrieved 108261 rRNA gene sequences that were clustered in 12783 OTUIDs belonging to 77 families (Figure 9a), 125 Genera, and 207 different species, while sample C2 retrieved 77507 rRNA gene sequences clustered into 10623 OTUIDs belonging to 69 families (Figure 9b), 111 genera and 181 different species. The rRNA gene sequences in sample C1 belonged to phylum *Ascomycota* (53950), *Basidiomycota* (17848), *Zygomycota*

**Figure 9. Bar graph depicting distribution of fungal diversity at family level from Arabian Sea sediments.**



(212), *Glomeromycota* (31),*Chytridiomycota* (94), *Rozellomycot*a (2), and Unidentified (36124). Among all the genera, the most abundant that were recorded were *Penicillium* sp. (18.8%) and *Aspergillus* sp. (13.3%). Similarly, the rRNA gene sequences in sample C2 belonged to phylum *Ascomycota* (41711), *Basidiomycota* (8281), *Zygomycota* (212), *Glomeromycota* (15), *Chytridiomycota* (69), and Unidentified (27219). The most abundant genera found were that of *Penicillium* sp. (23.6%) and *Aspergillus* sp. (14.9%). Further, Unidentified fungi accounted for 33.4 % in C1 and 35.1% in C2 samples respectively. Irrespective of the type of habitat, 32 genera were found to be common in all four samples (C1, C2, M1 and M2) while 38 genera were found only in mangrove sediments and 37 genera were found only in Arabian Sea sediments (Table 4). With the recent outbreak of high throughput meta-barcoding, it is now considered as a powerful tool to study fungal communities (Schmidt et al. 2013) as it bypasses the need for isolation and maintenance of cultures (Carola and Rolf, 2011). A DNA based analysis of ITS region has helped to understand the highly diverse fungal community persisting in the marine habitats. But, metagenomic data can only give the diversity in an area based on which functional roles played by individual species can be extrapolated established on existing literature. In order to prove a particular hypothesized function, the isolate needs to be cultured and studied which makes isolation techniques very important and essential. Although the aim of this objective was only to isolate and identify marine-derived fungi using culturing techniques, NGS analysis revealed the existence of many more species which could have many applications for the future.

**Table 4. List of genera found in mangrove and Arabian Sea sediments**

| Genera found in Mangrove sediments | Genera found in Arabian Sea sediments | Genera common to both marine habitats |
|---|---|---|
| *Ascobolus* | *Acaulospora* | *Acremonium* |
| *Bolbitius* | *Arthrobotrys* | *Alternaria* |
| *Botryosphaeria* | *Beauveria* | *Aspergillus* |
| *Cephaliophora* | *Bullera* | *Bipolaris* |
| *Chaetomium* | *Ceriosporopsis* | *Candida* |
| *Chalastospora* | *Chaetosartorya* | *Cercophora* |
| *Claroideoglomus* | *Chaetosphaeronema* | *Cladosporium* |
| *Clitopilus* | *Cladophialophora* | *Cochliobolus* |
| *Clonostachys* | *Collembolispora* | *Cryptococcus* |
| *Coniocessia* | *Colletotrichum* | *Cyphellophora* |
| *Coprinus* | *Corynespora* | *Debaryomyces* |
| *Dactylaria* | *Cunninghamella* | *Entoloma* |
| *Didymosphaeria* | *Devriesia* | *Eucasphaeria* |
| *Erythrobasidium* | *Didymella* | *Fusarium* |
| *Fusicolla* | *Engyodontium* | *Ganoderma* |
| *Geminibasidium* | *Exserohilum* | *Gibberella* |
| *Golovinomyces* | *Galactomyces* | *Glomerella* |
| *Hansfordia* | *Inonotus* | *Haematonectria* |
| *Lecythophora* | *Leptosphaerulina* | *Mortierella* |
| *Micronematobotrys* | *Leptoxyphium* | *Penicillium* |
| *Monographella* | *Lignincola* | *Periconia* |
| *Mycoleptodiscus* | *Lulworthia* | *Phoma* |
| *Myrmecridium* | *Marasmiellus* | *Podospora* |
| *Parasola* | *Massarina* | *Psathyrella* |
| *Phaeosphaeria* | *Microdiplodia* | *Pyrenochaetopsis* |
| *Piriformospora* | *Nectria* | *Rhodotorula* |
| *Plectosphaerella* | *Pandora* | *Savoryella* |
| *Powellomyces* | *Penidiella* | *Sporobolomyces* |
| *Pseudaleuria* | *Pestalotiopsis* | *Talaromyces* |
| *Remersonia* | *Phaeoisaria* | *Trichoderma* |
| *Schizothecium* | *Pisolithus* | *Trichosporon* |
| *Scolecobasidium* | *Rhodosporidium* | *Westerdykella* |
| *Scutellinia* | *Rigidoporus* | |
| *Spizellomyces* | *Scleroderma* | |
| *Stachybotrys* | *Stylonectria* | |
| *Thanatephorus* | *Teratosphaeria* | |
| *Volvariella* | *Torulaspora* | |
| *Zopfiella* | | |

# Chapter THREE

# Mycoremediation, a Budding Aspect of Heavy Metal tolerance Strategies

### 3.1 Heavy metal tolerance in fungi

Metals belonging to the transition element category in the periodic table having densities more than 5 gm/cm$^3$ are categorized as heavy metals (Beveridge et al. 1997). Anthropogenic activities associated with effluent discharge from industries such as electroplating, mining, tanneries, etc., lead to the release of high amounts of toxic as well as essential heavy metals into the environment, which in turn pollute groundwater and soil, and is also detrimental to all living beings (Ahmad et al. 2006; Hasan, 2007). Due to the high metal concentrations, even the essential heavy metals, which are required for proper metabolic functioning and participating as cofactors in many enzymes (Blust et al. 1987; Cervantes and Gutierrez-Corona, 1994; Hall, 2002), turn lethal to existing biota. One such essential heavy metal is copper which has atomic number of 29 and variable oxidation states (-2, +1, +2 and +4) with constant transition shifts from +1 to +2 states due to its redox-active nature (Bellion et al. 2006). Copper is the key metal in cytochrome c oxidase and is essential for oxidative respiration. Another toxic heavy metal is Cr with atomic number 24 and density of 7.19 gm/cm$^3$. It occurs in 9 oxidation states, with states +3 and +6 being the most stable, and the latter being 100 times more toxic and 1000 times more mutagenic than +3 state (Abbas et al. 2008). Cr (VI) has tetrahedral geometry [as opposed to the octahedral geometry of Cr (III)], which is similar to the molecular structure of sulphate, thereby giving it easy access to enter inside the cell via sulfate transport system as discussed earlier. The solubility of Cr (VI) in water being 1680 gm/L, in comparison to insoluble Cr (III), makes it even more dangerous and difficult to remove from contaminated sites. Such redox active metals, participate in the production of free radicals and reactive oxygen species that cause oxidative stress to cell organelles causing damage to DNA, proteins and lipids which is often fatal to organisms (Cervantes and Gutierrez-Corona, 1994; Borkow and Gabbay,

2005; Bellion et al. 2006). Metal contamination is, therefore, an issue of high concern. Various effluent treatment methods comprising of physical and chemical treatments (physical adsorption, solvent extraction, chemical precipitation) have been employed by industries which have their own set of drawbacks which include high costs, poor metal removal efficiencies and production of toxic by-products (Joshi et al. 2011; Bishnoi and Garima, 2005) and have been mentioned before. Besides metals being soluble under acidic pH, it's rather tough to eliminate/withdraw metal ions from contaminated sites/waters (Cuppett et al. 2006). Under such circumstances, bioremediation and such related approaches have been focused. Although a wealth of information is available on the application of microbes such as bacteria, fungi and other eukaryotes to bioremediate toxic wastes, more studies pertain to application of dead/heat killed biomass to adsorb metal. Fungi are found to be good candidates for metal waste remediation due to their adeptness in biosorption (Akthar et al. 1996; Lopez and Vazquez, 2003) along with their proficiency to solubilise metals better than bacteria (Thangavelu et al. 2006). Apart from prominent defence mechanism in the form of biosorption, fungi have been found to produce organic acids such as citric acids (Dave and Natarajan, 1981) and oxalic acid (Green and Clausen, 2003), for chelation of Cu (II) ions. In case of chromium, besides biosorption, fungi have been reported to enzymatically reduce the oxidation state of the metal (Cervantes et al. 2010).

Fungi are ubiquitous in nature and involved in the biogeochemistry of metals, and have proven to be an excellent bioremediating agent. But without detailed background study of biological as well as chemical reactions occurring during the mechanisms put forth by these fungi to tolerate and survive in the metal stress, often leads to failure of such promising applications. Hence it is very essential to understand the physiological response of fungi towards the said heavy metal before a relevant biotechnological

solution is devised to eradicate the heavy metal contamination problem (Jain, 1990).

**3.2 Materials & Methodology (<u>Objective TWO</u>: To check selected isolates for tolerance to metals such as Cr, Pb, Cd, Ni and Cu (one or two metals will be studied in detail))**

**3.2.1 Screening of selected isolates for heavy metal tolerance at varying concentrations**

All procured isolates from different marine habitats were sub-cultured on Czapek Dox agar (CDA) prepared using seawater (SW) for a week. Based on growth and colony morphology, fast-growing fungi were selected for metal screening on agar plates. Slow growers would take more incubation time for growth (15-20 days) and serve no benefit to use such cultures for further analysis. A total of 234 marine-derived fungal isolates were selected for primary/initial screening at 500 ppm of metal (Nickel, Copper, Chromium, Lead and Cadmium) concentration on CDA (SW). Filter sterilized metal stock solutions [$NiSO_4$ (1 M), $CuSO_4$ (1 M), $K_2Cr_2O_7$ (100 mM), Pb $(NO_3)_2$ (1 M) and $CdCl_2$ (2 M)] were added to autoclaved agar media to give a desired concentration of the concerned metal. Fungal isolates were initially screened at 500 ppm concentrations of all five metals to check for tolerance, in batches of 15 isolates at a time. Since the number of Cr- tolerant isolates were less at 500 ppm, the concentration was reduced to 250 ppm. One week old isolates previously grown on CDA (SW) were cut in the form of 7 mm discs using one mL tips and placed inverted on metal agar plates and incubated at room temperature (28°C) for ten days. The subsequent screening for selected 75 isolates that grew in previous screening was carried out at 750 ppm of Cu, Cd, Ni, Pb and 500 ppm of Cr. Criteria for selection was basically colony diameter of fungus in the metal plate as opposed to control. 16 isolates were screened at 1000 ppm of metals (Cu, Cd, Ni, Pb)

and 750 ppm of Cr and 15 isolates from these were screened at 1000 ppm of Cr and 1250 ppm of other metals. Metal tolerance index (MTI) in percent, was calculated for the selected 15 isolates using the following formula (Akhtar et al. 2013):

$$= \frac{\text{Colony diameter of isolate in presence of metal}}{\text{Colony diameter of isolate in absence of metal (control)}} \text{X } 100$$

Based on the MTI results, it was found that chromium was the most toxic metal amongst the three toxic heavy metals (chromium, cadmium and lead) while copper was found to be toxic amongst the essential heavy metals (copper and nickel). All the initial screenings were carried out on media prepared in SW as the isolates were marine-derived, and impact of pH on the bioavailability of metals was not considered. The metal screenings were carried out to shortlist a few isolates and carry out further analysis. Based on screening, six isolates were chosen to study Cr and Cu toxicity on fungi. Since metals are more bioavailable in acidic pH, further experiments were carried out in DW.

### 3.2.2 Studying the effect of pH on fungal morphology

Before subjecting the fungal isolates to heavy metal exposure under acidic pH, it was necessary to check the effect of acidic pH alone on fungal morphology to avoid false interpretation of results. Therefore three selected isolates (#NIOSN-SK56-S19, #NIOSN-SK56-S52 and #NIOSN-SK56-S76) were grown in CDB (DW) of pH ranging from 3 to 7.2 (adjusted using 6N HCl), to carry out morphological analysis of mycelia using scanning electron microscopy (SEM), so as to visualize if stress on mycelia was caused due to acidic pH. The samples were incubated at varying pH for a week at 28°C at 80 rpm. The protocol used for sample preparation was modified from Damare (2015) where the biomass was first fixed with 2% glutaraldehyde and dehydrated with series of

ethanol concentrations (10, 30, 50, 70, 90 and 100%) for 10 minutes each. The samples were sputtered coated with gold (SPI Module Sputter coater, USA) prior to observation using a scanning electron microscope (JSM 5800 LV SEM and EDS, Japan).

### 3.2.3 Determination of metal content in residual broth using atomic absorption spectroscopy (AAS)

Three isolates (#NIOSN-SK56-S19, #NIOSN-SK56-S52 and #NIOSN-SK56-S76) that showed tolerance to chromium (750 ppm screening) and three isolates (#NIOSN-M20, #NIOSN-M29 and #NIOSN-M98) that showed tolerance to copper (1250 ppm screening) on agar plates, were selected to estimate their respective metal removal efficiency from liquid broth. The starter inoculum for the experiment was prepared by incubating one 7 mm disc of isolate cut from the periphery of one-week old culture, in 100 mL Erlenmeyer flasks with 20 mL CDB (DW). Before the culture could start sporulating, the mycelia were broken into smaller bits by gently crushing it using glass beads and 1 mL of this suspension was inoculated in 20 mL of CDB (DW) containing increasing concentrations of metal [Cr (VI) and Cu (II)] (0, 50, 100, 200, and 300 ppm) in triplicates. The pH of the broth was adjusted to 5 using 6N HCl at the beginning of the experiment. After incubation of the cultures subjected to metal for one week at 80 rpm at 28°C, the contents of the flasks (duplicates) were filtered (0.45 µm Millipore filter paper) to separate broth from biomass (the third replicate was used for SEM analysis). The filtrate was directly acidified using 2% of Nitric acid ($HNO_3$, trace metal grade) and metal content in broth was estimated using AAS (Thermo Scientific Solar S series, Thermoelectron Corporation, Cambridge). Further, the separated biomass was lyophilised, weighed and acid digested with $HNO_3$ and perchloric acid ($HClO_4$) with a ratio of 1:1 (modified from Hseu, 2004) to check if biomass was involved in the removal

of metal. The remnants of digestion were dissolved in 2% $HNO_3$ and metal content was estimated using AAS.

### 3.2.4 Estimation of Cr (VI) reduction rate

All three Cr (VI) tolerant isolates were inoculated in 20 mL CDB (DW) in the form of a disc as starter inoculum and incubated under static condition till the onset of sporulation. The mycelial matt was crushed (as mentioned before) and 1 mL of this suspension was inoculated in 20 mL of CDB supplemented with 50 ppm of Cr (maximum biomass was obtained at this concentration from the previous experiment, therefore this concentration was chosen). The pH of the broth was adjusted with 6N HCl to 5 and incubated at 28°C for one week at shaking condition of 80 rpm. Diphenylcarbazide DPC method (Pfalaum and Howick, 1956) was used to spectrophotometrically estimate Cr (VI) reduction, at 540 nm (UV-2450, Shimadzu Corporation, Japan) every day. Further to detect any presence of nanoparticle formation, all three Cr (VI) tolerant isolates were inoculated in 250 mL Erlenmeyer flasks with 100 mL CDB (DW) amended with 50 ppm of Cr (pH adjusted to 5) and incubated for 20 days at 80 rpm at 28°C. Every alternate day 1 mL of broth was filtered using a syringe filter (0.22 µm) and spectrophotometrically analyzed using UV-VIS spectrophotometer (UV-2450, Shimadzu Corporation, Japan) for changes in absorption peaks.

### 3.2.5 Morphological analysis of biomass subjected to metal stress

The third replicate flask inoculated for estimating metal content in broth and biomass was used to study the mycelial morphology in the presence and absence of metal using SEM. The protocol for SEM analysis was modified from Damare (2015) and is mentioned before. The same set of samples were used for Electron Dispersive

Spectroscopy analysis.

### 3.3 Result and Discussion

#### 3.3.1 Heavy metal screening

Ecologically important marine habitats such as mangroves and mudflats are found to be organically rich (Holguin et al. 2001) and house significant fungal diversity (Raghukumar et al. 2014). Sediments from such niches contain recalcitrant humic substances that act as bio-filters for heavy metals and other toxic substances (Chakraborty et al. 2015), therefore the microfauna found in these regions can be expected to have tolerance to metal stress. Marine microbes are adapted to salinity stress via the mechanism of excluding sodium ions out of the system (Nayak et al. 2012) and this being the most effective means of tolerating high salt concentration, same could be expected under metal stress. Off-shore Arabian Sea sediment samples have apparently no or very less anthropogenic pollution compared to coastal areas. Since fungal response to heavy metal tolerance has never been addressed in these regions, the present study stands out as a first report.

