A

DISSERTATION

ON

Isolation and Characterization of Metal Resistance Genes by using Metatranscriptomic Approach

Submitted in partial fulfillment of the requirements
for the Award of the Degree of

MASTER OF TECHNOLOGY
IN
BIOTECHNOLOGY

Submitted
By

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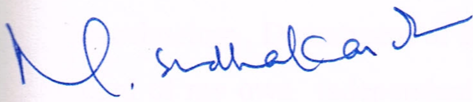
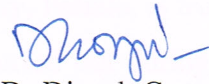
JULY 2014

CERTIFICATE

This is to certify that the dissertation entitled, **"Isolation and characterization of metal resistance genes by using metatranscriptomic approach"** submitted by Manpreet Kour in partial fulfilment of the requirement for the award of degree of Master of Technology in Biotechnology, Department of Biotechnology, Thapar University, Patiala is an authentic record of student's own work carried out by her under my supervision and guidance. The report has not been submitted for the award of any other degree or certificate in this or any other university.





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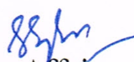

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Declaration of Original Work

I hereby declare that the work being presented in the dissertation entitled **"Isolation and characterization of metal resistance genes by using metatranscriptomic approach"** in the partial fulfillment of the requirement for the award of the degree of Master of Technology in Biotechnology, Department of Biotechnology, Thapar University, Patiala, is true and original record of my own independent and original research work carried out during the period of one year from July 2013 to June 2014, under the guidance of Dr.M.S.Reddy, Professor, Thapar University, Patiala. I have not submitted the matter embodied in this dissertation for the award of any other degree or diploma.

I have honoured the principles of academic integrity and have upheld the moral student code of academic conduct in the completion of this work.

Manpreet Kour

ACKNOWLEDGEMENT

This project has been possible by the bountiful mercy and grace of God. It is my great pleasure to express my thanks to the entire magnanimous person who rendered their full support to my work directly or indirectly

I would like to express my special and sincere gratitude to my dissertation guide, **Dr. M.S.Reddy**, Professor, Department of Biotechnology, Thapar University, Patiala, who has been constant in his valuable suggestions and encouragement apart from providing useful material and facilities for the completion of work. Without his inspiration and generous guidance, the work would not be successfully completed. I learned from him on this dissertation and that could lead me to success in my future career.

I express my special gratitude to **Dr. Dinesh Goyal**, Head, Department of Biotechnology, Thapar University, Patiala, for all his possible support in various facilities of the department for this work.

I am sincerely thankful to Dr. Gurdeep Kaur & Dr. Navdeep Kaur Dhami for their immense support, guidance and concern throughout the project work. I also wish to express my special thanks to all the Ph.D scholars in the lab Mr. Balwant, Mr. Sanjog & Ms. Bharti for their immense help and valuable suggestions.

A sincere token of gratitude is also paid to *my family, friends and colleagues* for their moral support and motivation. At last but not least I gratefully acknowledge my deep indebtedness to all other persons who helped me during the whole period of the work.

Manpreet Kour

ABSTRACT

Metatranscriptomics is a recent culture-independent approach in microbial ecology which gives a direct and simultaneous access to the genes expressed by all the microbial species, cultivable or not, living in a common environment. This approach has a strong potential in biotechnology to discover novel genes of interest for the bio-industry, in bioremediation and as biomarkers. In the present study, Functional metatranscriptomics approach was adopted to isolate and characterize metal resistance genes expressed by eukaryotic species in the heavy metal polluted environment. The total RNA was isolated from heavy metal polluted soil. The eukaryotic mRNAs, owing to their 3 poly-A tails were specifically converted into intron-less cDNAs. These cDNAs were size fractionated, cloned to generate environmental metatranscriptomic cDNA libraries, which are representative of the fraction of protein-coding genes expressed at the time of sampling. These cDNA libraries were constructed in the modified pFL61 yeast-*E. coli* shuttle vector. The metatranscriptomic libraries were screened for the presence of metal resistance genes by functional complementation of *Saccharomyces cerevisae* mutants. The mutant strains, copper sensitive *cup1*$^{\Delta}$ and cadmium sensitive *ycf1* were used in this study. Yeast transformants exhibiting metal resistance were identified by plating on medium supplemented with either copper or cadmium and further characterized by studying their growth curve in the presence of metal.

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Chapter 1
Introduction

Many prokaryotic species and eukaryotic microbes cannot be easily isolated from complex environmental matrices and/or cannot be grown *in vitro* (Bailly *et al*., 2007). To appreciate their true functional diversity and the activities they express *in situ* in the soil in response to different environmental constraints, it is necessary to develop new experimental approaches adapted to these microorganisms. Recently developed metagenomic approaches are shedding light on the diversity and ecology of unculturable microbes in diverse environmental habitats, unveiling their involvement in ecosystem processes (Dinsdale *et al*., 2008; Tringe and Rubin, 2005). Metagenomics has also been used successfully to search for novel biocatalysts (Daniel 2005; Steele *et al*., 2009). This strategy, however, largely favours the detection of archea and bacteria, as the genomes of eukaryotes are often large genomes and frequently include introns within gene coding regions. Therefore, screening for eukaryotic functions in environmental samples focuses on metatranscriptomic libraries and thus relies on transcriptionally expressed genes (Bailly *et al*., 2007; Warnecke and Hess 2009).

Numerous and often unknown eukaryotic microorganisms present in the environment potentially represent a rich source of genes encoding for novel enzymes, gene categories of interest in biotechnology and also to understand basic biological processes. In this respect, functional metatranscriptomics has been demonstrated as a powerful tool in discovery and study of these genes (Todaka *et al*., 2007; Kellner *et al*., 2011; Damon *et al*., 2011; Lehembre *et al*., 2013).

1.1 Metatranscriptomics

Like metagenomics, metatranscriptomics (or environmental transcriptomics) involves random sequencing of microbial community mRNA. Neither primers nor probes are needed, so there is no need to anticipate important genes beforehand and transcripts from microbial assemblages are sequenced with little bias. The approach is particularly amenable to an experimental framework in which gene expression is monitored while a biotic or abiotic parameter is manipulated. Experimental metatranscriptomics is one of the most powerful tools for understanding the timing and regulation of complex microbial processes within communities and consortia, as well as microbial dexterity in response to changing conditions.

A novel environmental genomic approach, the 'Functional Metatranscriptomics', allows the specific characterization of genes expressed by different eukaryotic microorganisms (eg. Fungi, Protists) directly in the environmental samples. This approach has a strong potential in biotechnology to discover novel genes of interest for the bioindustry, in bioremediation and as biomarkers. This approach allows characterization of genes implicated in adaptation to stressful conditions or involved in organic matter degradation.

1.2 Advantages and applications of Metatranscriptomics

The study of the eukaryotic metagenome faces several specific problems. The genome size of a eukaryote can be several orders of magnitude higher than the genome size of a bacterium. Among free-living unicellular eukaryotes it can vary from, for example, 13.8 Mpb for the yeast *Schizosaccharomyces pombe* with an estimated number of protein coding genes of 4800 (Wood *et al.*, 2002) to 69 Mpb for the ciliate *Paramecium tetraurelia* (39 600 gene models; Aury *et al.*, 2006). As a consequence, it is unlikely that a workable metagenomic library based on genomic DNA can capture a significant fraction of the gene content of a eukaryotic microbial community. Furthermore, the frequent presence of introns and lack of conservation of motifs in promoter sequences prevent expression of genomic copies of eukaryotic protein-coding genes not only in a bacterial cell but also in most eukaryotic host. Finally, as there is no established protocols to easily separate eukaryotic cells from bacteria and from a complex environmental matrix such as soil, a DNA-based metagenomic library that include eukaryotic DNA would also necessarily include prokaryotic sequences.

For eukaryotes, the use of RNA extracted from environmental samples could circumvent these problems. Owing to their 3′ poly-A tails, eukaryotic mRNA can indeed be specifically isolated from a complex RNA mixture and converted into intronless cDNAs that can be cloned to generate environmental metatranscriptomic cDNA libraries, which are representative of the fraction of protein-coding genes expressed at the time of sampling.

Metatranscriptomics provides a snapshot of transcriptional profiles that correspond to discrete populations within a microbial community at the time of sampling (Carvalhais *et al*., 2012).

Metatranscriptomics applied to environmental transcripts provides unique opportunities to reveal microbial activity in the environment and to discover novel enzymes of potential use in biotechnological applications (Kellner *et al*., 2011).

A powerful application of metatranscriptomics is in controlled experimental studies, in which microbial community gene expression can be measured in direct response to a defined manipulation (Moran, 2009).

1.2 Heavy metals: occurrence, chemical and physical properties and mode of their action

Environmental pollution by metals became extensive as mining and industrial activities increased in the late 19th and early 20th centuries. Mineral rock weathering and anthropogenic sources provide two of the main types of metal inputs to soils. According to Ross (1994) the anthropogenic sources of metal contamination can be divided to five main groups: (1) metalliferous mining and smelting (arsenic, cadmium, lead and mercury); (2) industry (arsenic, cadmium, chromium, cobolt, copper, mercury, nickel, zinc); (3) atmospheric deposition (arsenic, cadmium, chromium, copper, lead, mercury, uranium); (4) agriculture (arsenic, cadmium, copper, lead, selenium uranium, zinc); and (5) waste disposal (arsenic, cadmium, chromium, copper, lead, mercury, zinc).

Heavy metals are natural components of the earth's crust. They cannot be degraded or destroyed. Heavy metals are defined as metals with a density higher than 5 g cm^{-3}. Fifty three of the 90 naturally occurring elements are heavy metals (Weast, 1984), but not all of them are of biological importance. The chemical form (speciation) of heavy metals in soil solution is greatly dependent on the metal element concerned, pH and presence of other ions. Based on their solubility under physiological conditions, 17 heavy metals may be available for living cells and of importance for organism and ecosystems (Weast, 1984).

Among these metals, Fe, Mo and Mn are important as micronutrients. Some metals, such as calcium, cobalt, chromium, copper, potassium, magnesium, sodium, nickel and zinc, are essential, serve as micronutrients and are used for redox-processes; to stabilize molecules through electrostatic interactions; as components of various enzymes; and for regulation of osmotic pressure (Bruins *et al.,* 2000). Many other metals have no biological role such as silver, aluminium, cadmium, gold, lead and mercury, are nonessential (Bruins *et al.,* 2000) and potentially toxic to microorganisms (Fig1.1).

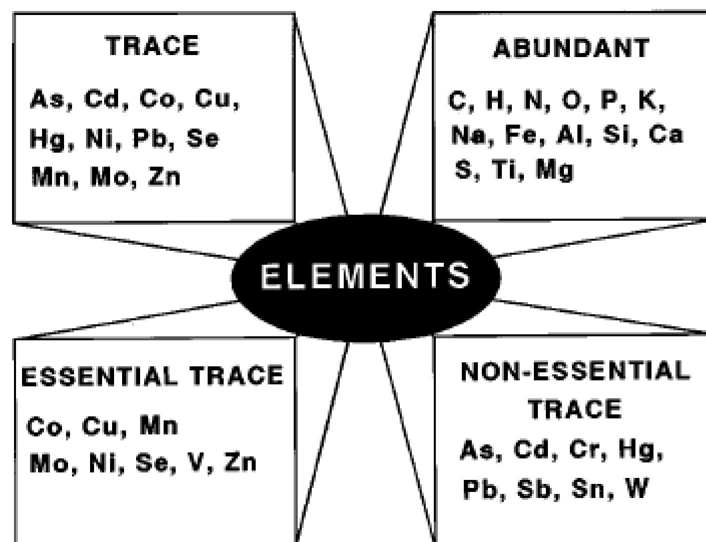

Figure 1.1: Metabolic and nutritional importance of elements and their classification (adapted from Prasad, 1998)

The toxicity symptoms seen in the presence of excess amount of heavy metals may be due to a range of interaction at the cellular/molecular level. Toxic metals can cause harmful effects in many ways, but principally as result of their strong co-ordinating abilities (Ochiali, 1987). Toxic effects include the blocking of functional groups of biologically important molecules (e.g. enzymes and transport system for essential nutrients and ions) the displacement and\or substitution of essential metal ions from biomolecules and functional cellular units, conformational modifications, denaturation and inactivation of enzymes and disruption of cellular and organellar membrane integrity (Ochiali, 1987). In addition, heavy metal excess may stimulate the formation of free radicals and reactive oxygen species, resulting in oxidative stress (Dietz *et al.,* 1999).