On exposing the marine-derived fungal isolates to different metals, interesting responses were observed. Screening of 234 fungal isolates to selected heavy metals at 500 ppm concentration on agar plates showed that the order of least toxic metal to most toxic metal was Pb < Ni < Cu < Cd < Cr. Most isolates showed lead tolerance (83%) with 66% of the tolerant isolates having colony diameters close to control plates (no metal) (Table 5). Next least toxic heavy metal was Ni (II) where 65% of the screened isolates were found to be tolerant to this metal followed by Cu (II) with 26%, Cd (II) with 23% and Cr (VI) with 0.06% of isolates being tolerant at 500 ppm concentration. Amongst all five metals, Cr (VI) and Cd (II) were found to be most toxic with most of its isolates having

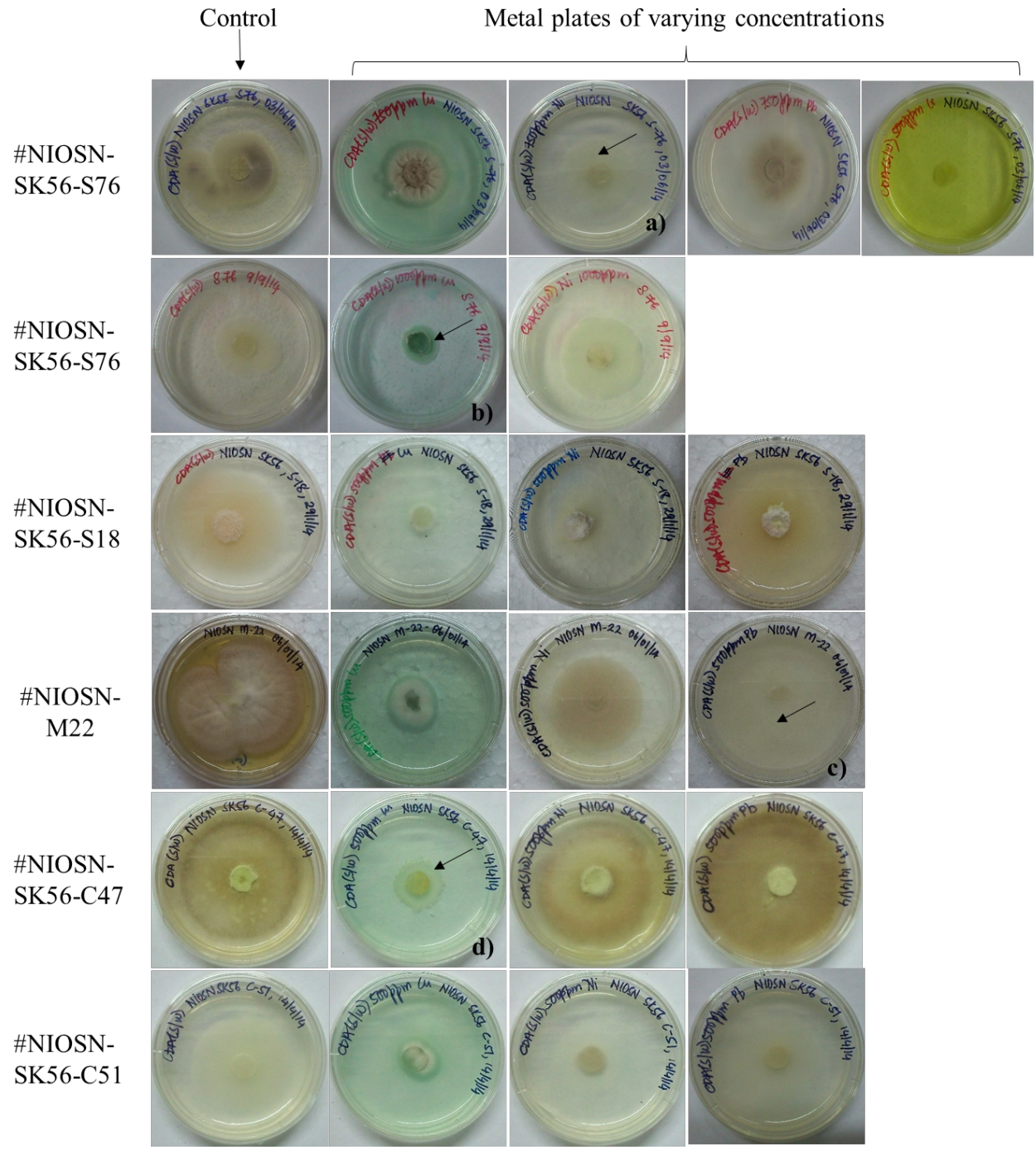
their colony diameters less than 20 mm in the presence of metal. To check for tolerance at 750 ppm concentration of each metal, 75 isolates that grew best at previous screening were selected. These included all the isolates that were tolerant to Cu (II), Cd (II) and Cr (VI), and the ones tolerant to Ni (II) and Pb, which showed colony diameters similar to control. It was observed that as the metal concentration increased the isolates tolerating the heavy metals decreased along with decrease in colony diameter. An exception was found in the case of Pb, where all 75 isolates showed tolerance at 750 ppm with 59% of its isolates having colony diameters similar to that of the control. The trend of least toxic metal to most toxic metal remained the same as before with Cr (VI) being the most toxic heavy metal having only 4 isolates tolerant to 750 ppm with less than 20 mm colony diameter. Amongst the essential heavy metals, Cu (II) emerged to be toxic with almost 54% of its isolates showing <20 mm colony diameters. Sixteen isolates that showed tolerance at 750 ppm metal concentration were selected for screening at 1000 ppm of all metals. Surprisingly, all 16 isolates were found to be tolerant to Pb at 1000 ppm while the numbers reduced to 13, 8, 3 and 1 for Ni (II), Cu (II), Cd (II) and Cr (VI) respectively. Fifteen isolates from the previous screening were screened at 1250 ppm metal concentration to finally shortlist cultures for further experiments. Here, Cd (II) and Cu (II) were found to be the most toxic metals with only 3 isolates each, tolerant to 1250 ppm concentration. Cr (VI) was not screened at 1250 ppm, as four isolates that grew at 750 ppm were already selected. In general, it was observed that as the metal concentration increased, numbers of tolerant isolates decreased, along with their colony diameters. Elevated concentrations of metals affected the fungi in terms of number of isolates tolerant to metal; their biomass production and reduction in sporulation, indicating towards adverse effects of metal on these organisms.

**Table 5. Numerical data denoting metal tolerance in terms of colony diameter.**

| Concentration of metal (ppm) | Colony diameter in mm | Number of isolates | | | | |
|---|---|---|---|---|---|---|
| | | Nickel | Copper | Lead | Cadmium | Chromium |
| | | ($NiSO_4$) | ($CuSO_4$) | ($Pb(NO_3)_2$) | ($CdCl_2$) | ($K_2Cr_2O_7$) |
| **500** | 36-50 | 70 | 7 | 131 | 0 | 0 |
| | 21-35 | 44 | 23 | 46 | 3 | 4 |
| | < 20 | 38 | 31 | 19 | 51 | 12 |
| | **Total** | **152** | **61** | **196** | **54** | **16** |
| **750** | 36-50 | 19 | 6 | 44 | 0 | 0 |
| | 21-35 | 30 | 10 | 26 | 2 | 0 |
| | < 20 | 20 | 19 | 5 | 6 | 4 |
| | **Total** | **69** | **35** | **75** | **8** | **4** |
| **1000** | 36-50 | 0 | 0 | 11 | 0 | 0 |
| | 21-35 | 9 | 2 | 5 | 0 | 0 |
| | < 20 | 4 | 6 | 0 | 3 | 1 |
| | **Total** | **13** | **8** | **16** | **3** | **1** |
| **1250** | 36-50 | 0 | 0 | 0 | 0 | * |
| | 21-35 | 5 | 0 | 8 | 3 | |
| | < 20 | 5 | 3 | 6 | 0 | |
| | **Total** | **10** | **3** | **14** | **3** | |

Number of isolates decreasing with increasing concentrations of metals is expected as not all fungal isolates are metal tolerant. Similar results were reported, where a subsequent increase in Pb concentrations on PDA, lead to decrease in lead-tolerant isolates (Joshi et al. 2011). Some fungi showed a reduction in sporulation in response to metal, while some isolates completely ceased to grow at higher concentrations (Figure 10). Distinct blue coloration was also observed in some isolates in response to Cu (II) exposure, while most isolates subjected to Pb showed no morphological difference from control plate. Interestingly, most isolates in the current study showed maximum colony diameters comparable to control when grown in the presence of lead (Figure 10c). As mentioned earlier, the bioavailability of metal is a very important aspect often neglected while studying the response of organisms to metals. Non-bioavailability of Pb to act on the organisms could be the possible reason for flourishing growth of isolates in the presence of Pb. In all the isolates exposed to different metals and their varying concentrations, a general trend was observed, where, as the metal concentration increased, the colony diameters of isolates for subsequent screenings decreased. It is known that the edges of a fungal colony are far more active in growth as compared to the center. Therefore the presence of essential heavy metals in growth media might initially act as growth stimulating factors as they are involved as cofactors for many important enzymes and proteins (Cervantes and Gutierrez-Corona, 1994). This observation was reflected from the results obtained from subjecting fungi to Ni and Cu (500 ppm), where the significant number of isolates had colony diameters ranging between 21-35 and 36-50 centimetres (Table 5). In a study carried out by Rajapaksha (et al. 2004), they reported that soil supplemented with copper led to the increase of fungal activity by seven folds in comparison to the control, indicating that copper could be acting as a growth stimulant. But at higher metal concentrations, the active growth of

**Figure 10. Photographic evidence of morphological differences in isolates subjected to metal stresses**



mycelia gets affected by the irreversible damages caused by the metal and is usually inhibited causing colony size restriction, which would be seen in the current study. Similar results have been reported previously where mycelial growth had been restricted due to the presence of metal and total inhibition of mycelial growth occurred on further increase of metal concentration (Babich et al. 1982; Akhtar et al. 2013). Reduction in sporulation of certain isolates in response to metals (Figure 10a and 10d) would be easily correlated with the results obtained by Abboud and Alawlaqi (2011), who reported fungal isolates *Aspergillus terreus* and *Alternaria alternate*, showed reduced sporulation under elevated Cu concentrations on CDA. Under such cases, only metal tolerant isolates manage to survive, while the rest cease to grow. Other responses such as changes in the mycelial colour under metal stress (Figure 10b) would be attributed to the metal accumulation inside the mycelia. Hefnawy and Razak (1998) reported Cu-tolerant *Fusarium oxysporum*, with its mycelia turned blue in the presence of 600 ppm of Cu. Blue coloration in mycelia can be caused as a result of the accumulation of copper oxalate crystals (Zapotoczny et al. 2007) or copper binding to the cell walls (Ezzouhri et al. 2009).

On calculating MTI % for 15 selected isolates (Table 6), it was observed that MTI decreased with increasing metal concentration, with some isolates (#NIOSN-SK56-S19, #NIOSN-M98) showing elevated metal tolerance at a higher metal concentration (750 ppm) with reduced tolerance at higher concentrations. Some isolates like #NIOSN-M20, #NIOSN-M29, #NIOSN-M110 and #NIOSN-SK56-S19 also showed 100 percent MTI, as the growth in metal plate exceeded growth in control plate. Discrepancies such as the better growth of isolates at higher metal concentration than the previous screening, were also reported by Valix and Loon (2003) with respect to tolerance index being more than 1.0 when a fungal isolate was subjected to adaptive tolerance to a metal. Although it is

**Table 6. Metal Tolerance Index of 15 selected isolates showing tolerance to different metals at varying concentrations**

| Isolate numbers | Metal tolerance index in % | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Ni (II) | | | | Cu (II) | | | | Pb (II) | | | | Cd (II) | | | | Cr (VI) | | | |
| | 500 | 750 | 1000 | 1250 | 500 | 750 | 1000 | 1250 | 500 | 750 | 1000 | 1250 | 500 | 750 | 1000 | 1250 | 250 | 500 | 750 | 1000 |
| **#NIOSN-M20** | **100** | **70** | **58** | **46** | **64** | **76** | **36** | **22** | **100** | **100** | **60** | **28** | **50** | **nil** | **nil** | **nil** | **nil** | **nil** | **nil** | **nil** |
| **#NIOSN-M29** | **100** | **70** | **36** | **40** | **64** | **80** | **38** | **22** | **100** | **48** | **208** | **34** | **40** | **36** | **38** | **46** | **nil** | **nil** | **nil** | **nil** |
| #NIOSN-M38 | 34 | 43 | nil | nil | 40 | 43 | nil | nil | 46 | 71 | 100 | 31 | 34 | 43 | nil | nil | nil | nil | nil | nil |
| #NIOSN-M56 | 36 | 24 | nil | nil | nil | nil | nil | nil | 100 | 100 | 100 | 28 | 22 | nil | nil | nil | 36 | 42 | nil | nil |
| #NIOSN-M84 | nil | 84 | 34 | 48 | nil | 60 | 52 | nil | 100 | 100 | 100 | 52 | 44 | 48 | 34 | 56 | 32 | nil | nil | nil |
| #NIOSN-M86 | 90 | 44 | nil | nil | 30 | nil | nil | nil | 100 | 100 | 100 | 26 | nil | nil | nil | nil | 30 | 42 | 34 | nil |
| **#NIOSN-M98** | **43** | **57** | **68** | **45** | **75** | **81** | **57** | **28** | **96** | **106** | **100** | **47** | **34** | **32** | **36** | **51** | **nil** | **nil** | **nil** | **nil** |
| #NIOSN-M110 | 100 | 82 | 48 | 50 | nil | nil | nil | nil | 100 | 100 | nil | 52 | nil | nil | nil | nil | 42 | 42 | nil | nil |
| **#NIOSN-SK56-S19** | **88** | **100** | **58** | **42** | **nil** | **nil** | **nil** | **nil** | **100** | **100** | **60** | **34** | **30** | **nil** | **nil** | **nil** | **34** | **40** | **22** | **32** |
| #NIOSN-SK56-S43 | 78 | 72 | nil | nil | nil | 68 | 30 | nil | 100 | 100 | 100 | 44 | 24 | nil | nil | nil | 22 | nil | nil | nil |
| **#NIOSN-SK56-S52** | **94** | **90** | **60** | **36** | **nil** | **nil** | **nil** | **nil** | **100** | **100** | **100** | **56** | **32** | **nil** | **nil** | **nil** | **46** | **46** | **32** | **nil** |
| #NIOSN-SK56-S54 | 75 | 55 | 34 | nil | 34 | 53 | 30 | nil | 106 | 106 | 82 | nil | 28 | nil | nil | nil | 36 | 32 | nil | nil |
| #NIOSN-SK56-S57 | 76 | 56 | 58 | 24 | 50 | 76 | 32 | nil | 100 | 54 | 182 | 54 | 26 | nil | nil | nil | nil | nil | nil | nil |
| **#NIOSN-SK56-S76** | **53** | **58** | **49** | **42** | **27** | **53** | **31** | **nil** | **56** | **67** | **nil** | **49** | **33** | **nil** | **nil** | **nil** | **42** | **36** | **29** | **nil** |
| #NIOSN-SK56-C15 | 94 | 72 | 64 | nil | nil | nil | nil | nil | 100 | 100 | 100 | 44 | 28 | nil | nil | nil | 32 | 40 | nil | nil |

unclear why this could occur, stimulatory effects of certain metals on fungi, that have been previously reported, should not be neglected. The screening of fungal isolates to different metals led to the recognition of few potential isolates that not only tolerate high concentrations of metal but also tolerant to more than one metal. Although literature has reported isolates being able to tolerate much higher concentrations of metals than what is reported here (Coogeevaram et al. 2007; Iskandar et al. 2011; Akhtar et al. 2013), it is very important to consider the media used for metal studies per se and consequences it can have. Chromium and cadmium were found to be the most toxic heavy metals as they had the least tolerance index in screened isolates, followed by copper. Therefore in accordance with the objective, most toxic metal from each, essential (copper) and non-essential (chromium) heavy metals, were chosen for further studies. The results for Pb gave rise to conflict whether the metal was actually toxic to the organisms or was not available to act on it. It was noted that, during the metal supplemented media preparation, on the addition of Pb $(NO_3)_2$ to molten CDA, always gave rise to white precipitate giving it a cloudy appearance. On replacing the compound Pb $(NO_3)_2$ with Pb $(CH_3COO)_2$, white precipitate still persisted. Although other metals such as Cu (II), Ni (II) and Cr (VI) also gave distinct colour to the media, no precipitate was formed. The precipitate could be formed as a result of metal reaction with seawater under alkaline pH. A similar result of lead being less toxic compared to other metal ions, followed by white precipitation in agar has been reported before (Ezzhouri et al. 2009). Therefore pH becomes an important factor in deciding whether a metal is actually available to act on a living organism and cause any damage. Therefore all further experiments were carried out in DW at an acidic pH.

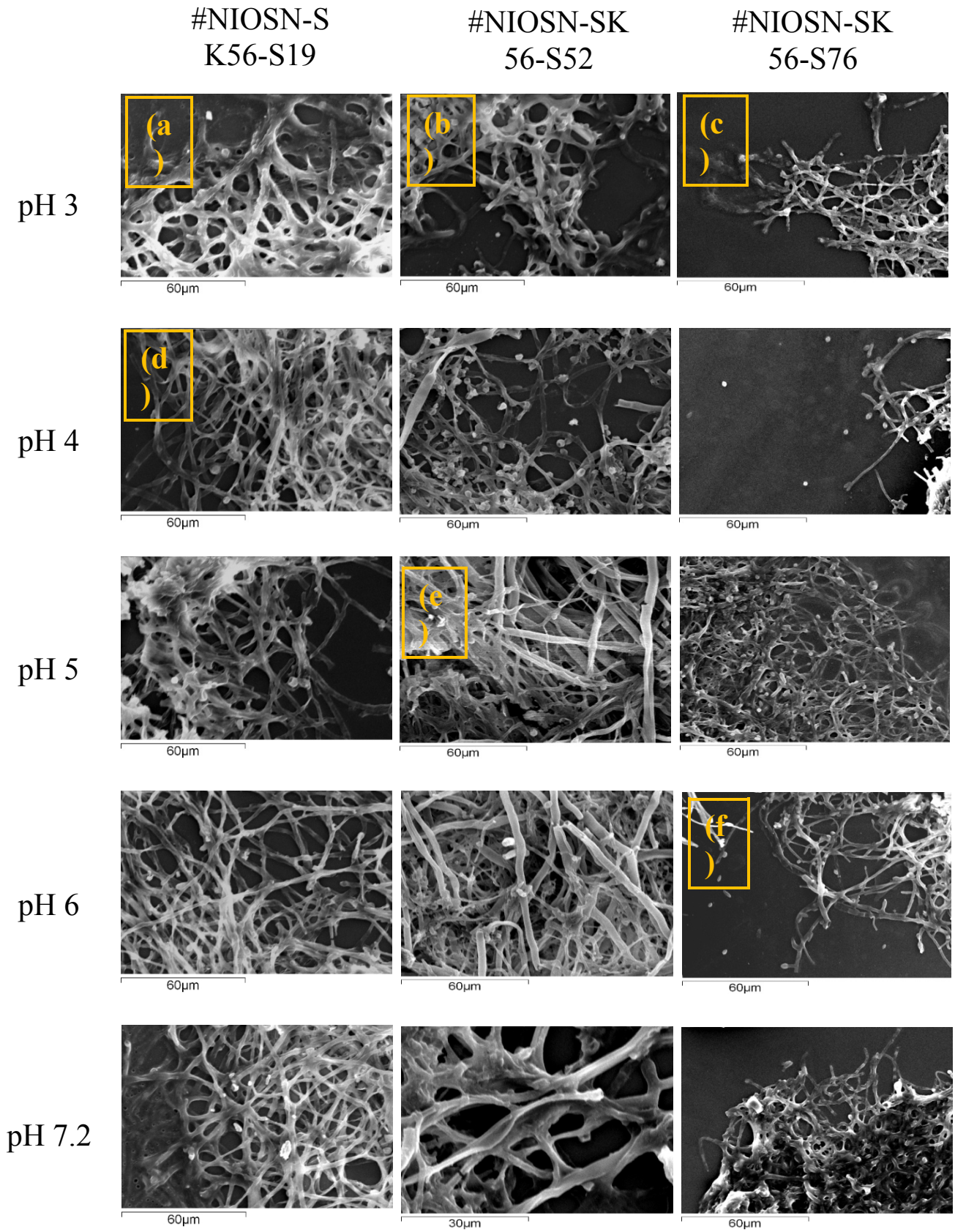
### 3.3.2 Effect of pH on fungal morphology

Effect of low pH alone, on the morphology of fungi is important to prove that the damage caused is solely due to metal stress. Acidic pH is essential in maintaining solubility of metal ions, besides, each unit decrease in pH approximately results in two-fold increase in the concentration and toxicity of metal (Giller et al. 1998). But solubility of metal is not the only concern, the growth of the test isolate under low pH alone should also be monitored as damage caused to the mycelial morphology due to extreme low pH could indicate towards the false interpretation of damage being caused by metal. Besides, at very low pH, abundant H+ ions start to compete with metal ions for binding sites (Coogeevaram et al. 2007). On growing three different fungi under varying acidic pH, it was observed that pH 3 appeared to have some deleterious effect on mycelia as seen in Figure 11 (a, b, c). While pH 4, 5 and 6 did not show much of a difference in morphology (Figure 11 d, e f) and hyphae appeared intact in comparison to hyphae present in control (pH 7.2, media pH). From this observation, it could be concluded that acidic pH pertaining to the range of 4-6, does not affect mycelial morphology, and any changes in mycelia that would occur due to response to metal in acidic pH, would solely be due to metal alone. Therefore pH of 5 was chosen for all experiments pertaining to broth studies after referring to literature (Coogeevaram et al. 2007; Borut et al. 2010; Sen, 2012).

### 3.3.3 Estimates of total metal content in biomass and residual broth

The selected fungal isolates, based on the metal tolerance screening were grown in broth supplemented with metal to study their metal removal efficiencies, morphological response and other physiological responses. All the Cr (VI) and Cu (II) tolerant isolates showed a similar trend of decreasing biomass production with an increase in metal

**Figure 11. Effect of pH on hyphal morphology**



concentration except for the isolate #NIOSN-SK56-S76, showing substantial production of biomass at all the concentrations, with increase in Cr (VI) concentration (Table 7). The inversely proportional relationship between biomass production and concentration could be attributed to the ceasing of growth or slow death due to metal stress. Isolates that showed considerable biomass production in the form of colony diameters on agar plates supplemented with metal, were severely affected in terms of biomass produced, when grown in broth (Table 7 and 8). A similar result was reported by Sandrin and Hoffman (2007), with regards to MIC (Minimum Inhibitory Concentration) for metal toxicity of several genera of bacteria being two to four-fold higher in liquid media in comparison to solid media. Agar used as solidifying agent in the media may play a role of chelator to form complexes with metals and therefore the toxicity of the metals might be reduced . Although the biomass production in all the isolates was not high, each of these isolates participated in metal removal from the broth. The total Cr content in biomass also decreased with the drop in biomass production at the elevated Cr (VI) concentrations, which suggested that the total Cr content in biomass is directly proportional to the active growth of culture and their biosorption capabilities. It was found that even minute quantities of biomass can cause the significant removal of metal from media. Isolate #NIOSN-SK56-S19 produced only 2.8 mg of biomass at 300 ppm of Cr (VI), but resulted in 26% removal of Cr content from broth with Cr removal efficiency of 0.01 mg Cr $mg^{-1}$ biomass. The same isolate with 25.1 mg biomass (50 ppm Cr VI), showed the removal of 22% with an efficiency of 0.007 mg Cr $mg^{-1}$ biomass. Isolate #NIOSN-SK56-S52 showed up to 40% removal of Cr (VI) from the broth with a Cr removal efficiency of 0.008 mg Cr $mg^{-1}$ biomass at 50 ppm. Further, isolate #NIOSN-SK56-S76 produced more biomass as compared to the other two isolates but interestingly did not show high percent removal of Cr (VI). At all Cr (VI)

concentrations, the biomass produced by this isolates exceeded 50 mg but the highest Cr removal efficiency achieved was 0.008 mg Cr $mg^{-1}$ biomass at 300 ppm where the percent removal by it was merely 14%.

In case of quantitative estimation of Cu (II) removal from the broth by Cu (II) tolerant isolates, all three isolates showed a decrease in biomass on increasing Cu (II) concentration depicting that high concentration of Cu had an adversee effect on growth (Table 8). Removal of Cu (II) by all three isolates, from broth, was seen at all the concentrations. Isolate #NIOSN-M98, when grown at 300 ppm concentration, showed up to 18% removal of Cu (II) content from broth, while isolates #NIOSN-M20 and #NIOSN-M29 showed removal up to 28% and 14% Cu content. Maximum removal of Cu (II) from the broth amounted to 32% by isolate #NIOSN-M98 at 100 ppm concentration. On acid digesting lyophilized biomass, Cu (II) content was found to be highest at 200 ppm concentration for isolate #NIOSN-M98 followed by isolate #NIOSN-M20 at 200 ppm and isolate #NIOSN-M29 at 100 ppm concentration of Cu (II) respectively. It was noted that all the isolates showed maximum biomass production at 50 ppm Cu (II) but did not show significant accumulation of Cu (II) in biomass. Isolate #NIOSN-M98 showed maximum Cu (II) removal efficiency of 0.008 mg Cu $mg^{-1}$ biomass while isolate #NIOSN-M20 and # NIOSN-M29 showed 0.006 mg Cu $mg^{-1}$ biomass and 0.007 mg Cu $mg^{-1}$ biomass efficiency respectively. Considering the experimental and instrumental error, it was observed that, on adding the metal content in broth and biomass, for both Cr (VI) as well as Cu (II) experiments, some amount of metal was left unaccounted, which could not be investigated further. In case of Cr (VI) tolerant isolates, they had better Cr removal efficiency than what has been previously reported, while Cu (II) tolerant isolates did not have significant Cu removal efficiencies as compared to terrestrial isolates.

**Table 7. Removal of Cr content from broth by the fungal cultures and Cr-assimilation on/in the mycelia**

| Isolate number | Concentration of Cr (VI) (ppm) | Absolute Cr content (mg) | Dry weight of biomass (mg) | Cr content in digested biomass (mg) | Cr content per mg of dried biomass (mg) | Residual Cr content (mg) in broth | % removal of Cr in broth |
|---|---|---|---|---|---|---|---|
| **#NIOSN-SK56-S-19 (*A. sydowii*)** | Control-0 | 0 | 141.8 | 0 | 0 | 0 | nil |
| | 50 | 1 | 25.1 | 0.186 ± 0 | 0.007 | 0.779 ± 0.011 | 22.1 |
| | 100 | 2 | 19.4 | 0.157 ± 0 | 0.008 | 1.947 ± 0 | 2.65 |
| | 200 | 4 | 1.6 | 0.007 ± 0 | 0.004 | 3.417 ± 0.023 | 14.575 |
| | 300 | 6 | 2.8 | 0.029 ± 0 | 0.01 | 4.450 ± 0.023 | 25.833 |
| **#NIOSN-SK56-S-52 (*A .terreus*)** | Control-0 | 0 | 73.2 | 0 | 0 | 0 | nil |
| | 50 | 1 | 50.4 | 0.39 ± 0 | 0.008 | 0.592 ± 0.016 | 40.8 |
| | 100 | 2 | 34.6 | 0.364 ± 0 | 0.01 | 1.975 ± 0.046 | 1.25 |
| | 200 | 4 | 6.5 | 0.053 ± 0 | 0.008 | 3.565 ± 0.023 | 10.875 |
| | 300 | 6 | 4 | 0.05 ± 0 | 0.013 | 4.598 ± 0.140 | 23.367 |
| **#NIOSN-SK56-S-76 (*P. lilacinum*)** | Control-0 | 0 | 169.1 | 0 | 0 | 0 | nil |
| | 50 | 1 | 76.6 | 0.15 ± 0 | 0.002 | 0.751± 0.032 | 24.9 |
| | 100 | 2 | 80.7 | 0.28 ± 0 | 0.004 | 2.134 ± 0.040 | error |
| | 200 | 4 | 50.6 | 0.264 ± 0.1 | 0.005 | 3.286 ± 0.040 | 17.85 |
| | 300 | 6 | 63.6 | 0.523 ± 0 | 0.008 | 5.154 ± 0.161 | 14.1 |

**Table 8. Removal of copper content from the broth by the fungal cultures and Cu-assimilation on/in the mycelia**

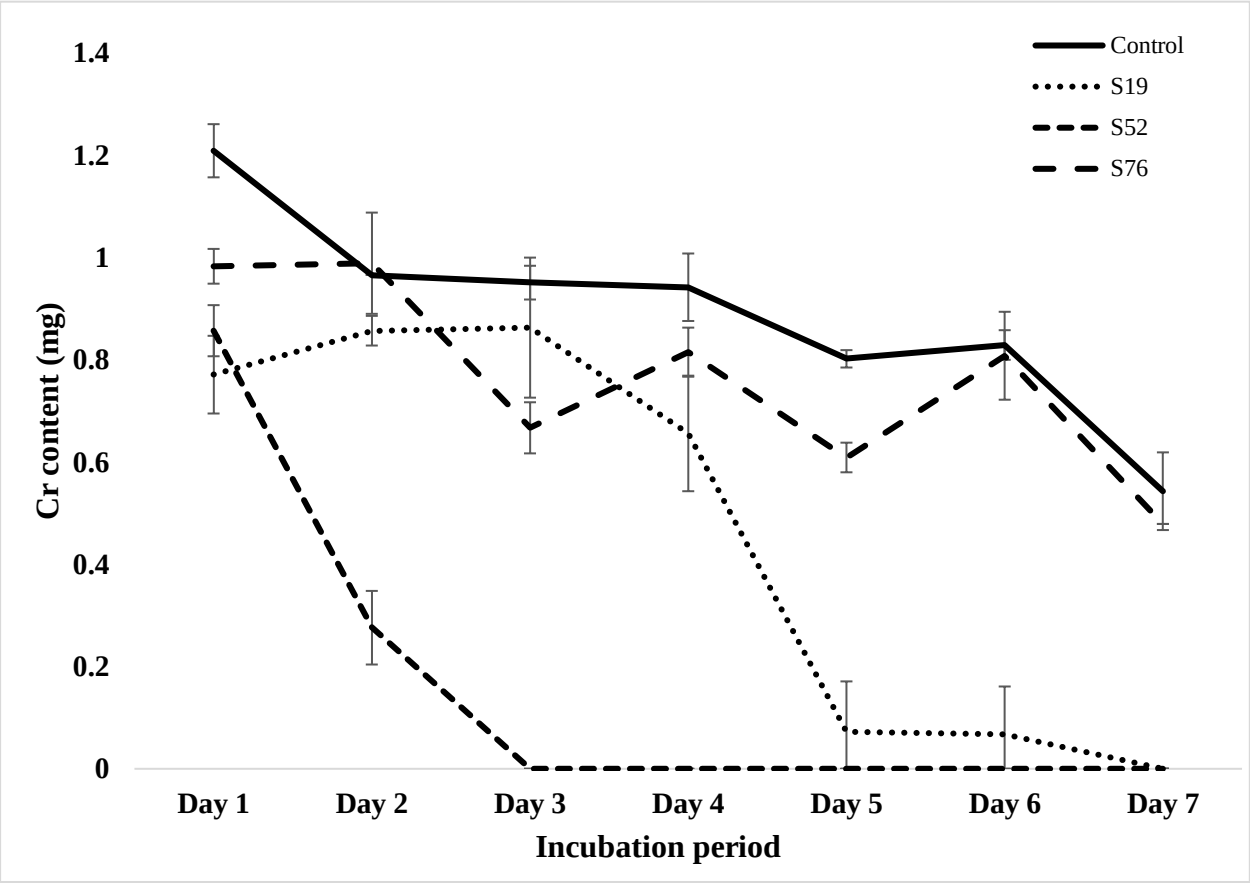
| Isolate number | Concentration of Cu (II) in ppm | Absolute Cu content (mg) | Dry weight of biomass (mg) | Cu content in digested biomass (mg) | Cu content per mg of dried biomass (mg) | Residual Cu content (mg) in broth | % removal of Cu in broth |
|---|---|---|---|---|---|---|---|
| **#NIOSN-M20 (*P. chrysogenum*)** | Control-0 | 0 | 153.66 | 0.024 ± 0.001 | 0.000 | 0.048 ± 0 | error |
| | 50 | 1 | 124.95 | 0.108 ± 0.001 | 0.001 | 0.958 ± 0.005 | 4.20 |
| | 100 | 2 | 101.32 | 0.121 ± 0.001 | 0.001 | 1.458 ± 0.001 | 27.10 |
| | 200 | 4 | 58.65 | 0.224 ± 0.001 | 0.004 | 2.843 ± 0.001 | 28.91 |
| | 300 | 6 | 11.81 | 0.067 ± 0.001 | 0.006 | 4.312 ± 0.001 | 28.14 |
| **#NIOSN-M29 (*P. chrysogenum*)** | Control-0 | 0 | 132.68 | 0.024 ± 0.001 | 0.000 | 0.048 ± 0 | error |
| | 50 | 1 | 98.46 | 0.102 ± 0.001 | 0.001 | 0.911 ± 0.005 | 8.93 |
| | 100 | 2 | 32.17 | 0.198 ± 0.001 | 0.006 | 1.551 ± 0.003 | 22.46 |
| | 200 | 4 | 13.06 | 0.089 ± 0.001 | 0.007 | 2.892 ± 0 | 27.70 |
| | 300 | 6 | 8.55 | 0.052 ± 0.001 | 0.006 | 5.136 ± 0.001 | 14.40 |
| **#NIOSN-M98 (*P. chrysogenum*)** | Control-0 | 0 | 97.59 | 0.024 ± 0 | 0.000 | 0.048 ± 0 | error |
| | 50 | 1 | 76.03 | 0.174 ± 0.003 | 0.002 | 0.904 ± 0.005 | 9.56 |
| | 100 | 2 | 61.08 | 0.29 ± 0 | 0.005 | 1.365 ± 0 | 31.73 |
| | 200 | 4 | 37.17 | 0.304 ± 0.001 | 0.008 | 2.8 ± 0.001 | 30.01 |
| | 300 | 6 | 17.39 | 0.132 ± 0.001 | 0.008 | 4.926 ± 0.006 | 17.89 |

Metal removal by the selected isolates from broth, irrespective of being Cr (VI)/ Cu (II), indicated towards survival strategy by these isolates. One of the most widely reported and most likely mechanism to occur is biosorption, which is a physical process and occurs in both live as well as dead biomass. Fungal cell walls contain huge proportions of oxygen-rich functional groups (carboxyl, hydroxyl) within cell wall components such as chitin, melanin and associated phenolic compounds, which easily adsorb metal cations (White et al. 1995; Akthar et al. 1996; Baldrian and Gabriel, 2003; Tan and Cheng, 2003). Further acidic pH gives rise to anionic species of Cr (VI) such as $Cr_2O_7^{2-}$, $HCrO^{4-}$ and $Cr_2O_4^{2-}$ that get attracted to $H^+$ ion coated fungal mycelia facilitating Cr adsorption on cell walls (Hawley, 2004; Coogeevaram et al. 2007; Sen, 2012).