Metals are directly or indirectly involved in all aspects of cell growth, metabolism and differentiation. This leads to expression of a detoxification mechanism for survival of organism. All organisms can achieve resistance to heavy metals by “avoidance” when the organism is able to restrict metal uptake, or by “tolerance” when the organism survives in the presence of high internal metal concentration (Joho *et al.,* 1985; Turnau *et al.,* 1996). The avoidance involves reducing the concentration of metal entering the cell by: extracellular precipitation, biosorption to cell walls, reduced uptake, or increased efflux. In the second situation, metals are chelated intracellularly through the synthesis of ligands such as metallothioenins, phytochelatins, polyphosphates and\or compartmentation within vacuoles.

1.4 Gaps in study

Several human activities, either industrial (mining and ore processing) or agricultural (use of Phosphate fertilisers) lead to a long- term contamination of soils by different heavy metals. The corresponding lands can no longer be used for agricultural purposes and must be revegetated to limit dispersal of the pollutants as dusts and/or their leaching to nearby rivers and water table. Although heavy metals show a general toxicity towards most living organisms, some metal tolerant/resistant species and ecotypes can tolerate relatively high concentrations of these compounds. As a result, most "moderately-polluted" soils host several species of metal-resistant microorganisms which carry out basic biological processes necessary to maintain soil fertility.

One current challenge in environmental science is to understand the diversity of mechanisms leading to heavy metal resistance. If a number of them (chelation, excretion, cellular compartmentation) have been described in a number of model species, they may not be representative of the diversity of mechanisms developed in the numerous metal resistant species. This study based on functional metatranscriptomics allows characterizing genes implicated in adaptation to heavy metal resistance.

1.5 Objectives

- Optimisation of RNA extraction protocol from metal contaminated soil
- cDNA synthesis, size fractionation and library construction
- Isolation and characterisation of metal resistanance genes by yeast complementation assay

Chapter 2

Literature Review

Soil borne microorganisms are one of the earth's greatest source of biodiversity (Curtis *et al*., 2002), with estimates ranging between 3000 and 11,000 microbial genomes per gram of soil (Schmeisser *et al*., 2007). The microbial diversity in most environments exceeds the biodiversity of plants and animals by orders of magnitude (Hoff *et al*., 2008). However, the classic microbiological approach of bringing microorganisms into pure culture fails for the majority of microbial organisms present in nature. It has been estimated that only ~1% of microbes are readily available by cultivation (Pace, 1997). A majority of microbial species including eukaryotic are uncultivable under laboratory conditions (Torsvik and Ovreas, 2002; Porter, 2008). Eukaryotic microbes play essential roles in functioning of the soil ecosystem. For example, saprotrophic fungi are responsible for the breakdown of plant biomass, while symbiotic (mycorrhizal) and pathogenic species affect either positively or negatively plant growth and health and therefore crop yield. Many protist species are efficient bacterial predators which regulate microbial population and release essential nutrients in the soil which get immobilized in the microbial biomass. It is increasingly recognized that a huge number of natural products exists in non-culturable microbes with chemical, biological, and functional activities for potential uses in various industrial and biomedical applications (Handelsman, 2004).

DNA microarray technologies are being employed as an important tool for analysis of complex microbial communities inhabiting various environments (Wang *et al*., 2009). A functional gene array, termed GeoChip (He *et al*., 2007), is a valuable tool in the assessment of microbial-community responses to a variety of environmental conditions, including metal pollution. Since this platform contains probes for genes with key biological functions (e.g. those involved in nitrogen, carbon, sulphur, and phosphorus cycling; metal reduction and resistance; and degradation of organic contaminants). It is also being used for assessing the recovery of soil-borne microbial communities from perturbations such as metal pollution, but the probes combine a non-specific binding, which is a major disadvantage with microarray data. Geochip also have many technical, experimental, and data analysis challenges that need to improve the sensitivity and quantitative accuracy of the arrays. Accordingly, the vast

majority of organisms cannot be studied by traditional techniques that have been designed over the last three centuries.

2.1 Culture independant approaches

Many prokaryotic species and eukaryotic microbes cannot be easily isolated from complex environmental matrices and/or cannot be grown *in vitro* (Bailly *et al*., 2007). So culture independent approaches are being used to study complex environmental matrices.

- Metagenomics
- Metatranscriptomics
- Metaproteomics

2.1.1 Metagenomics

Metagenomics have been successfully developed to address the issue of analysis of uncultured microbes. Metagenomics can be divided into sequence-based and function-driven analysis of uncultured microorganisms (Gabor *et al*., 2007). Sequence-based approaches involve screening clones for the highly conserved 16S rRNA genes for identification purposes and then sequencing the entire clone to identify other genes of interest, or large-scale sequencing of the complete metagenome to search for phylogenetic anchors in the reconstructed genomes (Riesenfeld *et al*., 2004; Hoff *et al*., 2008).

Functional metagenomics, on the other hand, involves screening metagenomic libraries for a particular phenotype, e.g. salt tolerance, antibiotic production or enzyme activity, and then identifying the phylogenetic origin of the cloned DNA (Dinsdale *et al*., 2008). Metagenomics is based on the direct extraction of whole DNA from environmental samples and it's cloning in appropriate vectors (eg. plasmids) and then propagated in domesticated bacterial cells (*E.coli*) (Dinsdale *et al*., 2008). The idea of cloning DNA directly from environmental samples was first proposed by Pace *et al*. (1985), and in 1991, the first such cloning in a phage vector was reported (Schmidt *et al*., 1991). These environmental DNA libraries contain therefore fragments of the genomes of all microbial species, cultivable or not, present in the original environmental sample. Screening of these libraries by different approaches is now becoming a standard approach for discovering novel genes of biotech potential, which for many of them originate from unknown, uncultured bacterial species (Rodon *et al*., 2000; Ferrer *et al*., 2009; Chistoserdova, 2010). Alternatively, metagenomic DNA or libraries can

be systematically sequenced, thus revealing the global gene content of the microbial community and also adaptation of these communities to specific, local environmental conditions (Gilbert *et al*., 2010).

Metagenomics is not; however, the most appropriate approach to study eukaryotic microorganisms. Firstly, examination of the sequence data from metagenome sequencing programs reveals that sequences are outnumbered by prokaryotic ones, and that therefore, metagenomic DNA library contain a low proportion of clones of eukaryotic origin. Secondly, due to frequent occurrence of introns in eukaryotic genes and non-conservation of transcription regulatory elements between eukaryotes and prokaryotes, the genomic copy of a eukaryotic gene is unlikely to be expressed in a bacterial host. However, as metagenomic DNA-based analyses cannot differentiate between expressed and non-expressed genes, it fails to reflect the actual metabolic activity (Sorek and Cossart, 2010).

To circumvent these problems, a new approach, called metatranscriptomics is being used.

2.1.2 Metatranscriptomics

It involves the extraction and analysis of metagenomic mRNA (the metatranscriptome) which provides information on the regulation and expression profiles of complex communities. It is based upon extraction of environmental RNA instead of DNA and on the purification by affinity chromatography of the eukaryote-specific polyadenylated messenger RNA from the total environmental RNA mixture. These poly-A mRNAs can be converted into cDNA, which can be cloned in appropriate expression vectors such as plasmids which allow expression of the cloned genes in the eukaryotic yeast *Saccharomyces cerevisiae* (Fig.2.1). These environmental cDNA libraries are therefore representative of the diversity of genes expressed by the different eukaryotic microorganisms present in the original soil sample. Thus, transcriptomics and metatranscriptomics are powerful tools to capture snapshots of the genes essential for the survival in particular niches (Falk and Matthias, 2009). This protocol, from soil to an environmental cDNA expressed in yeast, was first implemented for a *Pinus* forest soil and allowed for the isolation of histidine biosynthetic genes by complementation of a histidine auxotrophic yeast mutant (Bailly *et al*., 2007).

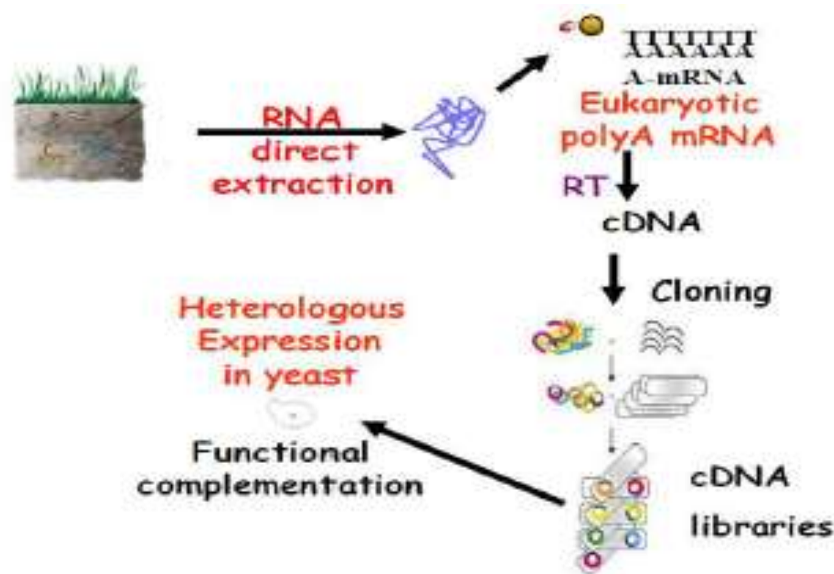

Figure 2.1: The functional metatranscriptomic approach

2.1.3 Metaproteomics

Metaproteomics is the study of all protein samples recovered directly from environmental sources. Technical issues of protein extraction, separation, and identification make metaproteomics more onerous, at least for now, than metatranscriptomics.

2.2 Applications of Metatranscriptomics

Metatranscriptomics tools were used to gain functional in-sights into the activities of environmental microbial communities by studying their mRNA transcriptional profiles (Mcgrath *et al*., 2008). The approach has been used to analyze microbial communities from ocean surface waters (Frias-Lopez *et al.,* 2008), coastal waters (Poretsky *et al.,* 2009), and soil samples (Urich *et al.,* 2008).

Warnecke *et al.* (2009) reported that metatranscriptomics is a promising tool for the sequence-based discovery of novel biocatalysts.

Holmes *et al.* (2009) deciphered the transcriptome of *Geobacter uraniireducens* strain growing in uranium-contaminated subsurface sediments. Several other ecosystems of various environmental niches have been explored, such as the investigations of ruminal bacterial

diversity (Edwards *et al*., 2004), acid mine drainage (Tringe *et al*., 2005), termite hindgut microflora (Warnecke *et al*., 2007), and permafrost-affected soils in the Arctic (Ganzert *et al*., 2007). These studies give insightful information on microbial ecology and evolution, and directions for harnessing novel genetic and biochemical information.

A comparative study by Poretsky *et al*. (2009) revealed that Bacterioplankton communities in oligotrophic North Pacific surface water show greater investments in energy acquisition and metabolism (photosynthesis, oxidative phosphorylation, and C1 compound metabolism) during the day and in biosynthesis (of membranes, amino acids, and vitamins) at night, according to an analysis of RNA transcripts from these periods.

Kellner *et al.* (2010) identified fungal genes encoding an acid phosphatase and an imidazoleglycerol-phosphate dehydratase in a metatranscriptomic library, which was obtained by reverse –transcribed polyA fraction of total RNA extracted from the organic layer of a sugar maple forest soil.