### 3.3.4 Estimation of Cr (VI) reduction rate in chromium tolerant fungi

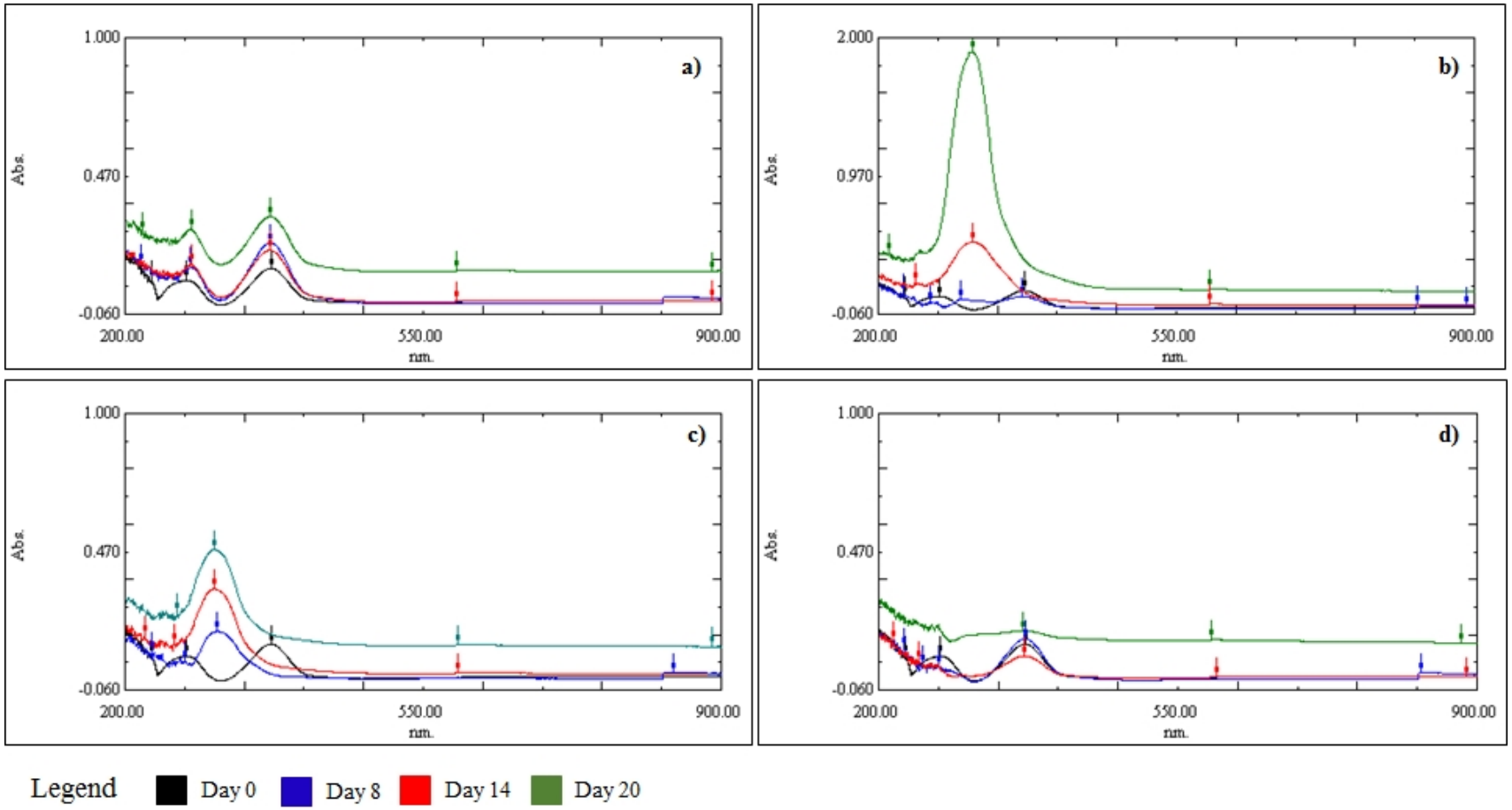
On determining the Cr (VI) reduction rate using DPC, it was observed that isolate #NIOSN-SK56-S52 was the most efficient (Figure 12) and reduced the Cr (VI) state to Cr (III) in a period of three days. Isolate #NIOSN-SK56-S19 reduced the same in 7 days. Reduction of Cr (VI) to Cr (III) can also occur due to chemical reaction and may not be due to the biological reaction. But, in comparison to the chemical reduction rate of Cr (VI) in control flask (without culture), the conversion of Cr (VI) to Cr (III) was not significant, and therefore chemical reduction of Cr (VI) was ruled out. This has been the first report to state that fungi also could cause direct reduction of chromium and to be detected by this method as opposed to the wide literature available on bacteria (Faisal and Haisan, 2004; Zahoor and Rehman, 2009). Conversion of Cr (VI) state to an undetectable form was inferred when a decrease in Cr (VI) concentration occurred over an incubation period of a week till no more Cr (VI) could be measured. Biological

**Figure 12. Determination of Cr (VI) reduction using Diphenylcarbazide (DPC) method.**



reduction of Cr in various living systems has been previously reported where two electrons participate in initial reduction stage of $Cr^{+6}$ to $Cr^{+4}$, which then gets further reduced to $Cr^{+3}$ in the second step (Borut et al. 2010). Alternatively, during the reduction of $Cr^{+6}$ to $Cr^{+3}$, $Cr^{+5}$ may be produced as an intermediate in a two-step reaction, which is highly unstable and generates toxic free radicals (Wang and Shen, 1995). Surprisingly, isolate # NIOSN-SK56-S76 did not participate significantly in Cr (VI) reduction, indicating the presence of an alternate mechanism for survival. To investigate the possible production of nanoparticles by the isolates as a strategy to overcome metal stress, filtered broth samples were subjected to UV-VIS spectroscopy. It was observed that an absorption peak at 370 nm was found in the control flask (no culture) at all days (Figure 13a) indicating no nanoparticle formation due to chemical redox reactions. Both the isolates (#NIOSN-SK56-S19, Figure 13b and #NIOSN-SK56-S52, Figure 13c) belonging to the *Aspergillus* genus, showed a shift in absorption peak from 390 to 310 nm. Isolate #NIOSN-SK56-S52, showed a gradual increase in peak intensity while #NIOSN-SK56-S19 exhibited a rapid increase in intensity by the $20^{th}$ day of incubation. The shift in the absorption peaks indicated towards change in oxidation state of Cr due to biological reduction (Figure 13b and 13c). Absorption peaks at 300 nm attribute to Cr nanoparticles and have been reported previously (Chandra and Kumar, 2013; Annamalai et al. 2014a, 2014b). In contrast to this, Mohite and his colleagues (2009) had reported that inter-band transitions of core electrons within chromium and chromium oxide gave an absorption peak at 445 nm that indicated towards the presence of $Cr_2O_3$ nanoparticles, while Jaswal and his colleagues (2014) reported $Cr_2O_3$ nanoparticles give absorption peak at 460 nm. The shift in absorption peak as well as its intensity could a result of highly oxidizing nature of Cr (VI), which

**Figure 13. Absorption spectra of cell-free culture broth obtained during growth of fungal isolates subjected to chromium using UV-VIS spectrophotometry.**

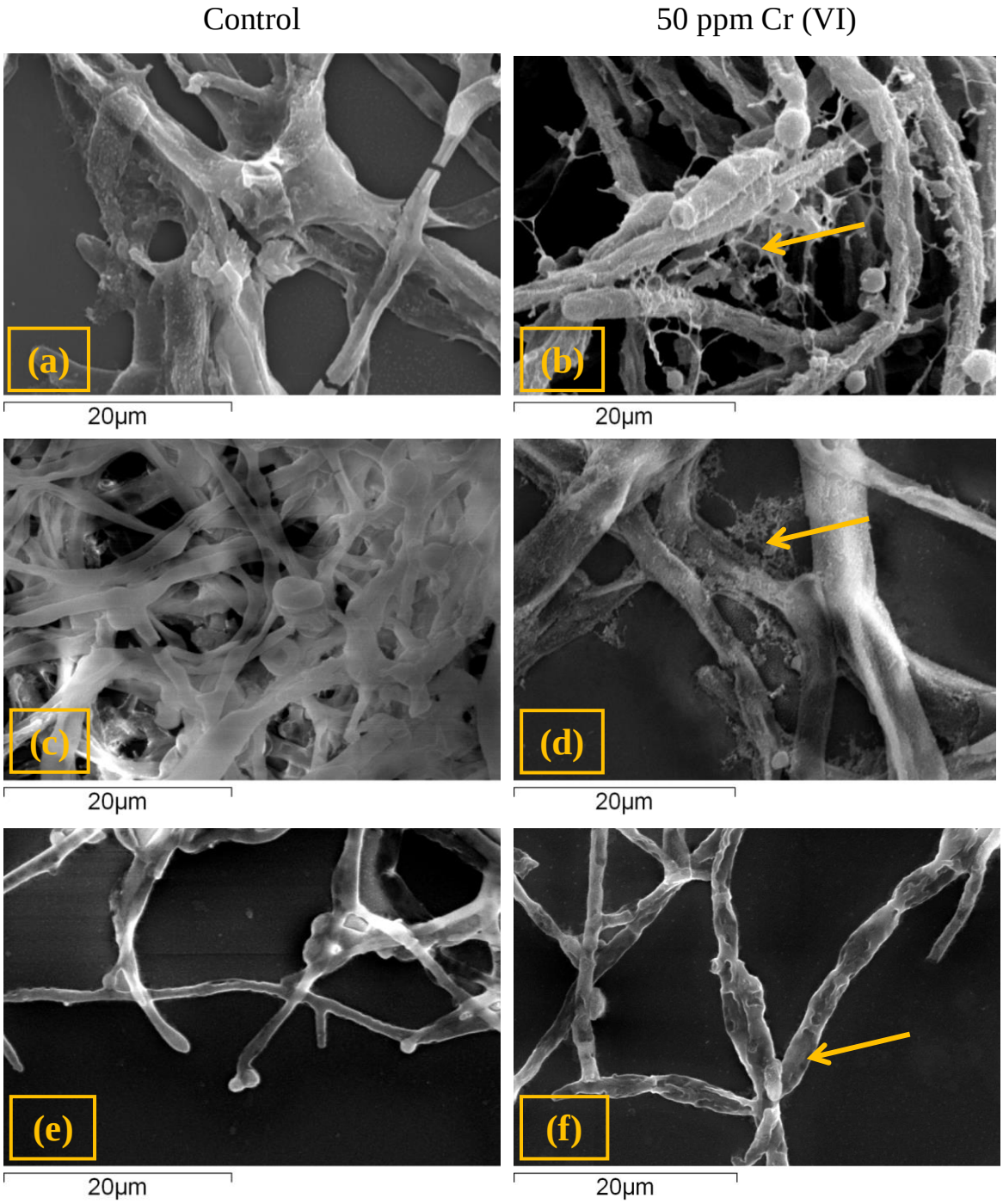


can easily get transformed into Cr (III) in the presence of organic matter (Focardi et al. 2013). Although this experiment could not support Cr nanoparticle production, it was evident now that these isolates had more than one metal tolerance mechanisms and therefore were able to survive in high concentrations of metals. As expected isolate #NIOSN-SK56-S76 (Figure 13d) did not display a shift in absorption peaks and was comparable to the control.

### 3.3.5 Effect of metal stress on fungal morphology

The response of marine-derived fungi to metal stress was studied based on the morphological appearance of hyphae using SEM. It was observed that isolate #NIOSN-SK56-S19 produced extracellular thread-like substances (Figure 14b) under Cr (VI) stress, which were otherwise absent under the control condition (absence of metal) (Figure 14a). Similarly, extracellular aggregation that appeared like fine mesh in between the mycelial network was seen in isolate #NIOSN-SK56-S52 (Figure 14d) under Cr (VI) stress which focused on an important point that such aggregation could be the sites for biosorption in these cultures. In addition to chitin and melanin, production of extracellular material mainly comprising of organic acids (oxalic acid, citric acid), or EPS (exopolysaccharides) are responsible for metal sorption (Hall, 2002; Green and Clausen, 2003; Bellion et al. 2006; Liu et al. 2006). The production of thread-like substances by isolate #NIOSN-SK56-S19 and extracellular aggregates by isolate #NIOSN-SK56-S52, with response to Cr (VI), led to the inference that biosorption of metals on cell walls, which is a generally occurring phenomenon, might not be the only mechanism for tolerance. The extracellular material produced by these cultures might be responsible in trapping of toxic metal ions so that less/no harm is further caused to the organism. Interestingly, isolate #NIOSN-SK56-S76, that efficiently produced more

**Figure 14. Effect of Cr (VI) on hyphal morphology of fungal isolates using SEM.**



biomass under Cr (VI) stress (Table 7), did not produce any extracellular material on its surface. SEM image indicated towards (Figure 14f) stressed mycelia which appeared damaged with deflation and pores caused due to Cr stress, while the mycelia in control were found to be intact (Figure 14e). Isolate #NIOSN-M20 was observed to show stressed and deflated mycelia in the presence of Cu (II) (Figure 15b), while the same showed healthy mycelia (Figure 15a) under control (without Cu). In contrast to the previous isolate, isolate #NIOSN-M29 showed absolutely no change in the appearance of mycelia in presence/absence of Cu (II) (Figure 15c and Figure 15d). While in case of isolate #NIOSN-M98, mycelia appeared to have got damaged in the presence of Cu (II) (Figure 15e), while in the absence of Cu (II), mycelia appeared to be long and slender as normally found (Figure 15f). Presence of pores and deformed mycelia was also reported by Subbaiah and Yun (2013) where mycelia were used as biosorbent in an aqueous solution of nickel. At acidic pH, metals are soluble, more toxic and available in free ionic states (Gadd and Griffiths, 1977). The reactions of such redox-active metal ions cause oxidative stress to cellular components and are known to cause lipid peroxidation leading to the formation of pores and disrupting membrane integrity as discussed earlier. Samples processed for SEM analysis were also used to conduct EDS analysis to understand the oxidation state of respective metal present within the biomass. In the case Cr (VI) tolerate fungi, the mycelia contained Cr (III) (Figure 16) which was evident from the compound $Cr_2O_3$ detected in biomass, in contrast to Cr (VI) state (chromium stock solution was $K_2Cr_2O_7$ which gives anionic species of oxidation state +6 under acidic condition) that was added initially. This further pointed towards the ability of Cr (VI) tolerant isolates to convert Cr from +6 to +3 state via a biological process indicating presence of multiple metal tolerance mechanisms.

**Figure 15. Effect of Cu (II) on hyphal morphology of fungal isolates using SEM.**

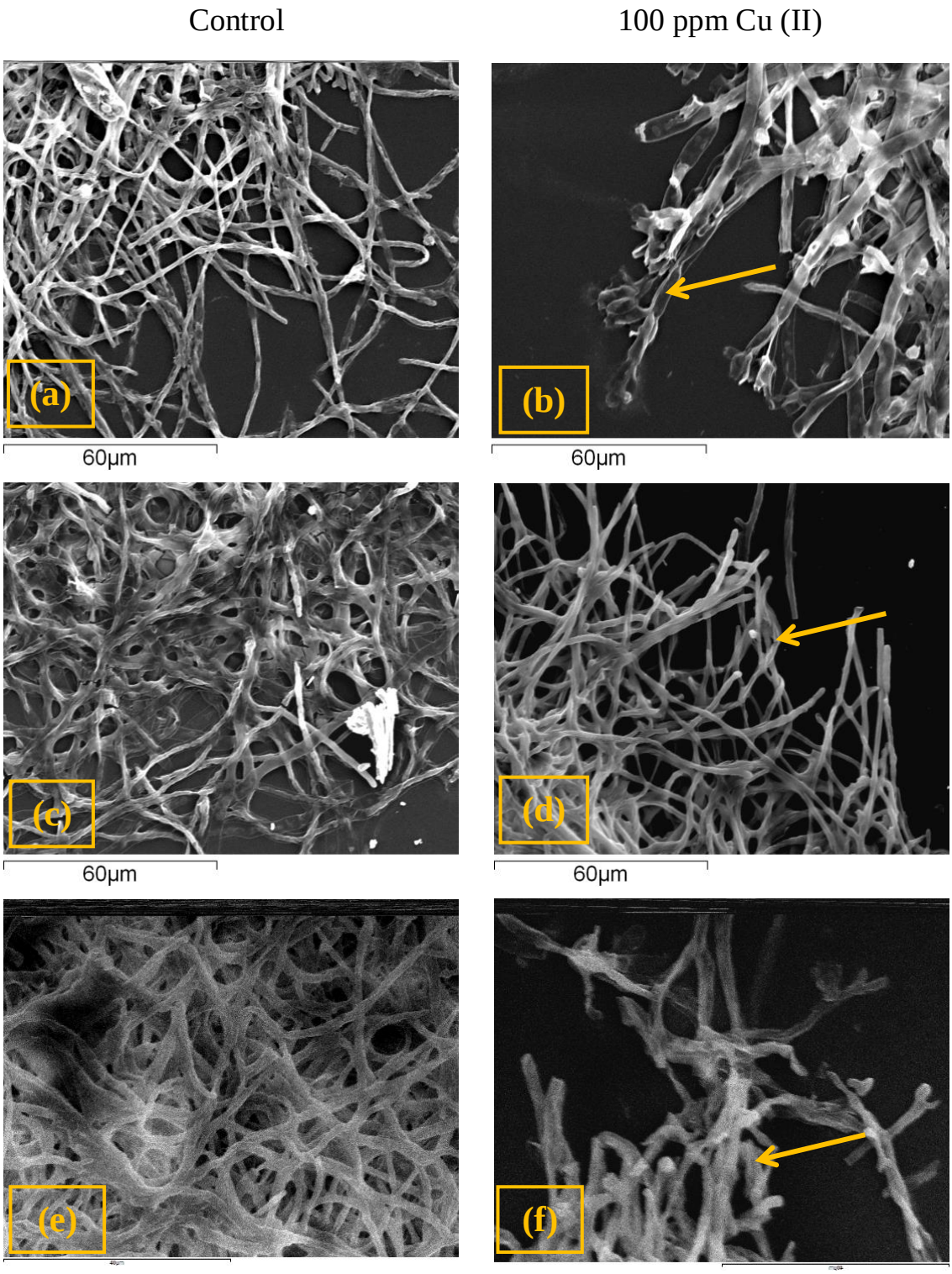
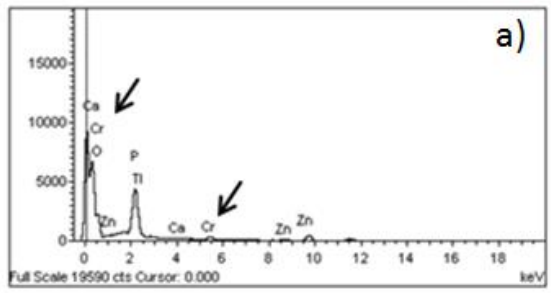
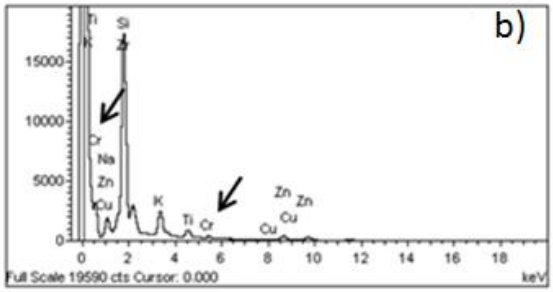


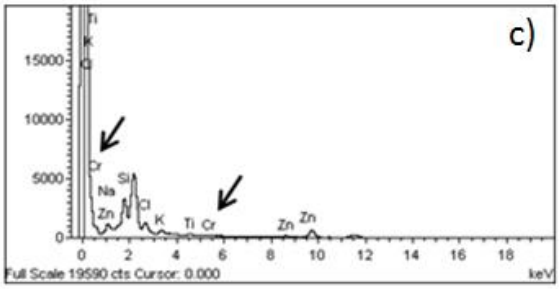
**Figure 16. EDS spectra of fungal isolates grown at 300 ppm of Cr (VI) showing presence of $Cr_2O_3$.**




| Element | Weight% | Atomic% | Compound% | Formula |
|---|---|---|---|---|
| P K | 15.11 | 15.30 | 34.63 | P2O5 |
| Ca K | 2.08 | 1.63 | 2.91 | CaO |
| Cr K | 16.57 | 9.99 | 24.22 | Cr2O3 |
| Zn K | 14.28 | 6.85 | 17.77 | ZnO |
| Tl M | 19.70 | 3.02 | 20.48 | Tl2O |
| O | 32.26 | 63.21 | | |
| Totals | 100.00 | | | |




| Element | Weight% | Atomic% | Compound% | Formula |
|---|---|---|---|---|
| Na K | 7.86 | 7.51 | 10.59 | Na2O |
| Si K | 31.79 | 24.89 | 68.01 | SiO2 |
| K K | 5.84 | 3.28 | 7.03 | K2O |
| Ti K | 2.51 | 1.15 | 4.19 | TiO2 |
| Cr K | 0.79 | 0.33 | 1.16 | Cr2O3 |
| Cu K | 0.02 | 0.01 | 0.02 | CuO |
| Zn K | 4.76 | 1.60 | 5.92 | ZnO |
| Zr L | 2.28 | 0.55 | 3.08 | ZrO2 |
| O | 44.16 | 60.68 | | |
| Totals | 100.00 | | | |




| Element | Weight% | Atomic% | Compound% | Formula |
|---|---|---|---|---|
| Na K | 12.74 | 12.99 | 17.17 | Na2O |
| Si K | 23.06 | 19.25 | 49.33 | SiO2 |
| Cl K | 11.13 | 7.36 | 0.00 | |
| K K | 5.38 | 3.22 | 6.48 | K2O |
| Ti K | 1.81 | 0.89 | 3.02 | TiO2 |
| Cr K | 1.80 | 0.81 | 2.63 | Cr2O3 |
| Zn K | 8.23 | 2.95 | 10.24 | ZnO |
| O | 35.86 | 52.54 | | |
| Totals | 100.00 | | | |

The fungal mycelia subjected to copper stress showed presence of only oxidation state +2. It is to be noted that Cu (I) and Cu (II) are in constant transition and does not reflect to a reduction in oxidation state. Therefore the EDS results for this experiment cannot be held significant.

One of the most interesting responses in this entire objective was that of isolate #NIOSN-SK56-S76 towards chromium. This particular isolate did not show any production of extracellular material to claim biosorption, nor did it participate in Cr (VI) reduction, as inferred from results obtained from the DPC method and UV-Vis spectrophotometry analysis. Yet this isolate was found to contain $Cr_2O_3$ inside its biomass which was detected in EDS analysis of the biomass sample. It was further surprising to note that the same isolate was able to produce maximum biomass under Cr (VI) stress in broth but did not participate in the significant removal of Cr (VI) from the same. Therefore the immediate question that arises is, how does this isolate manage to grow exceptionally well in metal stress? Further, all the isolates have been subjected to metal stress in broth prepared with DW. Since these isolates are marine-derived, how would they respond if another stress factor of varying salinity was introduced along with metal? To understand and find answers to these questions, mere physiological response was not sufficient. Apart from the cellular response, molecular response, especially study of proteins involved and the ones getting affected would shed more light on the survival strategies of these isolates.

# Chapter FOUR

# Proteomic Approach to elucidate Mechanisms involved in metal tolerance

### 4.1 Proteomics for the study of heavy metal tolerance in marine fungi

Heavy metals in higher concentrations are known to pose a significant threat to the human health as well as environment. Proteomic analysis attempts to obtain functional information regarding response of various microbial communities to heavy metals (Lacerda et al. 2007), and the same has been achieved here. Due to the structural and functional similarity between various genes in mammals and lower eukaryotes, cellular response of such toxic metals in lower organisms will provide a better understanding of mechanisms in human cells (Bae and Chen, 2004). Besides, modern proteomics tools, provide accurate and comprehensive information of the expressed proteins in an organism under stress at transcriptional as well as translational levels. Despite the estuarine occurrence of all the isolates in the current study, mechanisms of salt tolerance are seldom studied. Literature reports active efflux of cations and accumulation of osmolytes as widely documented mechanisms for salt tolerance in many organisms (Hernandez-Saavedra et al. 1995; Porcel et al. 2012; Ruppel et al. 2013), while cation diffusion facilitators (CDF), resistance nodulation cell division (RND) driven export and P-type ATPases have been reported previously to exclude divalent metal ions from the cell (Thilakaraj et al. 2007). Environment doesn't always exert a single type of stress, but comprises of multiple stresses acting at the same time. One such example would be tannery wastewaters, which are loaded with high concentrations of heavy metals as well as salts. Under such conditions, studying response of organisms subjected to multiple stresses becomes mandatory. Keeping this into consideration, the third objective attempts to study response of marine-derived fungi to individual metal and salinity stress as well as a combination of both. Such a study would give a better insight into the mechanisms adapted by lower eukaryotes to evade deleterious effects of stress.

**4.2 Materials & methods (Objective THREE:** To study protein profiles of selected fungal isolates with respect to salinity, temperature and heavy metal stresses.**)**

**4.2.1 Growth of selected isolates under varying salinities and temperatures**

Selected six isolates were grown in Erlenmeyer flasks (100 mL) in 20 mL of CDB (DW) and incubated at room temperature (28°C) till the onset of sporulation (3 days). Sterile glass beads were used to crush and break the newly developed mycelia into smaller bits and aliquots (1 mL) of this suspension were inoculated in CDB (20 mL) containing varying salinities (0, 35, 50, 100 and 250 PSU). The inoculated flasks were incubated at 28°C for a week under shaking condition (80 rpm). Another set of 100 mL Erlenmeyer flasks with 20 mL CDB (DW) was inoculated with the selected isolates separately and incubated at 20, 28 and 40°C under shaker condition, to study their growth in terms of biomass production at varying temperatures. After incubation, the contents were filtered through 0.45 µm Whatman filter and biomass was lyophilized prior to protein extraction.

**4.2.2 Protein extraction and sample processing**

Four isolates were selected for studying protein profiles under salinity, metal and temperature stress. All three Cr (VI) tolerant isolates (#NIOSN-SK56-S19, #NIOSN-SK56-S52, #NIOSN-SK56-S76) and one Cu (II) tolerant isolate (#NIOSN-M20) were grown till onset of sporulation in CDB (D/W) (20 mL) and broken to bits using sterile beads. Aliquots (1 mL) of this crushed mycelial suspension were inoculated into CDB prepared with varying salinities (0, 35 and 100 PSU), each comprising of varying Cr (VI)/ Cu (II) concentrations (0, 100 and 500 ppm) respectively and incubated at 80 rpm at 28°C for a week. After incubation, biomass from the flasks was harvested by filtration and lyophilized prior to weighing and protein extraction. To determine initial

biomass added, aliquots of crushed mycelial suspension were also added to pre-weighed tubes and lyophilized. To study protein profiling under temperature stress, aliquots of crushed mycelial suspension were inoculated in CDB (DW) and incubated at varying temperatures (20°C, 30°C, 40°C) for a week at 80 rpm shaking condition. The biomass were filtered from broth and lyophilized prior to protein extraction.

Approximately 30 mg of lyophilized biomass was taken from all conditions (nine) per culture for protein extraction using a previously standardized three buffer system (Krishnaswamy et al. 2018). To explain briefly, the biomass was suspended in buffer I (500 µL of) comprising of Tris HCl, CHAPS, $MgCl_2$ and dithiothreitol (DTT) and homogenized twice at 6.5 m/s for 60 seconds (FastPrep, MP Biomedicals, USA) using bead bashing tubes containing zirconia. The homogenized suspension was centrifuged at 14000 rpm (at 4°C), and the supernatant was collected in clean 1.5 mL tube and stored in ice. The homogenised biomass was further suspended in buffer II (500 µL) comprising of urea, CHAPS and DTT and once again homogenized. The suspension was centrifuged similarly and the resultant supernatant was collected in the same tube used. The sample tube was homogenized for the last time with 500 µL of buffer III containing urea, thiourea, CHAPS and DTT and centrifuged. The supernatant was mixed well with the previously collected supernatants in the tube. Protease inhibitor cocktail (5 µL) was added at all the homogenization steps to prevent degradation of proteins, apart from carrying out the entire procedure in ice. Pooled supernatants were thoroughly mixed and quantified using Folin Lowry method (Lowry et al. 1951). On quantification, 250 ng of protein was used to carry out SDS-PAGE on a 12% resolving gel with 6% stacking gel. The samples were electrophoresed at 90 V for two hours. The gel was stained using silver staining technique (Heukeshoven and Dernick, 1985) to check the quality of proteins before processing them further for MS analysis.

### 4.2.3 Tryptic digestion and LC-MS separation

The quality of protein sample was assessed based on visual appearance of protein bands on SDS gels and the sample was further processed for In-solution digestion using trypsin (modified from Kinter and Sherman, 2000). Prior to sample digestion, 100 µL of sample was precipitated using HPLC grade methanol which was then suspended in 6 M urea to dissolve the proteins. These proteins were reduced using dithiothreitol (DTT) and further alkylated using Iodoacetamide (IAA) before digesting them with trypsin. The tryptic digests (100 µL) were transferred into MS vials and analyzed for mass spectrometry using in- house LCMS QToF facility (6538 UHD Accurate Mass QTOF LC/MS, Agilent Technologies, USA). Eight µL of the digested samples in quadruplets were injected through auto-sampler using the 150 II 300A C18 150 mm column protein chip. The LC separations were carried out using aqueous phase (deionized water, 0.22 µm filtered and autoclaved) and an organic phase (HPLC grade acetonitrile) across a gradient (3–97%) using formic acid (0.1%) as an adduct in both the phases over 100 min. A range of 50 to 2000 m/z in positive mode was maintained for MS/MS of separated ions, at 2 ppm accuracy. The spectral data after MS/MS was acquired with the help of Mass Hunter software ver 5.0 (Agilent Technologies, USA) and was searched against the organism-specific database (#NIOSN-SK56-S52: NCBI txid 33178; #NIOSN-SK56-S76: NCBI txid 33203; #NIOSN-M20: NCBI txid 5076) in NCBI using Spectrum Mill MS Proteomics Workbench ver. B.04.01.141 (Agilent Technologies, USA) at an accuracy of 50–100 ppm. In case of isolate #NIOSN-SK56-S19, since species specific database was not available, the MS/MS data was searched against genus specific database and hence does not have a taxonomic ID. The MS searches were then auto-validated at a false discovery rate (FDR) of 1.2%, followed by generation of protein summary files and MPP files. The MPP files generated in Spectrum Mill were

processed in Mass Profiler Professional ver.13 (MPP13, Agilent Technologies, USA) for further analysis. Since this is a high throughput technique, samples were run as technical replicates as opposed to biological replicates and were grouped on the basis of salinities and metal concentrations.