He *et al.* (2010) employed metatranscriptomics to identify RNA-based regulation and expressed biological signatures in complex ecosystems.

A novel fungal family of oligopeptide transporters was identified by functional metatranscriptomics of soil eukaryotes. This family has a patchy distribution in the Basidiomycota and Ascomycota and is present in the genome of a *Saccharomyces cerevisiae* wine strain (Damon *et al*., 2011).

Damon *et al.* (2012) studied the diversity of genes expressed by eukaryotes in forest soils using metatranscriptomics. Specific annotation of plant cell wall degrading enzymes identified enzymes active on major polymers (cellulose, hemicelluloses, pectin, lignin) and glycoside hydrolases represented 0.5% (beech soil)–0.8% (spruce soil) of the cDNAs. Other sequences coding enzymes active on organic matter (extracellular proteases, lipases, a phytase, P450 monooxygenases) were identified, thus underlining the biotechnological potential of eukaryotic metatranscriptomes.

Mason *et al.* (2012) studied the microbial response to deep water horizon oil spill. This study based on metatranscriptomic sequencing revealed that genes for motility, chemotaxis and

aliphatic hydrocarbon degradation were significantly enriched and expressed in the hydrocarbon plume samples compared with uncontaminated seawater collected from plume depth.

Radax *et al*. (2012) studied the phylogeny and function of the active microbial community and the interaction with its host *Geodia barretti*, by using a metatranscriptomic approach**.** Archaeal genes of ammonia oxidation were identified as representing the most abundant transcripts.

Konopka *et al.* (2012) reported that metatranscriptomics approach can be used to reveal the limiting nutrient or environmental stressors impacting natural microbial populations.

Lehembre *et al*. (2013) applied metatranscriptomics to soil environments for mining eukaryotic heavy metal resistance genes. The work, based on the functional screening of soil eukaryotic metatranscriptomic libraries, revealed high degrees of diversity and novelty among the recovered sequences, demonstrating that this methodology is suitable and powerful for characterizing novel genes whose overexpression restored tolerance or even conferred heavy metal resistance in yeast.

Lejzerowicz *et al.* (2013) used metatranscriptomic approach based on RNA-derived (cDNA) sequences to study the diversity of the deep-sea benthic foraminifera in the Sea of Japan. The cDNA dataset was dominated by sequences of rotaliids and robertiniids, suggesting that these calcareous species are the most active component of foraminiferal community.

Lim *et al.* (2013) used microbial and viral metagenomics combined with microbial transcriptomics to characterize the dynamic polymicrobial communities found in CF (Cystic Fibrosis) airways, revealing both the taxa present and their current metabolic activities. These approaches can facilitate the development of individualized treatment plans and novel therapeutic approaches.

Loebus *et al.* (2013) studied metal coordination abilities of a metallothionein from an aquatic fungus *Heliscus lugdunensis* and concluded that Neclu_MT1 is the first solely cadmium-inducible metallothionein.

Leonhardt *et al.* (2014) screened an *R. atropurpurea* cDNA library in the Cd-hypersensitive *ycf1* yeast, for sequences encoding peptides capable of sequestering divalent heavy metals. They identified two cDNAs, RaZBP1 and RaZBP2, which protected the metal-sensitive yeast mutants against Cd and Zn, but not Co, Mn or Cu, toxicity.

Sacky *et al.* (2014) isolated and characterized HmMT1, HmMT2 and HmMT3 genes coding for different 5-kDa MTs of *H. mesophaeum* collected at a lead smelter site, through functional metatranscriptomic approach.

2.3 Mechanisms of resistance to metal toxicity

All microorganisms can achieve resistance to heavy metals by "avoidance" when the organism is able to restrict metal uptake, or by "tolerance" when the organism survives in the presence of high internal metal concentrations (Baker, 1987; Turnau *et al.,* 1996). The first mechanism involves reduced uptake or increased efflux, formation of complexes outside cells, biosorption to cell walls and organic acid release. In the second situation, metals are chelated intracellularly through the synthesis of ligands such as metallothioneins, phytochelatins, polyphosphates, and/or compartmentation within vacuoles (Gadd, 1993). Extracellular mechanisms are mainly implied in avoidance of metal entry, whereas intracellular systems aim to reduce metal burden in the cytosol (Fig. 2.2). Additional antioxidative detoxification systems, which allow the microorganisms to counteract the accumulation of reactive-oxygen species directly or indirectly, initiated by metals, may be part of tolerance mechanisms. The significance of these processes may vary as a function of the metal involved, its concentration, and the location of the primary lesion caused by the metal.

2.3.1 Extracellular chelation and cell-wall binding

2.3.1.1 Chelation by organic acids

Mycorrhizal fungi and certain plant hosts can excrete organic acids into the rhizosphere. The benefits of organic acid excretion might be to liberate base cations from soil minerals (Landeweert *et al.,* 2001), to mobilize phosphate from insoluble iron and aluminium phosphates, to counteract aluminium and iron toxicity by complexing their ions in soil solution, to mobilize trace metal cations by complexing them and to acidify the rhizosphere. Thus, organic acid exudation may either mobilize metal toxicants in soil, or they may immobilize, through precipitation with organic acids, or detoxify them by complexing them.

Organic acids such as citric acid, malic acid and oxalic acid are readily utilizable organic substrates, making the dynamics of these acids in the rhizospheres complex (Jones, 1998). Their affinities for particular metal ions are also important in their cycling and in their ability to mobilise-immobilise ions. These properties will also be pH dependent. Oxalic acid excretion into the rhizosphere was higher in mycorrhizal Scots pine compared to non-mycorrhizal controls when no toxic metals were present for a range of fungal isolates from contaminated sites. When exposed to aluminium there was a much greater rise in oxalic acid excretion in mycorrhizal plants compared to nonmycorrhizal plants for *Suillus variegatus* and *Rhizopogon roseolus* isolates, but not for *Paxillus involutus* isolates. Nickel and cadmium exposure did not result in organic acid production. Copper exposure enhanced oxalic acid production in both mycorrhizal and non-mycorrhizal trees (Ahonen-Jonnarth *et al.,* 2000).

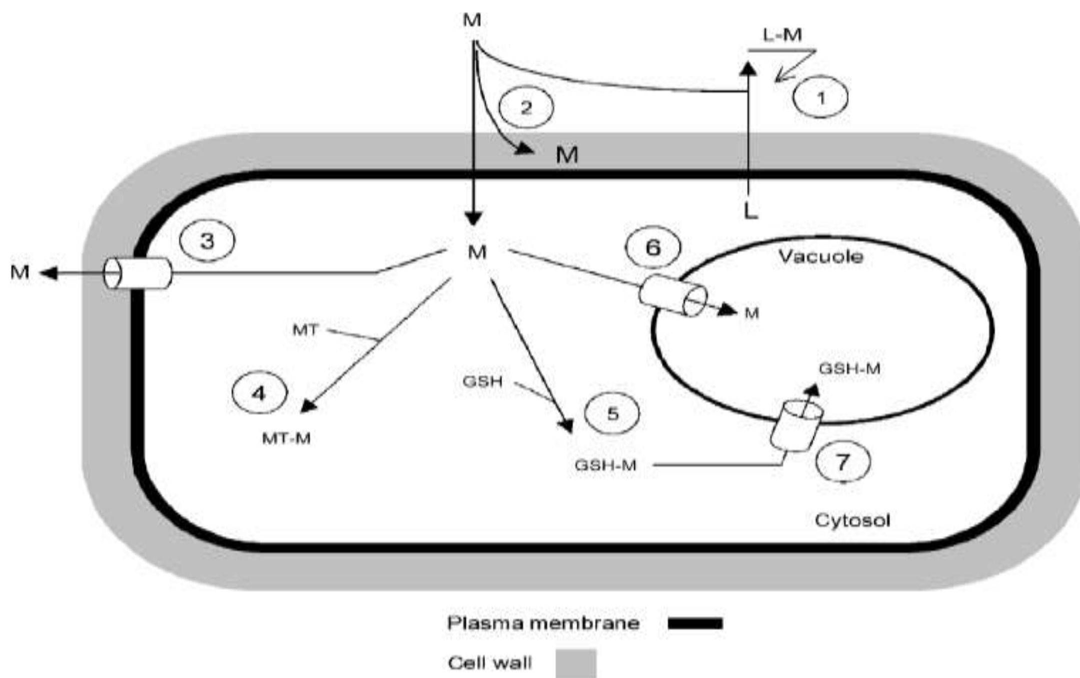

Figure 2.2 Schematic representation of cellular mechanisms potentially involved in metal tolerance in ectomycorrhizal fungi. (Adapted from Bellion *et al*., 2006)

M, metal-ion; 1,extracellular chelation by excreted ligands (L); 2,cell-wall binding; 3, enhanced efflux; 4, intracellular chelation by metallothionein (MT); 5, intracellular chelation by gluthathione (GSH); 6,subcellular compartmentation (vacuole or other internal compartments); 7, vacuolar compartmentation of GSH-Mcomplex (i.e. ycf1).

Different organic molecules particularly di- and tricarboxylic acids that do not belong to the matrix of the cell wall, are excreted by Eukaryotic cells to chelate metal ions, among other functions. In particular, citrate has been shown to be the most important Al^{3+} complex former in soil solution from podzolized forest soils (Landeweert *et al.,* 2001; Van Hees *et al.,*

2001).Exudation of organic acids may provide a source of protons for metal solubilization from metal-containing minerals, often resulting in soil acidification (Devevre *et al.,* 1996; Fomina *et al.,* 2005).A study showing that metal-tolerant ectomycorrhizal fungi grew and solubilized metal-containing minerals better than nontolerant species (Fomina *et al.,* 2005) confirm a possible relationship between tolerance to metals and extracellular chelation by extruded ligands. Isolates from copper mine spoils show enhanced resistance to copper and decreased free copper in solution to a greater extent. It is postulated that this enhanced precipitation is a tolerance mechanism (Adriaensen *et al.,* 2006).

2.3.1.2 Heavy metal binding in the extramatrical mycelium

Eukaryotes can effectively bind metals to cell walls or extracellular polysaccharides may have been suggested as a tolerance mechanism in eukaryotic cells (Gadd, 1993).

Frey *et al*. (2000) investigated zinc and cadmium localization in *Hebeloma crustuliniforme–Picea abies* root tips. They found that the cadmium was predominantly bound extracellularly in the Hartig net, while zinc accumulated mainly in cell walls of mantle hyphae, Hartig net hyphae and in cortical cells. They proposed that zinc accumulated in high quantities in fungal tissues, protecting the host plant. A range of metal elements were shown to accumulate in the fungal mantle and rhizomorphs in *Suillus luteus–Pinus sylvestris* associations collected from polluted soils (Turnau *et al.,* 1996). Gonzalez-Chavez *et al.* (2002) showed that a number of *Glomus* isolates from copper mine spoil absorbed copper to their external hyphae. *Glomus species* have a high capacity to bind cadmium, and zinc. Isolates from metal contaminated sites that exhibited metal resistance bound more cadmium and zinc to their surfaces (Joiner *et al.,* 2000).

2.3.1.3 Heavy metal binding by cell wall components

The cell wall has been suggested as the main barrier protecting against uptake of potentially toxic metal species. A large number of potential-binding sites are exhibited by free carboxyl, amino, hydroxyl, phosphate and mercapto groups present in cell wall polymers and proteins (Strandberg *et al.,* 1981). Binding to the wall, also called biosorption (Gadd, 1993), is a mechanism not depending on the metabolic activity of the cells. Biosorption of heavy metals may reduce the intracellular accumulation of metals and their effect on cytoplasmic processes (Brown and Wilkins 1985).