### 4.3 Results and discussion

The marine-derived fungi used in this study could be proficiently placed in the category of being 'Halotolerant' due to their ability to survive in 0 PSU as well as high salt condition of 100 PSU. Halotolerant fungi have been known to adjust and grow under varied salinities. All six isolates, (Cu/Cr tolerant), showed substantial growth at 100 PSU salinity (Table 9). In fact, showed better biomass production at higher salinities than what they were isolated from. On subjecting them to temperature variation, it was observed that these fungi preferred 30°C or close to room temperature (28°C) to produce maximum biomass (Table 10). The only exception was that of isolate #NIOSN-SK56-S19, which had maximum biomass production at 40°C, but was not significant enough from the biomass produced at 30°C, hence did not grab the attention. Although metabolic rate increases with increase in temperature, too high temperature can also be detrimental to growth. This was observed with the drastic drop in biomass production at 40°C. Since the Cu-tolerant fungal isolates did not show much variation in biomass production nor in experiments conducted before, only one isolate (#NIOSN-M20) was selected for further analysis.

**Table 9. Biomass production by marine-derived fungi subjected to varying salinities**

| CDB prepared in | Dry weight of biomass (g) | | | | | |
|---|---|---|---|---|---|---|
| | **S19** | **S52** | **S76** | **M20** | **M29** | **M98** |
| **Distilled water** | 0.172± 0.007 | 0.178± 0.011 | 0.175± 0.006 | 0.147± 0.015 | 0.103± 0.001 | 0.114± 0.013 |
| **Seawater** | 0.183± 0.042 | 0.22± 0.009 | 0.229± 0.009 | 0.192± 0.019 | 0.195± 0.008 | 0.183± 0.010 |
| **50 PSU** | 0.248± 0.007 | 0.231± 0.002 | 0.225± 0.015 | **0.245± 0.025** | 0.219± 0.028 | 0.228± 0.018 |
| **100 PSU** | **0.275± 0.004** | **0.267± 0.060** | **0.236± 0.045** | 0.238± 0.022 | **0.245± 0.010** | **0.243± 0.003** |
| **250 PSU** | 0.006± 0.0 | 0.008± 0.002 | 0.001± 0.0 | 0.005± 0.003 | 0.005± 0.0 | 0.004± 0.003 |

**Table 10. Biomass production by marine-derived fungi subjected to varying temperatures**

| | Dry weight of biomass (g) | | | | | |
|---|---|---|---|---|---|---|
| **Temperature** | **S19** | **S52** | **S76** | **M20** | **M29** | **M98** |
| **20°C** | 0.099± 0.028 | 0.117± 0.024 | 0.012± 0.002 | 0.182± 0.027 | 0.045± 0.004 | 0.217± 0.022 |
| **30°C** | 0.282± 0.005 | **0.266± 0.009** | **0.227± 0.005** | **0.246± 0.011** | **0.224± 0.016** | **0.237± 0.001** |
| **40°C** | **0.296± 0.031** | 0.252± 0.008 | No growth | 0.173± 0.055 | No growth | 0.002± 0.0 |

Protein profiling of selected isolates subjected to salinity and metal [Cr (VI) and Cu (II)] stresses, led to the identification of 4185, 659, 1412 and 919 expressed proteins across all nine conditions (0, 35 and 100 PSU at of 0, 100 and 500 ppm metal concentrations each) in isolates #NIOSN-SK56-S19, #NIOSN-SK56-S52, #NIOSN-SK56-S76 and #NIOSN-M20, respectively (Table 11), as a result of cellular response to salinity and metal. All the detected proteins could be divided into 17 categories (Figure 17) on the basis of their functional processes based on KEGG pathway sand metabolic processes. Majority of the proteins belonged to basic cellular functional processes such as carbohydrate metabolism, amino acid metabolism, cellular processes, environmental information processing, genetic information processing, etc. It is important to note that a significant amount of proteins in isolates #NIOSN-SK56-S19 (39% - Figure 17a), #NIOSN-SK56-S52 (32% - Figure 17b), #NIOSN-SK56-S76 (16% - Figure 17c) and #NIOSN-M20 (17% - Figure 17d) belonged to hypothetical proteins whose functions are currently unknown and may also be involved in subjected stress tolerance. Amongst all the proteins that were expressed by the isolates, 69 proteins in #NIOSN-SK56-S19, 53 proteins in #NIOSN-SK56-S52, 105 proteins in #NIOSN-SK56-S76 and 55 proteins in #NIOSN-M20, were commonly expressed in all the conditions, while a number of unique proteins were also found in each condition per isolate (Table 11). On combining the commonly expressed proteins amongst all the isolates, it was observed that most of these proteins belonged to carbohydrate metabolism (isocitrate dehydrogenase, pyruvate decarboxylases, phosphoglycerate kinase, transaldolases); transcription/translation proteins (elongation factors, ribosomal proteins, Ran GTPase); amino acid metabolism (aspartate aminotransferase, glutamine synthetase, adenosyl homocysteinase); Glutathione metabolism (glutathione reductase, ascorbate peroxidase, peroxiredoxins); proteins involved in oxidative phosphorylation (cytochrome c oxidases, ATP synthases)

**Table 11. Number of proteins expressed by marine-derived fungi subjected to varying salinities and metal stresses**

| Isolates | | NIOSN-SK56-S19 | | | | NIOSN-SK56-S52 | | | | NIOSN-SK56-S76 | | | | NIOSN-M20 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Conditions | | Number of proteins | | | | | | | | | | | | | | | |
| Salinity (PSU) | Metal conc in ppm (Cr/Cu) | A | B | C | D | A | B | C | D | A | B | C | D | A | B | C | D |
| 0 | 0 | | 915 | | 183 | | 168 | | 7 | | 453 | | 68 | | 282 | | 32 |
| | 100 | | 877 | | 151 | | 231 | | 6 | | 449 | | 48 | | 397 | | 37 |
| | 500 | | 509 | | 89 | | 212 | | 12 | | 454 | | 51 | | 105 | | 9 |
| 35 | 0 | | 771 | | 144 | | 159 | | 10 | | 426 | | 63 | | 418 | | 46 |
| | 100 | **4185** | 841 | **69** | 144 | **659** | 281 | **53** | 7 | **1412** | 380 | **105** | 32 | **919** | 275 | **55** | 9 |
| | 500 | | 826 | | 143 | | 297 | | 14 | | 544 | | 80 | | 409 | | 40 |
| 100 | 0 | | 921 | | 162 | | 216 | | 13 | | 426 | | 56 | | 312 | | 21 |
| | 100 | | 854 | | 123 | | 298 | | 14 | | 483 | | 49 | | 313 | | 26 |
| | 500 | | 842 | | 158 | | 334 | | 33 | | 41 | | 6 | | 352 | | 24 |

Legend:

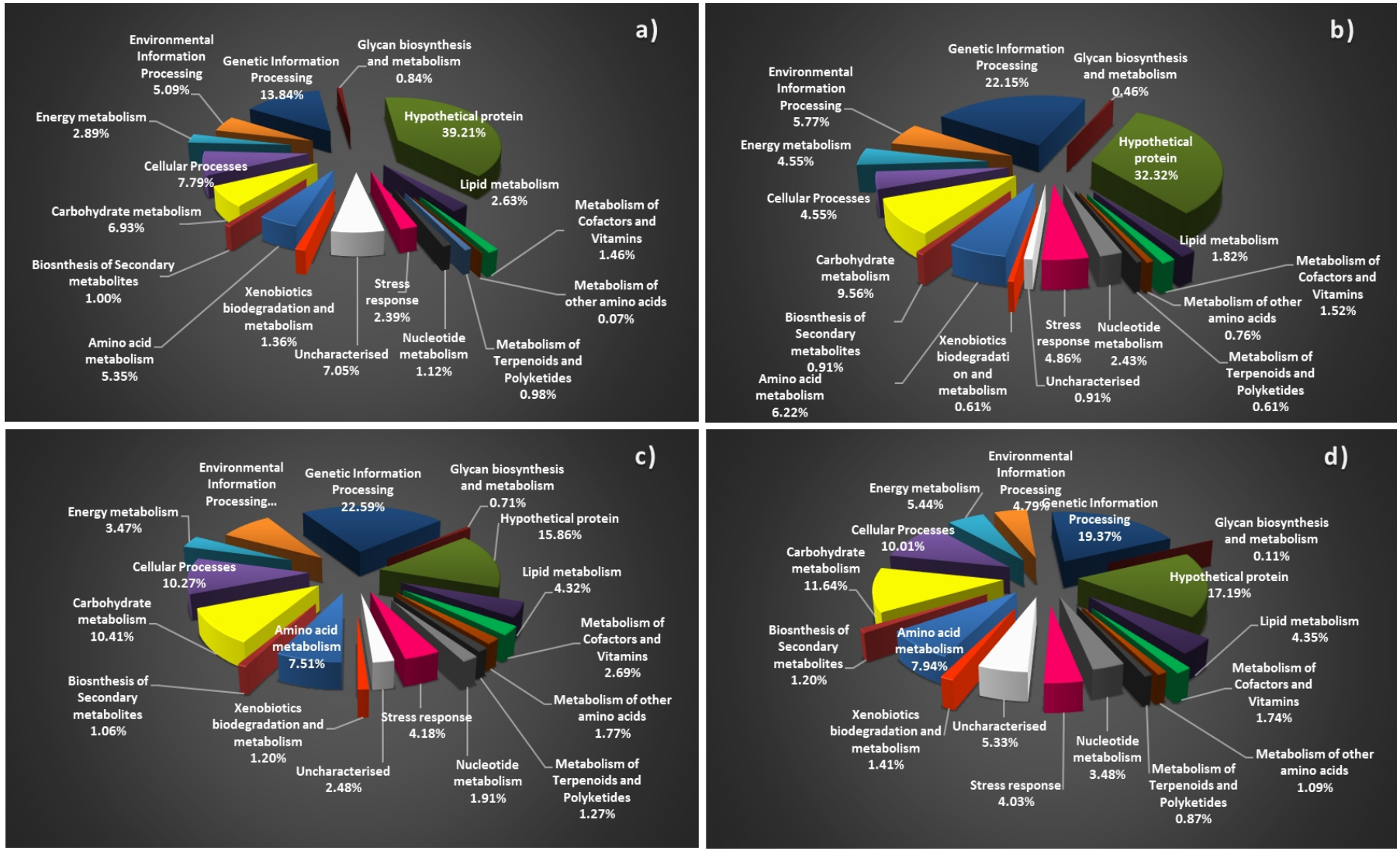
A: Total number of proteins

B: Number of proteins expressed in one condition

C: Number of proteins commonly expressed in all conditions

D: Number of unique proteins expressed in one condition

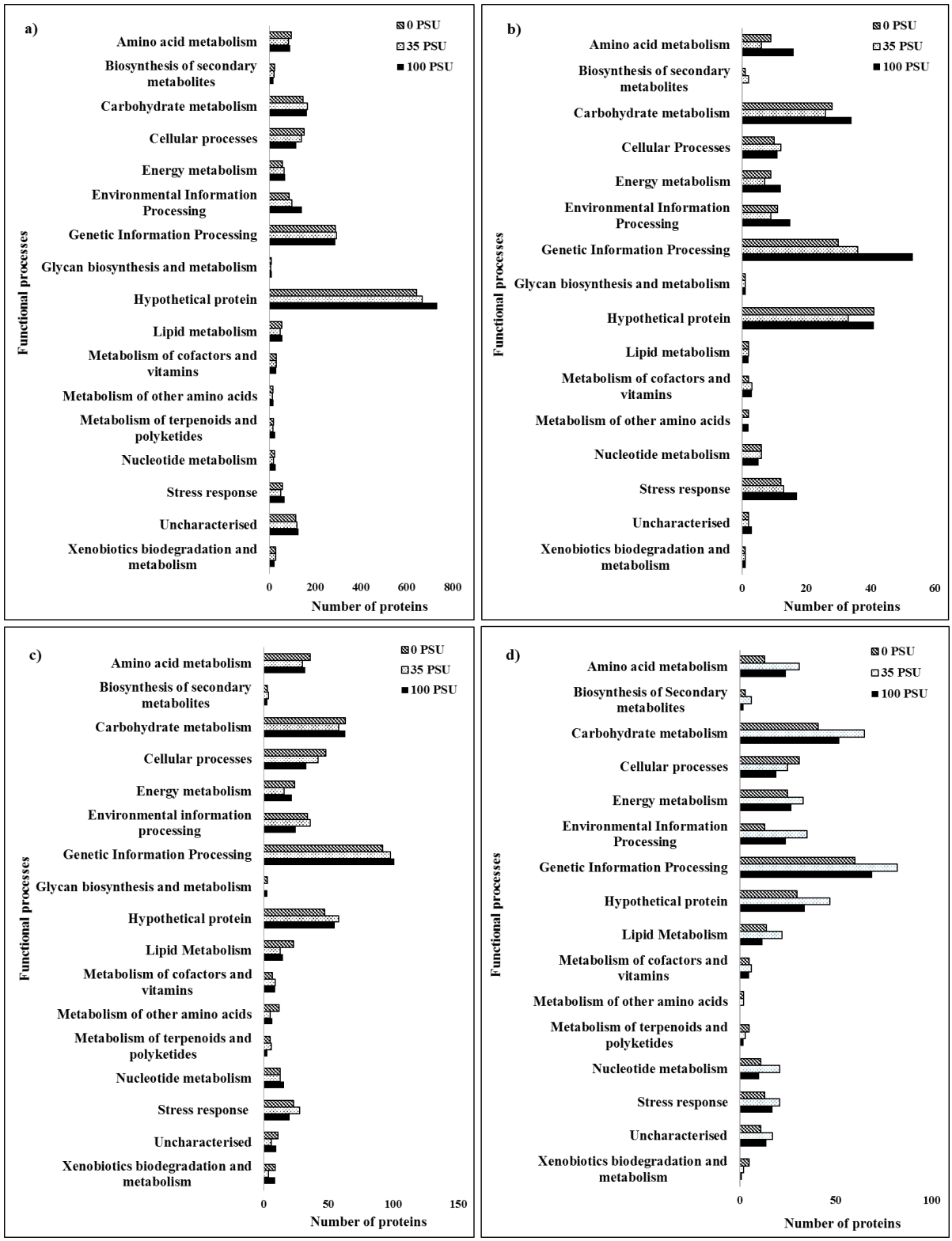
**Figure 17. Graphical representation of functional distribution of all proteins acquired under all condition for four selected isolates, S19 (a), S52 (b), S76 (c) and M20 (d).**



and also commonly occurring stress response proteins (heat shock proteins, peroxidases). To understand whether salinity alone caused any variability in protein profiles, proteins produced by each isolate across varying salinities (0, 35 and 100 PSU) were compared against each salinity (Figure 18). It was observed the number of proteins detected did not vary much across varying salinities for isolates #NIOSN-SK56-S19 and #NIOSN-SK56-S76, while noticeable variation in protein expression was observed in isolates #NIOSN-SK56-S52 and #NIOSN-M20 when subjected to different salinities. Isolate #NIOSN-SK56-S52 showed maximum biomass production at 100 PSU salinity (Table 9) which also coincided with the maximized protein expression at 100 PSU as compared to other salinities (Figure 18b). The maximum number of proteins in all the isolates belonged to the category of genetic information processing which involves processes such as DNA replication, repair, transcription, translation along with folding and sorting of proteins. Proteins involved in Glycolysis, TCA cycle, Pentose shunt, pyruvate, and sugar metabolism were equally expressed under 0, 35 and 100 PSU salinities for three isolates leaving out isolate #NIOSN-M20 where proteins involved in carbohydrate metabolism were found to be maximum at 35 PSU, followed by 100 PSU and then 0 PSU. *Penicillium chrysogenum*, was found to produce maximum biomass at 50 PSU salinity (Table 9) followed by 100 PSU. This result also coincided with the observation that this isolate showed more protein expression at 35 PSU followed by 100 PSU (Figure 18d).