Binding of Cd to cell walls was shown to represent a substantial fraction of the metal accumulation by *P. involutus* and may also be part of the mechanisms by which mycorrhizal fungi tolerate high amounts of metals (Blaudez *et al.,* 2000).

Interestingly, glomalin, a protein synthesized and excreted by arbuscular mycorrhizal fungi (Wright and Upadhyaya, 1998; Gonzalez-Chavez *et al.,* 2004) was shown to be able to sequester metal ions, especially Cu, Pb and Cd, found at high concentrations in polluted soils. Bhanoori and Venkateswerlu, (2000) have shown the formation of a complex between the Cd and chitin in *Neurospora crassa* cell walls and proposed a structure for the chitin–Cd complex based on the results of 13C-NMR spectroscopy, X-ray diffraction and infrared spectroscopy.

2.3.1.4 Transport mechanisms involved in metal tolerance

Metal transport proteins may be involved in metal tolerance either by extruding toxic metal ions from the cytosol out of the cell or by allowing metal sequestration into intracellular compartments (Hall, 2002). Gast *et al.* (1988) suggested a transport/regulation system at the cell membrane for essential elements such as Cu and Zn, and an exclusion mechanism for Cd for some ectomycorrhizal species such as *P.involutus*. Using radiotracer flux analyses, the significant accumulation of Cd found in the vacuolar compartment has been suggested as an essential Cd detoxification mechanism in the ectomycorrhizal fungus *P. involutus* (Blaudez *et al.,* 2000). A crucial step in Cd detoxification, certainly in fission yeasts and probably in higher plants, involves the accumulation of Cd-conjugated glutathione or Cd-conjugated phytochelatins in the vacuole. This process appears to be mediated by the ATP-binding cassette transporter *Hmt1* located at the tonoplast (Ortiz *et al.,* 1992).

The yeast cadmium factor (Ycf1) gene encodes a MgATP energized glutathione S-conjugate transporter responsible for the vacuolar sequestration of bis (glutathionate) cadmium (Li *et al.,* 1997) as well as bis (glutathionate) mercury (Gueldry *et al.,* 2003). The presence of this specific permease in the tonoplast of *P. involutus* could explain the high Cd content in the vacuole (Blaudez *et al.,* 2000).

Down regulation of transporter genes involved in the uptake of metal at the plasma membrane may also be part of tolerance mechanisms, (Eide, 2003; Clemens, 2001; Hall, 2002). Interestingly, an EST sequence showed a high similarity with the yeast transcription factor Zap1, involved in the regulation of numerous metal transporters in yeast (Zhao *et al.,*

1998). Zap1 plays a direct role in controlling Zn-responsive gene expression in yeast by binding to Zn responsive elements in the promoters of genes that it regulates. A search for EST sequences encoding metal transporters promisingly indicates the presence of potential genes belonging to the ATP-binding cassette (the Ycf1 Cd-conjugate ABC transporter), cation diffusion facilitator, natural resistance-associated macrophage protein (Smf1 Mn transporter) or P-type ATPase families. Members of these transporter families have been shown to actively participate in metal detoxification of cells in a broad range of organisms (Williams *et al.,* 2000).

2.3.2 Intracellular complexation by peptides

Despite extracellular chelation and cell wall binding capacities described above, large amounts of metal may enter into the cells. Chelation of metals in the cytosol by high-affinity ligands is potentially a very important mechanism of heavy-metal detoxification and tolerance. Potential ligands include amino acids and organic acids, and two classes of peptides, the metallothioneins and phytochelatins (Rauser, 1999; Clemens, 2001).

2.3.2.1 Metallothioneins

Metallothioneins (MTs) is a family of Cys-rich, low molecular weight (3500 to 14000 Da) proteins. MTs have the capacity to bind both physiological (Zn, Cu, Se) and xenobiotic (Cd, Hg, Ag) heavy metals through the thiol group of its cysteine residues, which represents nearly the 30% of its amino acidic residues.

MTs are thought to play roles in the intracellular fixation of the essential trace elements zinc and copper, in controlling the concentrations of the free ions of these elements, in regulating their flow to their cellular destinations, in neutralizing the harmful influences of exposure to toxic elements such as cadmium and mercury and in the protection from of a variety of stress conditions. The biosynthesis of many MTs is greatly enhanced *in vivo* by certain hormones, cytokines, growth factors, tumor promoters and many other chemicals.

Fungal metallothioneins have been characterized almost exclusively in yeasts. In yeasts, several resistance mechanism are known that are activated on exposure to toxic metals, and molecular genetic analysis has been used to identify some specific genes involved in heavy metal detoxification pathways. In baker's *Saccharomyces cerevisiae,* two distinct metallothioneins have been identified, encoded by *CUP1* and *CRS5* loci. The metallothionein

gene *CUP1* encodes a low-molecular-weight (6.6 kDa), cysteine-rich metal-binding protein, which play a predominant role in copper detoxification (Fogel and Welch, 1982) and the gene can be amplified up to 20 times or more (Butt *et al.,* 1984; Hamer *et al.,* 1985).

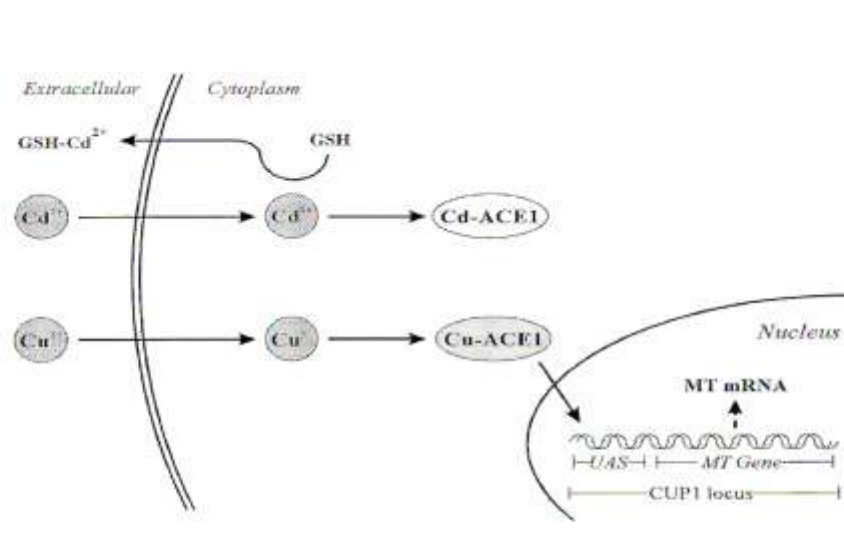

Figure 2.3: Metal detoxification pathways in yeast. *Saccharomyces cerevisiae* detoxify copper by sequestering it with the metal-binding protein metallothionein (MT). The synthesis of MT in *S. cerevisiae* is transcriptionally regulated via the binding of Cu^{+}, respectively, to the transcription factors ACE1 and AMT. The binding of copper as Cu^{+} to ACE1 produces a conformational change that increases the protein's affinity for the upstream activation sequence (UAS). The binding of cadmium by ACE1 does not lead to an active conformation. *S. cerevisia*e limited resistance to Cd^{2+} is due to its limited ability to export some Cd^{2+} as a glutathione (GSH) complex and to synthesize phytochelatins. (Adapted from Mehra *et al.*, 1991)

The multiple copy of gene allows *CUP1*-amplified strains to grow in medium with high levels of copper (Karin *et al.,* 1984), when over expressed it can also protect against Cd ions. However, it does not protect against the toxicity of Ni, Co, or Zn ions (Ecker *et al.,* 1986). Therefore, this defense mechanism is relevant to only a limited range of toxic metal ions. In contrast, the *CRS5* gene encodes a second MT, which is present as a single copy gene in yeast, provides minimal resistance to copper ion toxicity (Culotta *et al.,* 1994). In yeast, the metal-regulated transcription factor ACE1 (activator of CUP expression) regulates the transcription of the *CUP1* locus through an upstream activation sequence (UAS). Complexing of Cu^{+} or Ag^{+} produces a conformational change in ACE1 that increases its affinity for an UAS in the promoter of the metallothionein gene, leading to increased synthesis of metallothionein (Dameron *et al.,* 1993). ACE1 has little affinity for the UAS

when Cd^{2+} ions are bound (Dameron *et al.,* 1993) (Fig. 2.3). The metallothionein induction in *S. cerevisiae* is thus specific for Cu^{2+} ions. Although the *S. cerevisiae* metallothionein will bind a variety of transition metal ions *in vitro*, its synthesis is only induced *in vivo* by copper. The limited tolerance to cadmium of *S. cerevisiae* is postulated to be provided through a glutathione export mechanism (Mewes *et al.,* 1997).

Tamai et al. (1993) demonstrated that the yeast *CUP1* gene was transcriptionally activated when cells are grown in the presence of high oxygen tensions or during respiration, two conditions known to generate oxidative stress. Expression of the *CUP* 1-encoded MT suppresses a number of oxidative stress-induced growth defects of yeast strains lacking Cu, Zn superoxide dismutase. These observations demonstrate that yeast MT proteins are an important line of defense against oxidative stress.

2.3.2.2 Phytochelatins

Phytochelatins (PCs) are thiol-rich peptides whose production is induced by a range of metal (loid)s including arsenic, cadmium, copper and zinc (Grill *et al.,* 1985). They are synthesised from reduced glutathione (GSH) by the trans-peptidation of γ-glutamyl-cysteinyl dipeptides, through the action of the constitutive enzyme PC synthase (Vatamaniuk *et al*., 2000). These are mainly synthesized by plants and algae as well as in a range of filamentous fungi and yeasts (Grill *et al.,* 1985).

Phytochelatins act as chelators, and are important for heavy metal detoxification, especially cadmium metal. Although both induction of PCs *in vivo* and activation of PC synthase *in vitro* are conferred by a range of metal ions, there is little evidence supporting a role for PCs in the detoxification of such a wide range of metal ions. For metals other than Cd there are few studies demonstrating the formation of PC-metal complexes either *in vitro* or *in vivo*. PCs can form complexes with Pb, Ag, and Hg *in vitro* (Mehra *et al.,* 1996; Rauser, 1999). Phytochelatin seems to be transported into the vacuoles, so that the metal ions it carries re stored safely away from the proteins of the cytosol.

2.3.2.3 Antioxidative mechanism

The formation of free radical species, which can be initiated directly or indirectly by metals, can cause severe damage to different cellular components (Bellion *et al.,* 2006). Formation of metal-induced reactive-oxygen species could occur via several mechanisms. The Fenton or

Haber-Weiss reactions are catalysed by redox-active metals (e.g. Cu, Fe, Cr, V) and generate the highly reactive hydroxyl ($HO^{\bullet}$) radical from H_2O_2 and superoxide ($O_2^{-\bullet}$) substrates (Halliwell and Gutteridge, 1999). Redox-inactive metals such as Cd, Ni, Hg and Zn deplete glutathione and protein-bound sulphydryl groups, resulting in the production of reactive oxygen species. The successive reduction of molecular oxygen to H_2O yields the intermediates $O_2^{-\bullet}$, $HO^{\bullet}$ and H_2O_2, which are potentially toxic, because they are relatively reactive compared with O_2. Reactive oxygen species may lead to the unspecific oxidation of proteins and membrane lipids or may cause DNA injury. The control of oxidant levels is achieved by antioxidative systems. These defense systems are composed of metabolites such as ascorbate, glutathione, tocopherol, etc., and enzymatic scavengers of activated oxygen such as superoxide dismutases (SODs), peroxidases and catalases (Noctor and Foyer, 1998). Evidence for a role of reactive-oxygen species in metal-induced damage to yeast includes increased metal tolerance during anaerobicity, protection exerted by certain free radical scavengers, and the many overlaps in the molecular mechanisms used by yeasts to cope with oxidative and metal stress (Avery, 2001).

Chapter 3

Materials and Methods

3.1 Soil sampling

The soil samples were obtained from a mining area (Hindustan Zinc Limited, Zawar mines, Udaipur, 24.5800° N, 73.6800° E). The Twenty soil cores were collected in plastic bags and transported to lab in dry ice. The samples were sieved (2mm mesh size) to remove fine roots and large organic debris, and were then pooled to form a composite sample from which subsamples were taken and frozen at −80ºC until their use.

3.2 RNA extraction

3.2.1 Isolation of RNA

Soil samples were crushed in liquid nitrogen and further used for RNA isolation. The different methods (CTAB method, Trizol method, RNA PowerSoil® Total RNA Isolation Kit, kits E.Z.N.A. ® Soil RNA kit) were used to optimise protocol of RNA isolation from soil.

After optimisation of different protocols the best results were observed by using RNA PowerSoil® Total RNA Isolation Kit (Mo Bio laboratories, Carlsbad, CA).

Briefly, in the latter protocol, 2 g of soil were vortexed in presence of kit-provided silica carbide beads and solutions. Total RNA was isolated after phenol-chloroform extraction and precipitated by isopropanol. To remove residual impurities, RNA was captured on RNA capture column, washed and eluted with elution buffer. To completely remove the residual DNA contamination, the sample was treated with RNase free DNase I. Finally, the RNA was dissolved in nuclease free water.

3.2.2 Electrophoresis of RNA on agarose gel

RNA was loaded on agarose gels (0.8%) prepared in 0.5 X TBE, pH 8.0 (Appendix) using a 6 X loading dye (Appendix). Ethidium Bromide (0.5 µg/ml) was added to stain the gel prior to pouring. RNA samples were then eletrophoresed at 3 volts/cm for 45-60 minutes and visualised on a U.V. transilluminator.

3.3 Synthesis and size fractionation of cDNA

cDNA was synthesized by using the Mint-2 cDNA synthesis kit (Evrogen, Moscow, Russia) according to the manufacturer's instructions. Fig 3.1 represents the schematic outline of

cDNA synthesis by the kit. Briefly, three µg of total soil RNA were mixed with 10 µM of 3′-end CDS adapter containing an oligo(dT) sequence that anneals to poly(A) stretch of eukaryotic mRNA and 10 µM of 5′-end PlugOligo adapter containing an oligo(dG) sequence which, in presence of a specially tailored IP-solution, anneals to a complementary oligo(dC) stretch added to the 3′-end of the newly synthesized first-strand cDNA by Mint reverse transcriptase. The mixture was then incubated at 70 °C for 2 minute

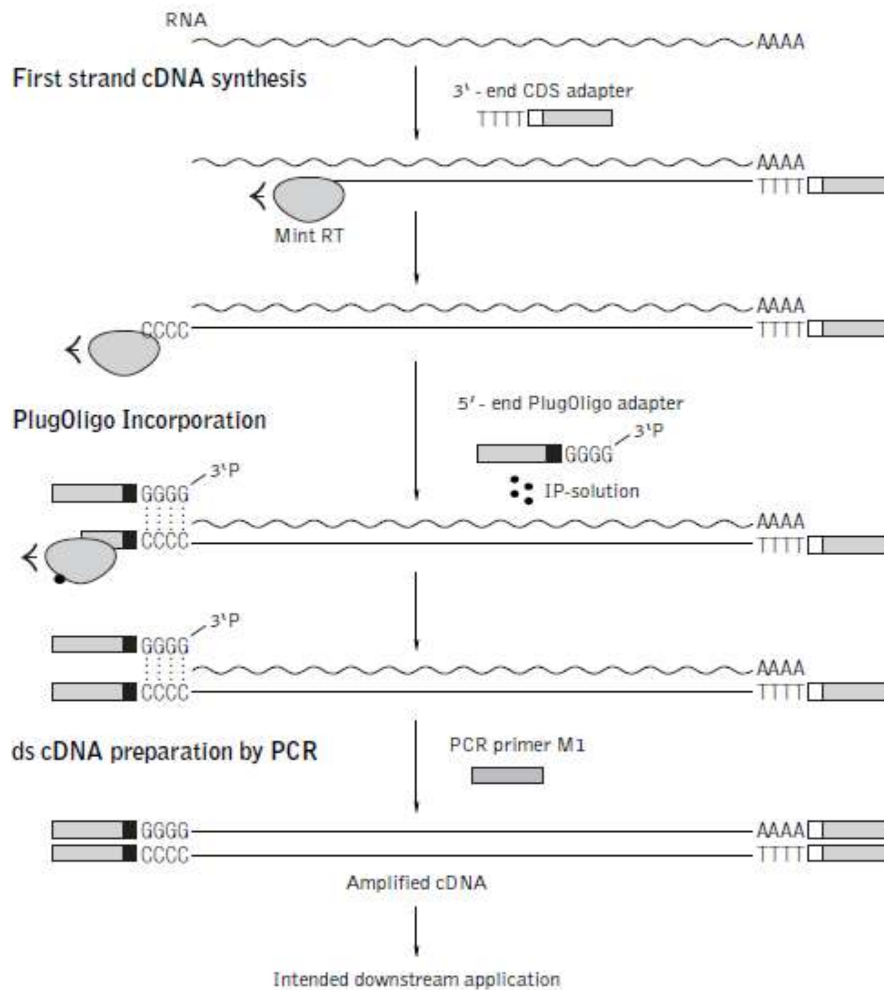

Figure 3.1: Schematic outline of Mint-2 cDNA synthesis. The rectangles represent the adapter and primer sequences and their complements. Within the rectangles, grey indicates the common external parts of the adapters, while black and white correspond to the internal parts that differ between the 3'-end and 5'-end adapters.

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First strand cDNA was synthesized at 42 °C by Mint reverse transcriptase in presence of dNTPs, DTT and first strand buffer in 10 µl of reaction volume. Second strand cDNA

synthesis and PCR amplification was carried out by Encyclo DNA polymerase (Evrogen) using the M1 primer (5'-AAGCAGTGGTATCAACGCAGAGT-3') which recognizes both the PlugOligo and CDS adapter sequences. Four µL from first strand cDNA reaction was used in second strand cDNA synthesis. A limited 3 cycle PCR amplification at 95 °C for 15 sec, 66 °C for 20 sec and 72 °C for 3 min was performed to get double stranded cDNA (ds cDNA). ds cDNA was purified by phenol-chloroform extraction and precipitated by 2.5 volume of ethanol and 1/10 volume of 3.0 M sodium acetate. To check the quality of cDNA preparation, it was amplified with EF1-α primer for the amplification of elongation factor 1-alpha gene fragments

For size fractionation of cDNA, the method described by Wellenreuther et al. (2004) was followed. Two separate but identical 0.7% (w/v) agarose gels were prepared and used for size fractionation of ds cDNA and a broad range DNA size standard respectively, at identical running conditions. After completion of electrophoresis, the gel lanes containing ds cDNA and DNA size standard were cut out. These gel lanes were rotated at 90° and placed into two separate but identical gel trays. 1.4% low melting point agarose gels were cast in both the trays and subjected to electrophoresis at 37 V for 10 h. The unstained gel containing cDNA was then superimposed over the ethidium bromide stained gel containing the size standard which was visualized using a Dark Reader trans-illuminator. Three gel slices having different cDNA size fractions (fraction A, 0.1 kb-0.5 kb; fraction B, 0.5 kb-1.0 kb and fraction C, 1.0 kb-4.0 kb) were cut out from the unstained gel. By using QIAEX II Gel Extraction Kit (Qiagen, Netherlands), cDNA was extracted from the gel slices which was then precipitated and amplified by PCR using primer M1. The number of PCR cycles which were performed on fractions A, B and C were 30, 26 and 22, respectively.

3.4 Cloning and library preparation

Restriction digestion of PCR-amplified cDNA fractions was done by using SfiI enzyme. This restriction endonuclease recognize SfiIA and SfiIB sites located in the sequences of primers PlugOligo and CDS, respectively. After digestion, cDNAs were extracted by phenol-chloroform and precipitated by 2.5 volume of cold ethanol and 1/10 volume of 3.0 M sodium acetate. cDNAs were then ligated downstream of the *S. cerevisiae* PGK promoter in a modified pFL61 yeast- *E.coli* shuttle vector containing the SfiIA and SfiIB restriction sites (Minet et al., 1992; Bailly et al., 2007) (Fig 3.2). Recombinant plasmids were introduced into

electro-competent *E. coli* cells (MegaX DH10B™ T1R Electrocomp™ cells, Invitrogen) by the following method:

1. 1 µl DNA was added to microcentrifuge tubes.

2. ElectroMAX™DH10B™ Cells were thawed on wet ice and mixed by tapping gently. 20 µl of cells was added to each chilled microcentrifuge tube containing DNA.

3. The cell/DNA mixture was pipetted into a chilled 2mm cuvette. The cuvette was gently tapped to ensure that the cell/DNA mixture made contact all the way across the bottom of the cuvette chamber. Formation of bubbles was avoided.

6. Electroporation was carried out in BioRad GenePulse® II electroporator, following electroporator conditions:

- Voltage: 2.5 kV
- Resistance: 650 Ω
- Capacitance: 25 µF

7. To the cells in the cuvette, 1 ml of S.O.C. medium (Appendix) was added and then the solution was transferred to a 15 ml falcon tube.

8. The tube was incubated at 225 rpm (37°C) for 1 hour.

9. 100-200 µl of the culture was spread on LA + Amp plates (Appendix). The plates were incubated overnight at 37°C.

At least 106 independent bacterial colonies growing on solid agar medium were collected to constitute each of the libraries.

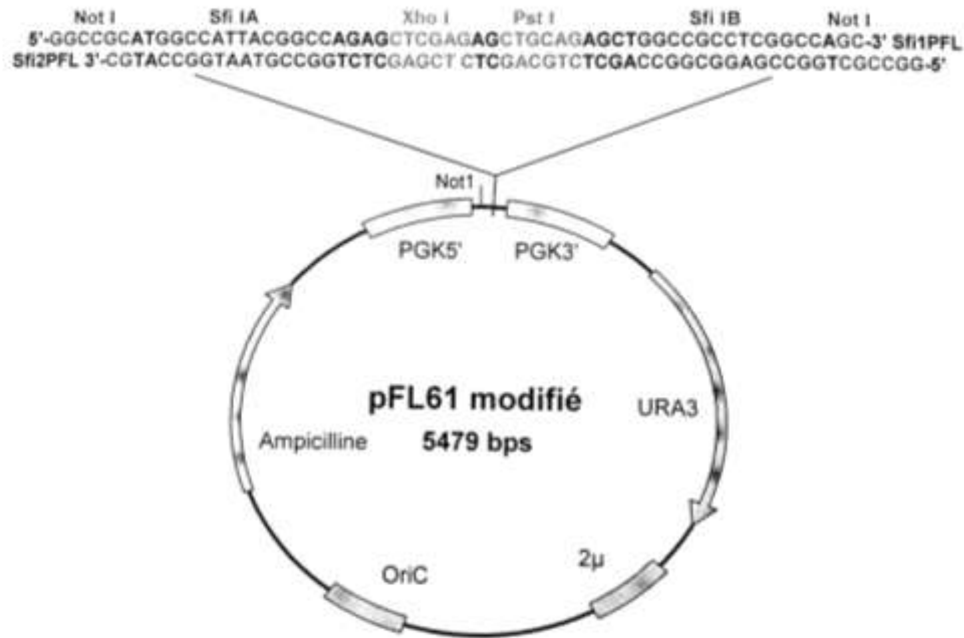

Figure 3.2: Modified pFL61 vector**.** OriC, origin of replication for multiplication in *E.coli ;* 2µ, origin of replication of plasmid 2µ of *S.cerevisiae* ; Ampicilline, selection marker for maintainance of plasmid in *E.coli* ; Ura 3, selection marker for maintainance of plasmid in *S.cerevisiae*

3.5 Plasmid isolation

The plasmid isolation was carried out from constructed libraries P1 (insert size 1-3 kb) and P2 (insert size < 1.0 kb) using QIAGEN Plasmid *Plus* Maxi Kit

1. Single colony was picked from a freshly streaked selective plate and inoculated in starter culture of 2–5 ml LB medium containing ampicillin. The culture was incubated for approximately 8 hr at 37°C with vigorous shaking (approx. 300 rpm).
2. The starter culture was diluted 1/500 to 1/1000 into 3 ml selective LB medium and incubated at 37°C for 12–16 hr with vigorous shaking (approx. 300 rpm).
3. The bacterial cells were harvested by centrifugation at 6000 x g for 15 min at 4°C.
4. The bacterial pellet was resuspended in 0.3 ml of Buffer P1.
5. 0.3 ml of Buffer P2 was added, mixed thoroughly by vigorously inverting the sealed tube 4–6 times, and incubated at room temperature (15–25°C) for 5 minutes.
6. 0.3 ml of chilled Buffer P3 was added, mixed immediately and thoroughly by vigorously inverting 4–6 times, and incubated on ice for 5 min.