Accumulation of intracellular solutes has been the preliminary response of fungi grown in presence of salts in order to generate positive turgor pressure and maintain osmotic balance (Gadd et al. 1984). Some of the frequently reported osmo-protective compounds in fungi are polyols (glycerol), sugars (trehalose, mannitol, erythritol, dulcitol, ribitol, inositol), amino acids (glutamine, proline, glycine), amino acid

**Figure 18. Graphical representation of functional distribution of proteins acquired under varying salinity for four selected isolates, S19 (a), S52 (b), S76 (c) and M20 (d).**



derivatives (peptides, N-acetylated amino acid) and other organic solutes (betaine, ectoines) (Ravishanker et al. 2006; Abu-Seidah, 2007; Porcel et al. 2012; Ravindran et al. 2012). In the current study, the fungal isolates subjected to salinity stress led to the expression of Phosphatidyl glycerol specific phospholipase, polyamine transporter (#NIOSN-SK56-S19, Table 12), glucose-regulated protein precursor, glycerol kinase, phosphatidyl inositol phospholipase C which are involved in inositol metabolic process and sugar1, 4- lactone oxidase proteins (#NIOSN-SK56-S76, Table 14), in the presence of 35 and 100 PSU salinity which possibly play a role in osmoregulation. In addition to this, MAPK (Mitogen-Activated Protein Kinase) signaling protein, proteins involved in transport such as $Mg^{+2}$ transporter protein, vacuolar import and degradation protein 27 and ABC transporters, were also expressed in #NIOSN-SK56-S76. Fungal cells have been reported previously to exclude $Na^{+2}$ ions out of their cells for osmotic adjustment along with accumulation of osmolytes and activation of HOG (High Osmolarity Glycerol) and MAPK pathways under elevated osmotic stress (Abu-Seidah, 2007, Nikolauo et al. 2009). ABC transporters which are known to have multiple functions, one of which is transport of xenobiotic compounds across cell membranes (Eyckmans et al. 2012), have been reported in cotton roots in response to salinity stress (Li et al. 2015). Oxidative stress caused as a result of high salt concentration, leads to the production of ROS (Reactive Oxygen Species) which then activates the assembly of antioxidant enzymes and free radical scavengers.

Enzymes such as catalase, superoxide dismutase, peroxidase participate in detoxification of $H_2O_2$ and $O^{2-}$ radicals, while oxidized antioxidants such as glutathione (GSH), glutathione reductase (GR) and ascorbate help in elimination of $H_2O_2$ from cellular compartments (Porcel et al. 2012, Ravindran et al. 2012). Apart from these, other enzymes such as Glutathione oxidoreductase Glr1 (Table 12), thioredoxin, monothiol

glutaredoxin-4 (Table 13), peroxiredoxin-5 and glutaredoxin (Table 15), were also expressed in the current study. Proteins involved in DNA/ Protein damage control, such as, heat shock proteins, DNA repair proteins, DNA mismatch repair proteins, were expressed by these cultures under salinity stress. Further, a number of autophagy and cell senescence proteins, besides stress/DNA repair proteins, were expressed by #NIOSN-SK56-S52 and #NIOSN-SK56-S76 under salt stress. In short, proteins involved osmoregulation, combating oxidative stress and repair were expressed by all isolates under salinity stress.

Under metal stress, it was observed that besides ROS scavenging proteins and repair mechanisms, these isolates produced metal chelating proteins with a number of proteins involved in transport as well as proteins involved in enzymatic reduction of metals, specially the chromium tolerant ones (Table 12, 13, 14 and 15). On comparing all four isolates and each of the nine conditions, it was observed that in isolates, #NIOSN-SK56-S19, # NIOSN-SK56-S52 and #NIOSN-M20, the least number of proteins were found under 500 ppm concentration of metal [Cr (VI) / Cu (II)] at 0 PSU salt concentration with an exception of isolate #NIOSN-SK56-S76 (Table 11). Although the isolate, #NIOSN-SK56-S76 has been found to grow remarkably well at 100 PSU, the decline in proteins expressed could be ascribed to stress caused by Cr (VI) at 500 ppm. Interestingly, at the same concentration of Cr (VI) under 35 PSU salinity, the protein numbers were not affected in comparison to presence/absence of salinity or Cr (VI) stress condition. This indicated that high salt concentrations along with metal stress can have a synergistic toxic effect as inhibitors for growth. On the contrary, the three isolates that showed substantial amount of biomass at 100 PSU, demonstrated close to equivalent expression of proteins in both conditions of 100 PSU, with/ without metal which could not be explained.

**Table 12. Proteins expressed by #NIOSN-SK56-S19 (*A. sydowii*) subjected to varying stresses**

| Type of Stress | Mechanism involved | Proteins expressed |
|---|---|---|
| **Salinity** | DNA/ Protein Damage Control | DNA mismatch repair protein (Mlh3) |
| | | DNA mismatch repair protein Msh1 |
| | | Damage-specific DNA binding protein |
| | | Heat shock protein/chaperonin HSP78 |
| | ROS Scavenging | Glutathione oxidoreductase Glr1 |
| | | Superoxide dismutase |
| | | Catalase |
| | Osmoregulation | Phosphatidylglycerol specific phospholipase |
| | | Polyamine transporter |
| | | Glycerol Uptake protein 1 |
| **Chromium** | Metal chelation | 2Fe-2S iron-sulfur cluster binding domain protein |
| | | Heavy metal tolerance protein precursor |
| | Enzymatic Reduction | Cytochrome c oxidase polypeptide IV |
| | | Cytochrome c oxidase polypeptide VI |
| | | Cytochrome P450 |
| | ROS Scavenging | Superoxide dismutase |
| | | Flavodoxin and radical SAM domain protein |
| | Transport/ metal efflux | ABC drug exporter AbcA |
| | | ABC drug exporter AtrF |
| | | Cation transporter |
| | | Efflux pump antibiotic resistance protein |
| | | MFS transporter (Seo1) |
| | | MATE efflux family protein |
| **Salinity + Chromium** | DNA/ Protein Damage Control | DNA repair protein Pso2/Snm1 |
| | | DNA mismatch repair protein msh3 |
| | | DNA mismatch repair protein msh-2 |
| | | DNA damage inducible protein |
| | ROS Scavenging | Zinc-binding oxidoreductase ToxD |
| | | Glutathione peroxidase Hyr1 |
| | Transport/ metal efflux | Zinc-regulated transporter 1 |
| | | Putative HC-toxin efflux carrier TOXA |
| | | MFS multidrug transporter |
| | | Golgi transport complex component Cog5 |

**Table 13. Proteins expressed by #NIOSN-SK56-S52 (*A. terreus*) subjected to varying stresses**

| Type of Stress | Mechanism involved | Proteins expressed |
|---|---|---|
| **Salinity** | DNA/ Protein Damage Control | Heat shock 70 kDa protein<br>DNA damage checkpoint protein rad24 |
| | Early Senescence | WD-repeat protein mip1 |
| | ROS Scavenging | Catalase B precursor<br>Thioredoxin<br>Superoxide dismutase<br>Monothiol glutaredoxin-4 |
| **Chromium** | DNA/ Protein Damage Control | DNA damage checkpoint protein rad24<br>Heat shock 30 kDa protein<br>Heat shock 70 kDa protein<br>Heat shock protein 82 |
| | Enzymatic Reduction | Glutathione reductase<br>Coproporphyrinogen III oxidase<br>Cytochrome c peroxidase<br>Cytochrome P450 monooxygenase |
| | ROS Scavenging | Thioredoxin<br>Catalase B precursor<br>Peroxiredoxin PRX1<br>Superoxide dismutase |
| | Transport/ metal efflux | ABC transporter CDR4<br>High-affinity nickel transport protein |
| **Salinity + Chromium** | DNA/ Protein Damage Control | Heat shock protein 60<br>Heat shock protein SSC1<br>DNA damage checkpoint protein rad24<br>Coproporphyrinogen III oxidase<br>Cytochrome c oxidase |
| | Enzymatic Reduction | Cytochrome c oxidase polypeptide VIb<br>Cytochrome c peroxidase<br>Peroxiredoxin PRX1<br>Glutathione peroxidase |
| | ROS Scavenging | Glutathione reductase<br>Catalase B precursor<br>Superoxide dismutase<br>ABC transporter CDR4<br>Calcium-transporting ATPase 1 |
| | Transport/ metal efflux | High-affinity nickel transport protein |

**Table 14. Proteins expressed by #NIOSN-SK56-S76 (*P. lilacinum*) subjected to varying stresses**

| Type of Stress | Mechanism involved | Proteins expressed |
|---|---|---|
| **Salinity** | Osmoregulation | Glucose-regulated protein precursor<br>Glycerol kinase<br>Phosphatidylinositol phospholipase C<br>Sugar 1, 4- lactone oxidase proteins |
| | ROS Scavenging | Superoxide dismutase<br>Catalase<br>Peroxidase<br>Glutathione (GSH)<br>Glutathione reductase (GR) |
| | Transport/ metal efflux | MAPK signaling protein<br>$Mg^{+2}$ transporter protein<br>ABC transporters |
| **Chromium** | DNA/ Protein Damage Control | Hsp90 co-chaperone<br>Heat shock proteins |
| | Enzymatic Reduction | Cytochrome c oxidase polypeptide Vib<br>Flavin reductase FMN-binding protein |
| | ROS Scavenging | Superoxide dismutase<br>Catalase<br>Peroxidase<br>Glutathione (GSH) |
| | Transport | PX domain<br>Tubulin-specific chaperone c<br>Protein EFR3<br>Calcium-proton exchanger |
| **Salinity + Chromium** | DNA/ Protein Damage Control | DNA mismatch repair proteins<br>Universal stress proteins<br>Heat shock proteins |
| | Metal Chelation | Pyoverdine/dityrosine biosynthesis protein |
| | Transport/ metal efflux | F1F0- ATP synthase regulatory factor Stf2<br>GDP/GTP exchange factor Sec2p<br>Siderochrome- iron transporter |

**Table 15. Proteins expressed by #NIOSN-M20 (*P. chrysogenum*) subjected to varying stresses**

| Type of Stress | Mechanism involved | Proteins expressed |
|---|---|---|
| **Salinity** | DNA/ Protein Damage Control | DNA repair and recombination protein |
| | | DNA mismatch repair protein |
| | | Heat shock 70 kDa protein |
| | ROS Scavenging | Catalase B |
| | | Peroxiredoxin-5 |
| | | Glutathione reductase |
| | | Glutaredoxin |
| | | Superoxide dismutase |
| | | Mitochondrial peroxiredoxins |
| | | Thioredoxin |
| **Copper** | DNA/ Protein Damage Control | Heat shock 70 kDa protein |
| | | Hsp90 co-chaperone |
| | Metal Chelation | Glutathione S-transferase |
| | | Calcium-binding mitochondrial carrier protein Aralar2 |
| | ROS Scavenging | Catalase B |
| | | Peroxiredoxin-5 |
| | | Glutaredoxin |
| | | Superoxide dismutase |
| | | Thioredoxin |
| **Salinity + Copper** | DNA/ Protein Damage Control | UV excision repair protein |
| | | DNA mismatch repair protein |
| | | Heat shock 70 kDa protein |
| | Osmoregulation | Polyol transporter |
| | ROS Scavenging | Glutaredoxin |
| | | Superoxide dismutase |
| | | Mitochondrial peroxiredoxins |
| | Transport/ metal efflux | Putative copper-transporting ATPase |
| | | Zinc transporter |

Since metals cannot be synthesized nor degraded, their homeostasis can be achieved via transport in/out of the cell, chelation and sequestration (Clemens, 2001; Chandrangsu et al. 2017). Production of molecular chaperones and heat shock proteins/stress proteins has been one of the most widely reported responses towards heavy metal stresses. Chaperones demonstrate the primary function of prevention of incongruous protein aggregation and degradation of mis-folded or damaged proteins (Eyckmans et al. 2012; Morris et al. 2013), besides delivering metal ions to organelles and metal requiring proteins (Clemens, 2001). Heat shock proteins such as HSP70 and HSP90 group are an evolutionarily conserved set of proteins that assist in many processes such as protein folding, transport, maturation and degradation (Hildebrandt et al. 2007; Morris et al. 2013), hence have been considered as useful universal biomarkers for stress. The current study detected the expression of metal chelating proteins by isolate #NIOSN-SK56-S76, such as pyoverdine/tyrosine biosynthesis protein along with F1F0-ATP synthase regulatory factor Stf2 involved in the transport of $H^+$ ions and GDP/GTP exchange factor Sec2p involved in vesicular transport, at 500 ppm Cr (VI) under 35 PSU salinity. Similarly, 2Fe-2S iron-sulfur cluster binding domain protein was expressed by isolate #NIOSN-SK56-S19, at 100 ppm Cr (VI) under 0 PSU salinity, while isolate #NIOSN-M20 expressed glutathione S-transferase at 100 ppm of copper at 0 PSU salinity. Metal chelators have been previously reported in literature where a transition metal ion chelator, picolinic acid, (Kapoor, 1986), hemopexin, free radicals scavenger glycoprotein (Eyckmans et al. 2012), glomalin, an insoluble glycoprotein that binds to toxic heavy metals (Hildebrandt et al. 2007), have been expressed in the presence of metals. Apart from these, metallothioneins and phytochelatins which have metal chelating abilities, decrease metal toxicity by using Cys-thiol groups, thus forming stable metal–organic complexes (Kawakami et al. 2006; Zhang et al. 2009). The

production of metal chelators by these isolates, coupled with production of various transporter proteins, directs towards the mechanism of chelation of metal and then transporting it to vacuoles. This could also be achieved by enzymatically reducing metals into non-toxic or lesser toxic forms and transporting them to the vacuoles. In bacterial cells, the enzyme systems participating in electron transport chain, such as cytochrome P-450, complex I and IV systems, are involved in chromium reduction (Casadevall and Kortenkamp, 2002). Puzon and his collegues (2002) characterized reduction of chromium by flavin reductases in *E.coli*. They reported that the end product of this reaction was soluble Cr (III)-$NAD^+$ complex instead of an insoluble precipitate. Following up with the work, it was further reported that in bacterial enzyme systems, when NADH is used as a reductant, soluble Cr (III)-$NAD^+$ complex is formed while cytochrome c leads to the formation of Cr (III) adducts (Puzon et al. 2005). Therefore, chromium reduction in presence of cellular organic metabolites could lead to formation of both, soluble and insoluble chromium complexes. The final step of heavy metal detoxification is sequestering of the metal ions or metal complexes into cell vacuoles which are usually mediated by P-type ATPases, ABC transporters and tonoplast-bound cation/proton exchangers (Hall, 2002; Hossain and Komatsu, 2013).

On studying the protein expression and its regulation across various conditions in all four isolates, it was observed that amongst the commonly up-regulated proteins, proteins such as peroxidases, peroxiredoxins which are involved in free radical scavenging, showed significant fold change values. ROS and its subsequent products formed during cascading reactions have been reported to be counteracted by both non-enzymatic (tocopherols, ascorbic acid, GSH, phenolics) and enzymatic (catalase, superoxide dismutase, guaiacol peroxidases) antioxidants (Anjum et al. 2014; Zhou et al. 2017). Enzymes responsible for direct removal of ROS e.g., glutathione peroxidase, glutathione

reductase as well as glutathione dehydrogenase have been expressed at 100 and 500 ppm Cr (VI) by #NIOSN-SK56-S76, under varying salinities. Peroxiredoxin (Prx) which is an important antioxidant and plays multiple roles such as detoxification of hydroperoxides, cell signaling, enzyme activation and redox sensing (Hossain and Komatsu, 2013), was found to be up-regulated in the presence of 100 and 500 ppm of Cr (VI) under 0 and 35 PSU salinity (Table 18). A similar trend was seen with respect to S-(hydroxymethyl) glutathione dehydrogenase, isocitrate dehydrogenase, RNP domain protein, ATP synthase subunit 4, in this isolate. Isolates #NIOSN-SK56-S19 and #NIOSN-SK56-S52 showed up-regulation of heat shock proteins as well as cytochromes in presence of Cr (VI), (Tables 16, 17), while isolate #NIOSN-M20 expressed up-regulation of ribosomal proteins under Cu (II) stress (Table 19). Different types of stresses hamper the normal metabolic functioning of the organism. Oxidative stress can often affect carbohydrate metabolism, amino acid metabolism and also affect pathways in oxidative phosphorylation. Fructose-bisphosphate aldolase involved in carbohydrate metabolism was one such protein that was down-regulated in all Cr (VI) tolerant isolates at both Cr (VI) concentrations across varying salinities.

On the basis on expression of down-regulated proteins in all isolates, it was fair to state that metal stress along with salinity affected carbohydrate metabolism by down-regulating proteins such as ATP citrate synthase, glucose-6-phosphate isomerase, phosphoglycerate kinase, which are involved in important pathways such as glycolysis, TCA cycle and pentose phosphate shunt. Replacement of three important glycolytic enzymes by sulphur depleted isozymes was reported in *S. cerevisiae* which allowed channelling of sulphur containing amino acids towards the synthesis of glutathione, which is a known metal chelator (Yin et al. 2009).