7. Centrifuged at maximum speed in a microcentrifuge for 10 minutes. Supernatant containing plasmid DNA was removed promptly.
8. A QIAGEN-tip 20 was equilibrated by applying 1 ml Buffer QBT, and the column was allowed to empty by gravity flow.
9. The supernatant from step 7 was applied to the QIAGEN-tip 20 and allowed it to enter the resin by gravity flow.
10. The QIAGEN-tip 20 was washed with 2 x 2 ml Buffer QC.
11. DNA was eluted with 0.8 ml Buffer QF.
12. To precipitate the eluted DNA 0.7 volumes (0.56 ml per 0.8 ml of elution volume) of room-temperature isopropanol was added, mixed properly and centrifuged immediately at ≥10,000 rpm for 30 min in a microcentrifuge. The supernatant was carefully decanted.
13. DNA pellet was washed with 1 ml of 70% ethanol and centrifuged at 10,000 rpm for 10 minutes. The supernatant was carefully decanted without disturbing the pellet.
14. The pellet was air dried for 5–10 minutes, and the DNA was redissolved in a suitable volume of buffer (TE buffer, pH 8.0).

3.6 Colony PCR

A pin-point amount of bacterial cells was added in 5 microliters of ultrapure water, mixed and incubated at 95ºC (in PCR) for 10 minutes. The tubes were then quick chilled in ice and vortex for 5 seconds. Then they were quick centrifuged and 3 µl of supernatant was transferred in fresh tube. PCR mastermix was added in the tube. The PCR was performed with primers pFL61 L1 (5'-CTTCTAACCAAGGGGTGGTTTAGTTTAG-3') and pFL61 R1 (5'-CTGCATAAAGGCATTAAAAAGAGGAGCG-3'). The PCR program was carried out as follows: initial denaturation at 94ºC for 5 minutes, followed by 25 cycles for 30 seconds at 94ºC, 1 minute at 55ºC annealing temperature, 1 minute at 72ºC and final a extension at 72ºC for 10 minutes. Amplified products were visualized on a 0.8% (w/v) agarose gel.

3.7 Yeast Transformation

3.7.1 Yeast and culture conditions

The *S. cerevisae* strains, copper- sensitive DTY4 (*MATα trp1-1 leu2-3 112his3*$^{\Delta}$*1 gal1 ura3-50, cup1*$^{\Delta}$*::URA3*$^{+}$), cadmium sensitive *ycf1* (*MATα his3D1 leu2D met15D0 ura3D YDR135::kanMX4*) and zinc sensitive *zrc1* (*MATα can1-100 his3-11,15 leu2-3,112 trp1-1*

ura3-52 zrc1::His3) derived from the wild type strain BY4741 (*MATα his3D1 leu2D0 met15D0 ura3D0*) were used in this study. These yeast strains were maintained on YPD agar medium (Appendix) or appropriate SD agar medium at 30ºC (Appendix). For complementation studies, transformed cells were selected by their capacity to grow in complete synthetic medium (SD), without Ura (pFL61 vector selection marker).

3.7.2 Transformation

These yeast strains were transformed with plasmids isolated from constructed libraries (P1 & P2), control vector pFL61 separately by using the standard lithium acetate method.

1. 1 colony of the yeast strain was inoculated in 5ml YPD medium and incubated at 200 rpm, 30 ºC for 5-6 hrs.
2. 400 µl of this culture was inoculated in 40 ml YPD medium and incubated overnight at same conditions.
3. Fresh 50 ml YPD media was taken and its $O.D._{600nm}$ was set at 0.5 by inoculating from previous culture. It was then incubated at same conditions till the $O.D._{600}$ reaches 0.7-0.8.
4. The culture was centrifuged in 50 ml falcon tube at 3000 rpm for 5 minutes.
5. The supernatant was discarded and pellet was resuspended with 25 ml autocalved distilled water and again centrifuged at same parameters.
6. The pellet was resuspended with 1 ml autocalved distilled water.
7. The yeast suspension was transferred in a 1.5 ml eppendorf tube and centrifuged for 30 seconds with the bench centrifuge. The supernatant was discarded.
8. The pellet was resuspended with 1 ml autocalved distilled water.
9. The cells were distributed 100 µl/transformation in 1.5 ml eppendorf tubes, centrifuged for 30 seconds with the bench centrifuge. The supernatant was discarded.
10. The following mixture was added in each tube.

PEG 3500 50% w/v	240 µl
LiAc 1M	36 µl
Salmon sperm DNA 10mg/ml (boiled 5 min and put immediately on ice)	10 µl
DNA + H_2O	74 µl
Total	360 µl

11. The eppendorf tubes were incubated in a water bath for 1 hour at 42ºC, centrifuged for 30 seconds with the bench centrifuge and the supernatant was discarded.
12. The pellet was resuspended with 1ml autocalved distilled water.
13. The yeast cells were spread on selective Petri Dishes and incubated for 2/3 days at 30 ºC.

3.8 Yeast functional complementation assays

For functional complementation experiments, cultures of *ycf1* and $cup1^{\Delta}$ yeast cells carrying either pFL61 or plasmids from library P1/P2 were grown in each respective medium at 30 ºC and 200 rpm. Yeast cultures were adjusted to OD_{600} =1.0 and 5 µl serial dilutions were spotted on SD plates and on SD supplemented with 150µM $CuSO_4$ and 40µM $CdSO_4$ plates. Plates were incubated for 3days at 30 ºC and photographed.

In parallel experiments, Falcon jars containing 20 ml of fresh SD-Ura media were inoculated with mid-log precultures of *ycf1* and $cup1^{\Delta}$cells containing pFL61, plasmids from library P1/P2 to attain a starting optical density of 0.02 at 600nm. Cells were grown at 30 ºC and 200 rpm and $CuSO_4$ (150µM) and $CdSO_4$ (40µM) were added 5 hours after inoculation. The optical densities of the cultures were measured at 2-3 hours interval for 42 hours.

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Chapter 4

Results and Discussion

4.1 RNA isolation

Soil sample was crushed in liguid nitrogen and RNA isolation was done by using Trizol method, CTAB method and by commercial kits E.Z.N.A. ® Soil RNA kit, RNA PowerSoil® Total RNA Isolation Kit. Best yield of RNA with good quality was obtained by RNA isolation through commercial kit, RNA PowerSoil® Total RNA Isolation Kit. Isolated RNA was checked on 0.8% (w/v) agarose gel and visualized by ethidium bromide staining under U.V light (Fig. 4.1).

Figure 4.1: Total RNA isolation from Soil. Lane1: Ladder, Lane 2, 3, 4: Total RNA

4.2 cDNA synthesis from total soil RNA

mRNA constitutes only a small portion of the total RNA in the cell. So when total RNA is isolated from soil, the quantification of poly-mRNA is expected to be lower. Further purification of µg amounts of poly-A mRNA, as recommended in most cDNA library construction protocols, can hardly be met. Therefore protocol implemented in the Mint-2 kit (Evrogen) was adopted, which authorizes the use of few µg of total RNA as starting material and includes long range PCR amplification of the synthesized cDNAs. This protocol allowed obtaining ds cDNA of eukaryotic cDNAs from 3 µg of total RNA. To check the quality of cDNA preparation, it was amplified with EF1-α primer for the amplification of elongation factor 1-alpha gene fragments (Fig.4.2).

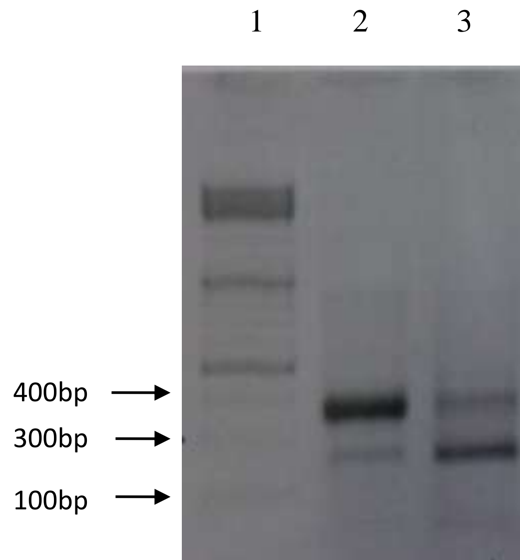

Figure 4.2: cDNA amplified with EF1-α.primer. Lane 1: 100 bp ladder, Lane 2, 3: cDNAs amplified with EF1-α

4.3 Size fractionation and PCR amplification of eukaryotic cDNA

One of the main problems encountered in cDNA library construction is getting low number of clones having long full-length inserts. As a result, a large number of genes of interest are often only represented by truncated cDNAs. This problem can arise when the initial RNA sample is partially degraded, when reverse transcription stops prematurely, or when a PCR amplification step is introduced in the protocol as it will bias against longer cDNAs (Karrer *et al*., 1995). Furthermore, high number of PCR cycles to enhance the quantity of long cDNAs is not recommended because of the error rates of polymerases (Kunkel, 2004). In addition, preferential ligation of small inserts during cloning also occurs. Size fractionation of cDNA before cloning to get sized cDNA libraries can circumvent this problem. In the current protocol, ds cDNAs, amplified through only three PCR cycles, were separated by bi-dimensional agarose gel electrophoresis to maximize complete separation of cDNAs on the basis of their sizes and minimize cross-contamination of size fractions. The cDNA containing gel was left unstained to prevent the adverse effects of ethidium bromide staining but also because of the very low quantity of loaded cDNA which prevented their direct visualization. Three different cDNA size fractions; 0.1-0.5 kb (Fraction A), 0.5-1 kb (fraction B) and 1-4 kb (fraction C) were recovered and amplified separately by PCR using between 22 and 30 cycles.

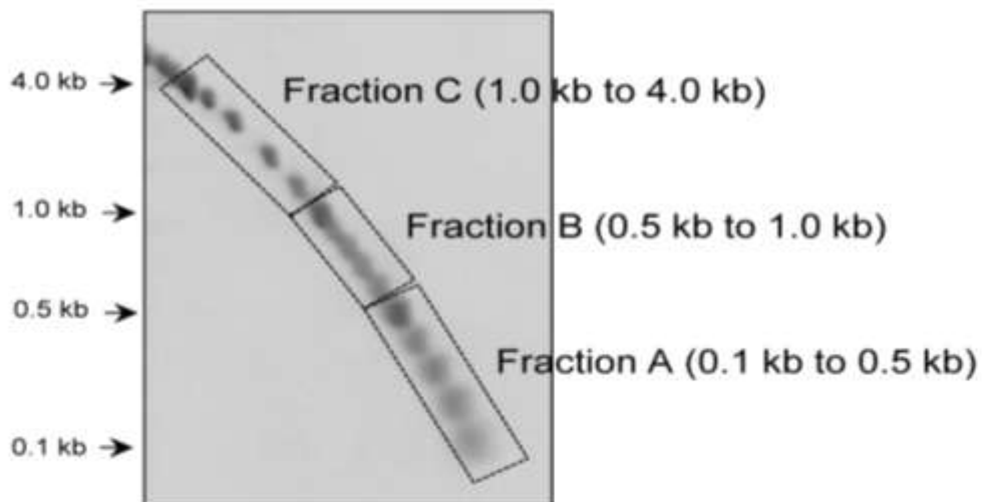

Figure 4.3: Size fractionation of eukaryotic cDNA

4.4 Colony PCR:

To confirm the construction of the cDNA libraries, colony PCR was performed. The bacterial colonies were picked up randomly from LA+ Amp plates for both cDNA libraries P1 (insert size 1-3 kb) and P2 (insert size < 1.0 kb), amplified with primers pFL61 L1 and pFL61 R1. PCR products were separated on 0.8% (w/v) agarose gels and visualized by ethidium bromide staining under U.V light (Fig. 4.1)

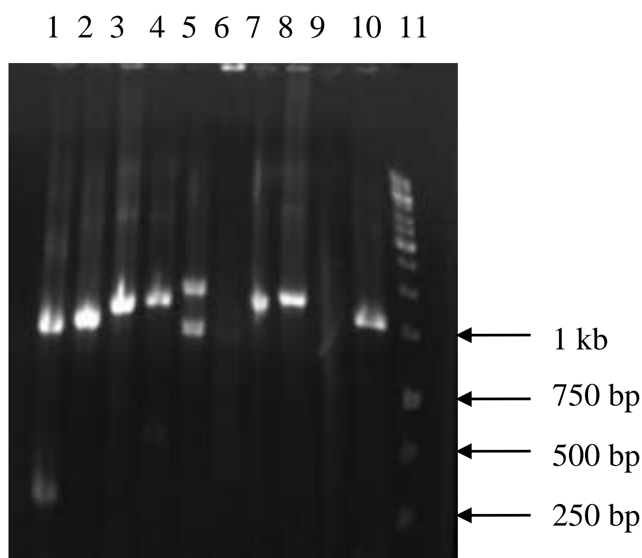

Figure 4.4: Colony PCR of P1 library. Lane 1-10: PCR products form 10 randomly picked colonies, Lane 11: 1 kb ladder

The bands were observed in size range of 1-3 kb and below 1.0 kb after separation of PCR products from both libraries on separate gels (Fig. 4.4 , Fig. 4.5), establishing that cDNA libraries have been successfully constructed.

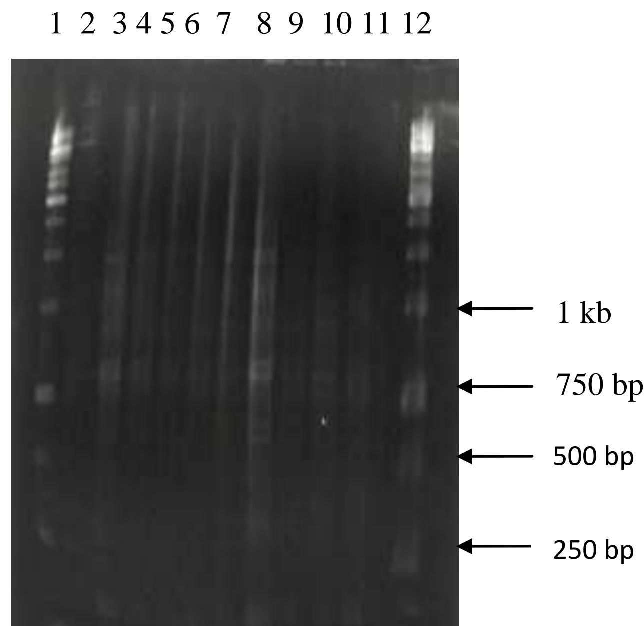

Figure 4.5: Colony PCR of P2 library. Lane 1, 12: 1 kb ladder, Lane 2-9: PCR products form 10 randomly picked colonies,

4.5 Yeast complementation studies

To identify the cDNAs that were able to increase copper/cadmium or zinc tolerance, the *cup1*$^{\Delta}$ and, *ycf1, zrc1* yeast strains were transformed with the two environmental cDNA libraries described above. Transformations were performed using the standard lithium acetate method. Metal tolerant ura+ transformants were selected after plating the transformants on SD-Ura medium supplemented with that particular metal.

4.5.1 Copper studies

Yeast transformation results showed that empty pFL61 transformed *cup1*$^{\Delta}$ cells were unable to grow at 150 µM copper supplemented media. In case of transformation of *cup1*$^{\Delta}$ cells with plasmids from library P1, two copper tolerant ura+ transformants were obtained which were randomly named as A1 and A2. Similarly a single copper tolerant ura+ transformant was obtained in the transformation with plasmids from library P2 and it was named as B1.

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These three transformants were streaked on SD-Ura plates supplemented with 150µM $CuSO_4$ to confirm their growth. After getting the positive results, drop out assay of these

transformants was done. *cup1*$^{\Delta}$ cells carrying pFL61 was grown in SD medium at 30 ºC and 200 rpm. Similarly three transformants (A1, A2 and B1) and wild type strain BY4741 were grown in SD-Ura medium at the same conditions. These yeast cultures were adjusted to OD_{600}=1.0 and 5 µl serial dilutions were spotted on SD−Ura plates and on SD−Ura supplemented with 150µM $CuSO_4$ plates. Plates were incubated for 3days at 30ºC and photographed (Fig. 4.6).

The *cup1*$^{\Delta}$ containing empty pFL61, the transformants A1, A2 and B1 were tested for resistance to 150 µM of copper in a liquid medium growth assay for 48 hours. The results showed that the *cup1*$^{\Delta}$ containing empty pFL61cells were unable to grow on 150 µM copper containing media and their growth was completely inhibited, whereas in case of A1,A2 and B1 growth was increased significantly with increasing time of incubation (Table 1, Fig.4.7). The present results indicated that insert in the plasmid was responsible for copper tolerance.

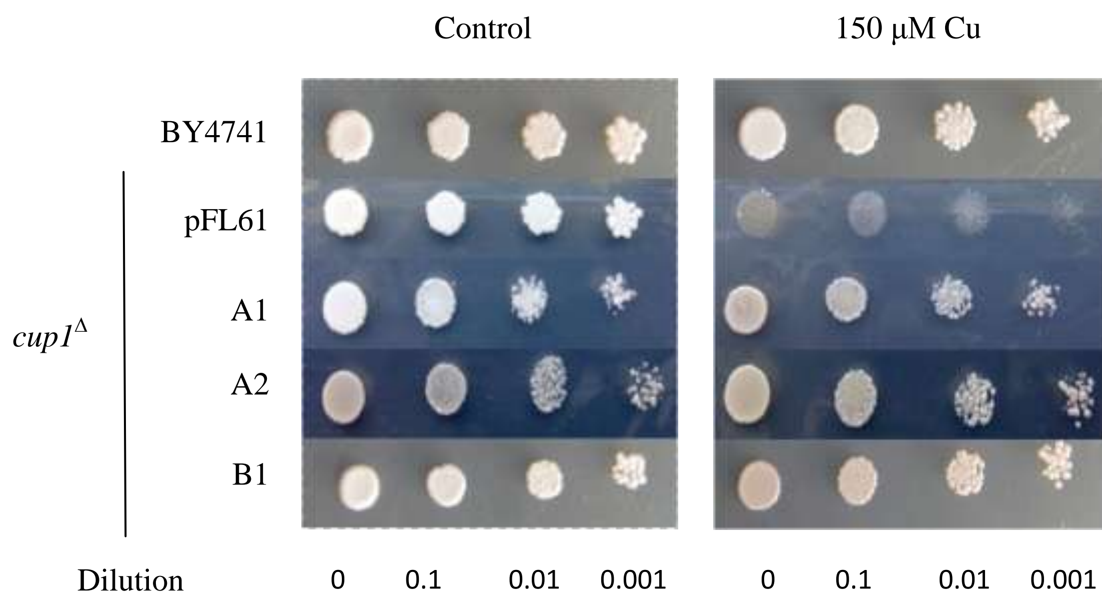

Figure 4.6: Functional complementation of yeast mutants on SD-Ura medium supplemented (or not) with 150 µM Cu. *cup1*$^{\Delta}$ cells harbouring the control vector pFL61 were unable to grow on Cu containing media, where as A1,A2 ,B1 and wild type strain BY4741 cells were grown well on Cu medium.

Table 4.1 Growth curve of copper tolerant ura+ transformants (A1,A2 and B1) and *cup1*$^{\Delta}$ cells transformed with the control vector pFL61 in SD−Ura medium supplemented (or not) with 150 μM Cu.

Time (hours)	pFL61	pFl61+Cu	A1+Cu	A2+Cu	B1+Cu
8	1.340±0.002	1.286±0.006	1.386±0.031	1.264±0.010	1.287±0.018
12	1.373±0.005	1.293±0.006	1.441±0.024	1.358±0.001	1.414±0.017
16	1.404±0.006	1.295±0.005	1.491±0.004	1.482±0.014	1.459±0.015
19	1.421±0.010	1.297±0.005	1.597±0.006	1.621±0.011	1.669±0.026
22	1.484±0.039	1.303±0.003	1.610±0.005	1.639±0.009	1.689±0.027
25	1.569±0.071	1.307±0.003	1.621±0.004	1.650±0.006	1.716±0.025
28	1.657±0.057	1.313±0.003	1.626±0.003	1.670±0.013	1.727±0.028
32	1.725±0.068	1.313±0.003	1.635±0.004	1.676±0.014	1.796±0.035
48	1.692±0.055	1.310±0.006	1.632±0.004	1.594±0.056	1.754±0.031

Mean±SEM

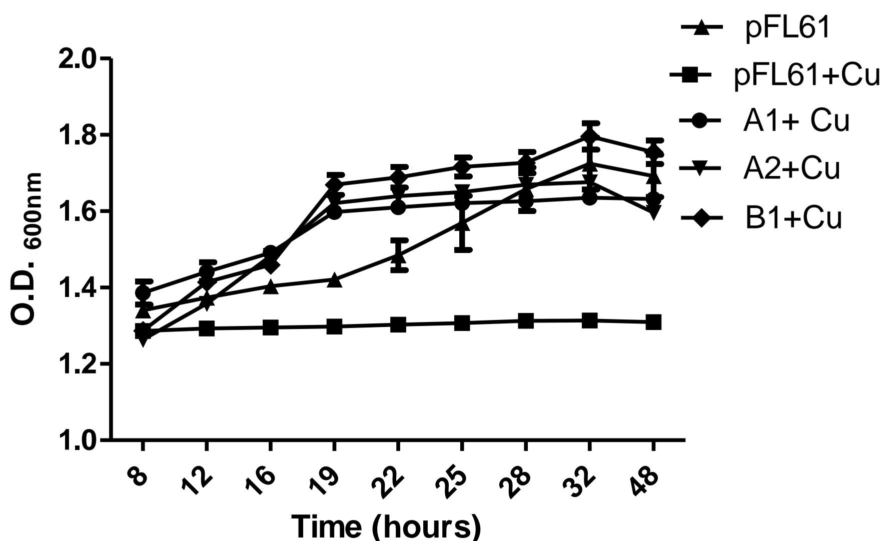

Figure 4.7: Growth curve of copper tolerant ura+ transformants (A1, A2 and B1) and *cup1*$^{\Delta}$ cells transformed with the control vector pFL61 in SD−Ura medium supplemented (or not) with 150 μM Cu.

4.5.2 Cadmium Studies

The *ycf1* mutant cells transformed with plasmids from P1 library showed no cadmium tolerant ura+ transformants on SD−Ura plates supplemented with 40 µM cadmium, while in case of transformation with plasmids from P2 library, 3 such transformants were observed. These transformants were randomly named as C1, C2, and C3.