**Table 16. Regulation of proteins under Chromium stress for #NIOSN-SK56-S19 (*A. sydowii*).**

| | Up-regulated proteins | | | Down-regulated proteins | | |
|---|---|---|---|---|---|---|
| **Condition** | **Protein expressed** | **FC[a]** | **FC[b]** | **Protein expressed** | **FC[a]** | **FC[b]** |
| **0 PSU** | 1,3-beta-glucanosyltransferase Gel1 | 15.79 | 14.77 | Cytochrome P450 | -2.87 | -2.43 |
| | 60S ribosomal protein L10a | 16.75 | 16.01 | **Fructose-bisphosphate aldolase** | -0.11 | -3.15 |
| | ATP synthase Delta/Epsilon chain beta-sandwich domain protein | 16.88 | 15.04 | Transaldolase | -1.29 | -3.62 |
| | Dihydrosphingosine 1-phosphate phosphatase | 17.76 | 17.38 | **Triosephosphate isomerise** | -1.88 | -3.65 |
| | **Inosine-5'-monophosphate dehydrogenase** | 15.84 | 15.54 | S-adenosylmethionine synthetase | -1.29 | -3.69 |
| **5 PSU** | **Heat shock protein 60** | 1.83 | 5.43 | WD40 repeat protein | -1.49 | -1.62 |
| | **Cytochrome P450** | 1.87 | 2.71 | Probable ATP-citrate synthase subunit 2 | -0.35 | -1.53 |
| | **Heat shock protein SSC1** | 0.78 | 2.18 | **UDP-glucose 4-epimerase** | -1.13 | -2.6 |
| | Sulfate adenylyltransferase | 1.1 | 1.79 | Woronin body major protein | -0.7 | -1.37 |
| | D-3-phosphoglycerate dehydrogenase 2 | 1.5 | 3.03 | **Pyruvate kinase** | -0.33 | -1.5 |
| | ATP synthase gamma chain | 1.9 | 3.11 | Glutamine synthetase | -0.22 | -1.17 |
| **100 PSU** | **Heat shock protein 60** | 1.26 | 2.77 | **Aldehyde dehydrogenase** | -2.93 | -5.13 |
| | **Heat shock protein Hsp88** | 1.73 | 1.14 | **Transketolase 1** | -1.54 | -2.11 |
| | NmrA-like family protein | 5.6 | 4.76 | **Triosephosphate isomerise** | -1.54 | -1.95 |
| | Dihydrolipoyl dehydrogenase | 1.59 | 2.37 | Outer mitochondrial membrane protein porin | -3.74 | -4.49 |
| | Elongation factor Tu | 2.13 | 2.87 | Protoplast secreted protein 2 precursor | -2.95 | -4.6 |

Legend:

FC[a] : Fold change values for proteins expressed under 100 ppm Cr VI concentration in comparison to 0 ppm Cr VI concentration (control)
FC[b] : Fold change values for proteins expressed under 500 ppm Cr VI concentration in comparison to 0 ppm Cr VI concentration (control)

**Table 17. Regulation of proteins under Chromium stress for #NIOSN-SK56-S52 (*A. terreus*).**

| | Up-regulated proteins | | | Down-regulated proteins | | |
|---|---|---|---|---|---|---|
| **Condition** | **Protein expressed** | **FC[a]** | **FC[b]** | **Protein expressed** | **FC[a]** | **FC[b]** |
| **0 PSU** | 40S ribosomal protein S5 | 2.77 | 0.74 | Adenosyl homocysteinase | -2.5 | -1.26 |
| | S-adenosylmethionine synthetase | 2.13 | 3.61 | **Alcohol dehydrogenase I** | -1.26 | -2.19 |
| | **Thioredoxin** | 1.05 | 1.37 | **Enolase** | -0.77 | -1.17 |
| | Dihydrolipoyllysine-residue acetyltransferase | 2.72 | 1.1 | **Phosphoglycerate kinase** | -0.88 | -1.69 |
| | Ketol-acid reductoisomerase | 1.38 | 0.46 | **Pyruvate decarboxylase** | -1.08 | -1.86 |
| **35 PSU** | 40S ribosomal protein S0 | 2.51 | 3.05 | Catalase B precursor | -0.74 | -1.27 |
| | 40S ribosomal protein S12 | 1.12 | 2.26 | hypothetical protein ATEG_07892 | -2.96 | -0.71 |
| | **Heat shock 70 kDa protein** | 1.58 | 1.97 | hypothetical protein ATEG_02219 | -1.69 | -0.24 |
| | **Heat shock protein 82** | 4.51 | 3.64 | hypothetical protein ATEG_04940 | -1.15 | -3.18 |
| | **Cytochrome c oxidase polypeptide IV** | 3.28 | 3.78 | - | - | - |
| | Glucose-6-phosphate isomerase | 2.41 | 3.28 | - | - | - |
| **100 PSU** | 40S ribosomal protein S22 | 2.39 | 3.12 | Adenosyl homocysteinase | -1.79 | -0.44 |
| | 60S ribosomal protein L11 | 1.45 | 3.28 | **Aldehyde dehydrogenase** | -1.1 | -0.51 |
| | **Heat shock protein 60** | 1.13 | 2.93 | **Fructose-bisphosphate aldolase** | -0.33 | -1.11 |
| | **Heat shock protein 82** | 0.44 | 1.83 | **Glucose-6-phosphate isomerase** | -1.04 | -0.17 |
| | **Thioredoxin** | 2.36 | 1.84 | **Inorganic pyrophosphatase** | -0.64 | -2.34 |

Legend:

FC[a] : Fold change values for proteins expressed under 100 ppm Cr VI concentration in comparison to 0 ppm Cr VI concentration (control)
FC[b] : Fold change values for proteins expressed under 500 ppm Cr VI concentration in comparison to 0 ppm Cr VI concentration (control)

**Table 18. Regulation of proteins under Chromium stress for #NIOSN-SK56-S76 (*P. lilacinum)*.**

| | Up-regulated proteins | | | Down-regulated proteins | | |
|---|---|---|---|---|---|---|
| **Condition** | **Protein expressed** | **FC[a]** | **FC[b]** | **Protein expressed** | **FC[a]** | **FC[b]** |
| **0 PSU** | **Heat shock protein 70** | 1.17 | 1.14 | **Glucan 1,3-beta-glucosidase** | -2.23 | -1.21 |
| | **Heat shock protein SSC1,** | 0.24 | 1.04 | **Glucose-6-phosphate isomerase** | -1.68 | -1.48 |
| | **Peroxidase/catalase 2** | 2.83 | 0.76 | Glutamine synthetase | -1.81 | -3.51 |
| | **Peroxiredoxin type-2** | 4.04 | 4.48 | Glutathione reductase | -0.53 | -1 |
| | Woronin body major protein | 1.46 | 1.29 | Catalase B precursor | -2.41 | -0.57 |
| **35 PSU** | **Heat shock protein 70** | 0.12 | 1.42 | **2,3-bisphosphoglycerate-independent phosphoglycerate mutase** | -1.28 | -1.21 |
| | **$Mg^{2+}$ transporter protein,** | 0.45 | 2.69 | 26S protease regulatory subunit 6A-B | -0.99 | -1.01 |
| | **Peroxidase/catalase 2** | 1.8 | 1.89 | Acyl coA binding protein | -2.23 | -1.29 |
| | **Peroxiredoxin type-2** | 4.65 | 4.7 | **Alcohol dehydrogenase I** | -1.64 | -1.48 |
| | **Superoxide dismutase** | 2.06 | 2.47 | **Alpha-glucosidase** | -1.72 | -1.48 |
| | Woronin body major protein | 0.72 | 1.44 | Histone H3 | -2.53 | -0.26 |
| **100 PSU** | O-methyltransferase, | 19.97 | 17.64 | Cytochrome c oxidase polypeptide IV | -2.47 | -3.32 |
| | Protein kinase | 17.02 | 13.72 | Elongation factor 1-alpha | -0.3 | -5.96 |
| | Ankyrin repeat- protein | 13.82 | 14.72 | **Fructose-bisphosphate aldolase** | -1.28 | -5.93 |
| | - | - | - | **Glyceraldehyde-3-phosphate dehydrogenase** | -1.17 | -5.97 |
| | - | - | - | Histone H2A | -1.69 | -0.75 |

Legend:

FC[a] : Fold change values for proteins expressed under 100 ppm Cr VI concentration in comparison to 0 ppm Cr VI concentration (control)
FC[b] : Fold change values for proteins expressed under 500 ppm Cr VI concentration in comparison to 0 ppm Cr VI concentration (control)

**Table 19. Regulation of proteins under Copper stress for #NIOSN-M20 (*P. chrysogenum)*.**

| | Up-regulated proteins | | | Down-regulated proteins | | |
|---|---|---|---|---|---|---|
| **Condition** | Compound | FC[a] | FC[b] | Compound | FC[a] | FC[b] |
| **0 PSU** | 60S ribosomal protein L10-A | 22.26 | 16.39 | Mannitol-1-phosphate 5-dehydrogenase | -1.98 | -19.21 |
| | **Glutaredoxin** | 18.39 | 13.95 | Mitochondrial amidoxime reducing component | -0.9 | -17.6 |
| | Hexokinase | 19.47 | 14.36 | **Mitochondrial peroxiredoxin** | -2.52 | -20.33 |
| | Histone H2A | 3.21 | 1.69 | Retinol dehydrogenase | -0.33 | -17.09 |
| | Importin subunit beta-1 | 16.48 | 14.43 | Septin-like protein | -1.76 | -20.35 |
| **35 PSU** | 1,3-beta-glucanosyltransferase | 1.1 | 1.78 | ATP synthase subunit | -1.66 | -0.79 |
| | 26S protease regulatory subunit 6A | 1.01 | 1.97 | **Cell division control protein** | -2.06 | -0.56 |
| | 40S ribosomal protein S6-B | 1.66 | 2.63 | **Citrate synthase** | -1.29 | -1.73 |
| | 60S ribosomal protein L14-A | 0.8 | 1.31 | **Fructose-bisphosphate aldolase** | -3.31 | -2.44 |
| | 60S ribosomal protein L4-A | 0.75 | 1.26 | Plasma membrane ATPase | -1.12 | -3.7 |
| | Woronin body major protein | 0.84 | 1.75 | Spermidine synthase | -3.42 | -0.99 |
| **100 PSU** | **Catalase B** | 2.38 | 1.05 | Aconitate hydratase | -1.38 | -3.12 |
| | **Cytochrome c peroxidase** | 1.93 | 0.79 | **Bifunctional purine biosynthesis protein** | -1.24 | -0.68 |
| | **Cytochrome c1, heme protein** | 0.31 | 3.58 | D/L-glyceraldehyde reductase | -1.13 | -1.26 |
| | **Superoxide dismutase** | 0.75 | 1.88 | **Elongation factor 1-beta** | -0.6 | -1.83 |
| | Versicolorin reductase | 6.16 | 5.89 | Protein disulfide-isomerase | -0.14 | -1.08 |

Legend:

FC[a] : Fold change values for proteins expressed under 100 ppm Cu II concentration in comparison to 0 ppm Cu II concentration (control)
FC[b] : Fold change values for proteins expressed under 500 ppm CuII concentration in comparison to 0 ppm Cu II concentration (control)

Dramatic physiological changes in gene expression and the patterns of protein synthesis of an organism, is often a result of diverse stresses, which helps the organisms to survive in an altered environment (Kapoor, 1986; Baby and Jini, 2010). Stresses such as presence of heavy metals and elevated salt concentrations are habitually detrimental to organisms. High sodium concentrations can lead to water deficient cells which inhibit metabolic and cellular activities (Ravindran et al. 2012). Production of ROS triggering oxidative stress and alterations in lipid patterns, are also an outcome of increased salinity stress (Turk et al. 2004; Baby and Jini, 2010). Redox-active heavy metals, pose a serious threat due to its toxicity and intrinsic persistence in environment. They have strong binding affinities to thiol or histidyl moieties (sulfhydryl groups of proteins), which results in the displacement of essential metal ions from biomolecules, disruption of cellular integrity, inactivation of enzymes or conformational modifications, along with formation of ROS and free radicals (Bae and chen, 2004; Labra et al. 2006; Hossain and Kotmasu, 2013). Inevitably cellular proteins end up absorbing 70% of these radicals along with its derivatives which lead to reversible/irreversible changes in amino acid side chains resulting in protein inactivation, aggregation, or degradation (Ilyas et al. 2014). The ROS generated inside cells participate in deleterious reactions, for e.g., peroxidation of lipids, causing disruption of membrane integrity and also affecting synthesis of nucleic acids, ribosome and enzyme systems in organisms (Bar et al.2007; Porcel et al. 2012; Ilyas et al. 2014). Therefore microorganisms need to formulate robust cellular responses to overcome such life-threatening toxic effects. For doing so, individual isolates have to embrace multiple mechanisms to survive the combination of multiple stresses. Experiment, results and its analysis revealed that stress responsive mechanisms put forth by these isolates were intracellular chelation of metal, enzymatic scavenging of free radicals, activation of repair mechanisms, elevated expression of

transporter proteins as well as possible vacuolar accumulation of metal complexes. Although this study has provided an insight into the various responses in terms of protein expression under stressful conditions by individual isolates, a lot more research supported by experimental evidence is required to prove the existence of these mechanisms.

# Chapter FIVE

# Significance of this Study

## 5.1 Conclusion

Fungi are ubiquitous, found in both terrestrial and aquatic environments. Marine-derived fungi particularly were found to be abundant in coastal as well as deep-sea environment, but the isolation and culturing techniques being insufficient and semi efficient, have led to poor cultivable counts as compared to the total existing diversity in these regions. It is therefore essential to improvise and improve on the current techniques to isolate and identify novel species of marine fungi. Isolates procured in this study could have wide range of applications. This study highlighted the metal tolerance ability of these isolates, while some of these isolates have also been tested for hydrocarbon degrading ability (Barnes et al. 2017). Therefore no isolate should be neglected and needs to be preserved for further exploration. All the isolates from the current work have been sub-cultured on Czapek Dox agar plates, but for long term storage and to avoid inevitable problems of contamination associated with continuous sub-culturing, these isolates were lyophilized. The technique used was very simple, where fungal spores or mycelia were suspended in skimmed milk and frozen overnight at -80°C in previously sterilized glass cryovials (quadruplets) covered with cotton plugs. The frozen samples were then lyophilized under vacuum and the tips of the vials were sealed used flame. These samples could then be stored at 4°C for long periods. In order to revive an isolate, the seal of the vial needs to be broken and the powered sample needs to be suspended in a culture broth aseptically and incubated for 2-4 days at room temperature.

The results of the current study have helped to observe and derive conclusions such as a single isolate can be tolerant to multiple metals and not necessarily to a single one, and can react differently to different metals and their varying concentrations. Further, single isolate

can have one, two or multiple metal tolerance mechanisms pertaining to the number of stresses it is exposed to. Experiments conducted on the isolates subjected to metal stress and the subsequent results helped to hypothesize that mechanisms such as intracellular metal accumulation, biosorption and enzymatic reduction of metal could be simultaneously occurring. But such morphological and preliminary metal tolerance experiments are insufficient to draw satisfactory conclusions regarding metal tolerance mechanisms.

Proteomic approach to address possible metal tolerance mechanisms has given an insight to the various proteins being produced under stress. Up-regulation of stress proteins, transporter proteins, ROS scavenging proteins and other defence systems, focuses on the cell's strategy to overcome stressful condition and its attempt to survive. Down-regulation of proteins involved in carbohydrate metabolism, amino acid metabolism and other important metabolic functions is because the cell is actively producing proteins required for metal detoxification or tolerance. Under stressful conditions, active cell growth and proliferation is no longer the priority, instead the main focus is to survive. Expression of autophagy and senescence proteins also takes place in some cells, as it is unable to overcome the stressful conditions and prepares for death phase. Besides this, expression of hypothetical proteins (proteins which are not functionally annotated) and their regulation patterns are of great importance and needs to be addressed as these proteins might play a role in defence mechanisms against heavy metals in fungal cells

### 5.2 Future Prospects

The current study provides tremendous scope for using the fungal isolates as bioremediation agents in heavy metal contaminated sites. These isolates especially the

chromium tolerant ones, have the potential for metal removal via intracellular accumulation as well as detoxification of Cr (VI) to Cr (III) through enzymatic reduction.

Moving a step ahead with proteomics, selected hypothetical proteins which have frequently been found under metal stress could be studied in detail and annotated to know their function. Such unique proteins can then be studied to find the genes responsible for coding and can be used to prove its function using gene deletion and complementation studies. Further, on confirmation of the roles such proteins play, they could be purified and further studied for their structure and metal binding abilities using crystallography and kinetic studies.

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