The complementation studies of *ycf1* on Cd containing medium showed that *ycf1* transformed with empty pFL61 vector was unable to grow at 40 µM cadmium, whereas the Cd-sensitive phenotype of the *ycf1* mutant was fully complemented in case of C1, C2 and C3 (Fig. 4.10) . These transformants were further characterised by liquid medium growth assay.

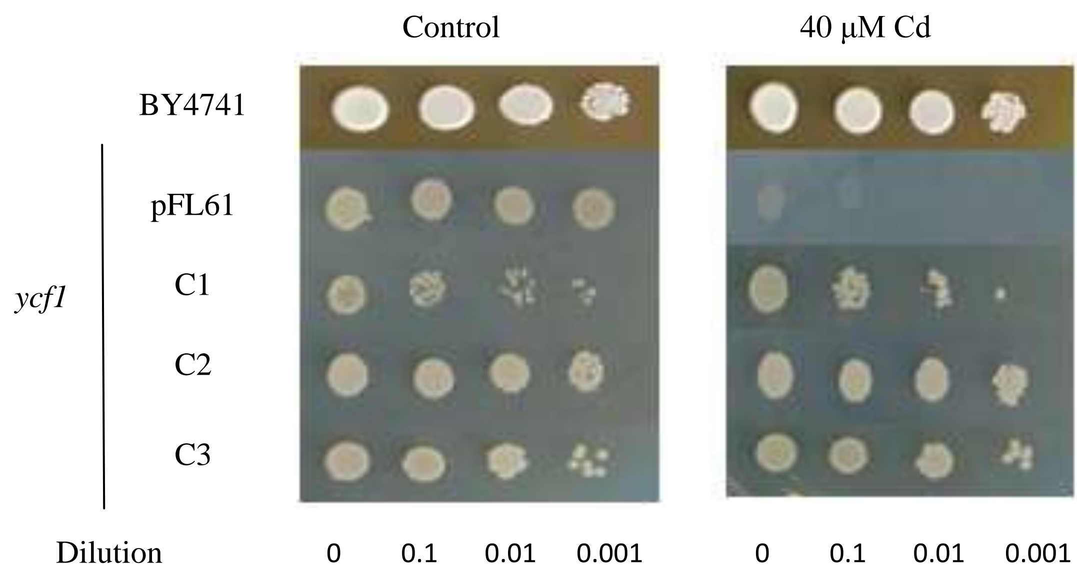

Figure 4.8: Functional complementation of yeast mutants on SD-Ura medium supplemented (or not) with 40 µM Cd. *ycf1* cells harbouring the control vector pFL61 were unable to grow on Cu containing media, where as A1,A2 B1 and wild type strain BY4741 cells were grown well on Cu supplemented medium.

Figure 4.7: Growth curve of cadmium tolerant ura+ transformants (C1, C2 and C3) and *cup1*$^{\Delta}$ cells transformed with the control vector pFL61 in SD−Ura medium supplemented (or not) with 40 µM Cd.

Time (hours)	pFL61	pFl61+Cd	C1+Cd	C2+Cd	C3+Cd
8	1.340±0.002	1.293±0.006	1.2496±0.004	1.263±0.009	1.287±0.018
12	1.373±0.004	1.313±0.013	1.326±0.004	1.358±0.001	1.414±0.016
16	1.404±0.005	1.317±0.013	1.384±0.005	1.495±0.005	1.459±0.014
19	1.428±0.004	1.326±0.014	1.439±0.008	1.621±0.011	1.669±0.026
22	1.552±0.002	1.329±0.014	1.513±0.004	1.639±0.009	1.689±0.027
25	1.593±0.052	1.332±0.014	1.575±0.002	1.650±0.006	1.716±0.027
28	1.657±0.057	1.329±0.013	1.625±0.002	1.677±0.005	1.727±0.027
32	1.725±0.067	1.325±0.012	1.656±0.002	1.686±0.004	1.796±0.034
48	1.782±0.002	1.315±0.007	1.642±0.001	1.669±0.001	1.754±0.030

Mean±SEM

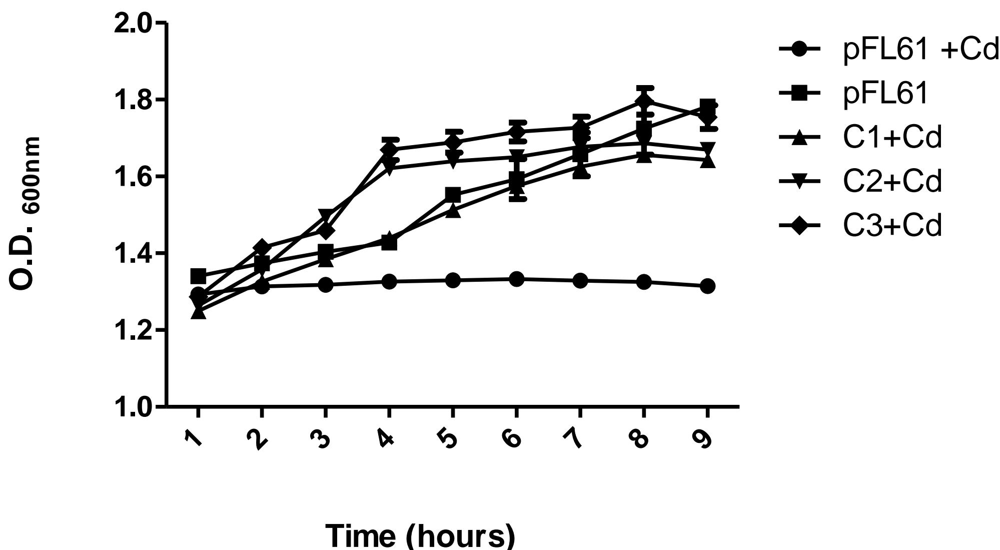

Figure 4.9: Growth curve of cadmium tolerant ura+ transformants (C1, C2 and C3) and *cup1*$^{\Delta}$ cells transformed with the control vector pFL61 in SD−Ura medium supplemented (or not) with 40 µM Cd.

4.5.3 Zinc studies

Several colonies were observed in case of *cup1*$^{\Delta}$ cells transformed with plasmids from library P1, P2 on SD-Ura plates supplemented with 6mM zinc. But *cup1*$^{\Delta}$ cells transformed with pFL61 vector were also able to grow on SD-Ura also, which may be due to flocculation of zinc in SD medium at pH 5.9. When the pH of the medium was decreased to avoid this flocculation, cells were not able to grow at the acidic conditions. So optimisation of zinc concentration and pH is required for the further studies.

In the present study, transformants were selected for their ability to rescue the metal (Cu or Cd) sensitive phenotype of yeast mutants by functional complementation studies. The cDNA insert in the plasmid which was transformed in mutant yeast cells may be responsible for providing this phenotype. The recent studies by following the same approach indicated that the metal resistance was caused by genes showing homology to metallothioneins (Lehembre *et al.,* 2013, Leonhardt *et al.,* 2014). Metallothionein-like peptides are involved in sequestration of Zn in the Zn-accumulating ectomycorrhizal fungus *Russula atropurpurea.* Screening of an *R. atropurpurea* cDNA library for sequences encoding peptides capable of sequestering divalent heavy metals, conducted in the Cd-hypersensitive *ycf1* yeast allowed identification of two cDNAs, RaZBP1 and RaZBP2, which protected the metal-sensitive yeast mutants against Cd and Zn, but not Co, Mn or Cu, toxicity (Leonhardt *et al.,* 2014).

Other studies showed that presence of metal ions induced the expression of metal specific metallothioneins (Gross *et al.,* 2000*;* Loebus *et al.,* 2013).

Regarding metal tolerance, functional analysis of the recovered genes and their protein products suggest that several of them (i) originated from unknown taxonomic groups and (ii) could act by interfering with cellular processes that have been poorly studied in the context of heavy metal homeostasis. In this respect, these genes could be used to probe these processes and to evaluate their contribution to the overall capacity of organisms to cope with heavy metals. These genes could find applications as biomarkers either to detect heavy metals in the environment or to manipulate the tolerance/ resistance levels of species of interest for the bioremediation or revegetation of polluted lands (Lehembre *et al.,* 2013).

Conclusion

In the present study, cDNA libraries were successfully constructed from the RNA isolated from metal contaminated soil. Transformation of yeast mutants was done with the plasmids isolated from both libraries. Two copper tolerant ura+ transformants were obtained from the transformation with plasmids from library P1. While in case of library P2, one copper tolerant ura+ transformant and three cadmium tolerant ura+ transformants were selected. These all transformants were further characterised by drop out assay and liquid growth assay. The growth curve studies showed that metal sensitive phenotype of yeast mutants was fully complemented in these transformants and they showed significant growth in medium supplemented with metal. For the further studies, plasmids from these metal tolerant transformants need to be isolated and the sequencing of cDNA inserts need to be done to identify and characterise the genes conferring metal resistance.

This study demonstrates the feasibility of the metatranscriptomic approach, from soil-extracted RNA to the recovery of functional cDNAs expressed in a eukayotic heterologous host cell (*S. Cerevisiae*). In this respect, this approach developed for eukaryotic micro-organisms has the same potential as the traditional metagenomic approach for the cloning of single genes, coding enzymes of interest, from the environment. In conclusion, the screening strategy used here appears useful not only for detecting functional transcripts from unknown microbes (especially eukaryotes) but also for heterologous production of the corresponding enzymes, including biotechnologically relevant ones.

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Appendix

TBE Buffer (10x)

Tris-HCl 0.09 M (pH 8)

Boric acid 0.9 M

EDTA 0.02 M (pH 8)

Agarose gel loading dye (6X)

Bromophenol blue 0.25%

Xylene cyanol FF 0.25%

Glycerol in water 30.0%

Luria-Bertani (LB) Medium

Ingredient	Quantity (g/l)
NaCl	10.0
Beef extract	5.0
Tryptone	10.0

pH adjusted to 8.0 with 1N NaOH, sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 min.

LB+ Amp plates

Prepared LB broth as above. Agar (15 g/l) was added, media was autoclaved, and cooled to 50°C. Ampicillin 50 µg/ml was added. Poured plates and stored at 4°C.

YPD Medium

Ingredient	Quantity (g/l)
Peptone	20
Yeast extract	10
Dextrose	20
Agar (for plates only)	20

pH adjusted to 6.5, sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 min.

Buffers used in Plasmid Isolation:

Buffer	Composition
Buffer P1 (resuspension buffer)	50 mM Tris·Cl, pH 8.0 10 mM EDTA 100 µg/ml RNase A
Buffer P2 (lysis buffer)	200 mM NaOH, 1% SDS (w/v)
Buffer P3 (neutralization buffer)	3.0 M potassium acetate pH 5.5
Buffer QBT (equilibration buffer)	750 mM NaCl 50 mM MOPS, pH 7.0 15% isopropanol (v/v); 0.15% Triton® X-100 (v/v)
Buffer QC (wash buffer)	1.0 M NaCl 50 mM MOPS, pH 7.0; 15% isopropanol (v/v)
Buffer QF (elution buffer)	1.25 M NaCl 50 mM Tris·Cl, pH 8.5 15% isopropanol (v/v)
TE	10 mM Tris·Cl, pH 8.0 1 mM EDTA

SD medium

Yeast nitrogen base (without amino acids)	6.7 g/L
Agar (for plates only)	20 g/L
10X Dropout Solution	100 ml

10X Dropout Solution

Nutrient	10X Concentration
L-Adenine hemisulfate salt	200 mg/L
L-Arginine HCl	200 mg/L
L-Histidine HCl	200 mg/L
L-Isoleucine	300 mg/L
L-Leucine	1000 mg/L
L-Lysine HCl	300 mg/L
L-Methionine	200 mg/L
L-Phenylalanine	500 mg/L
L-Threonine	2000 mg/L
L-Tryptophan	200 mg/L
L-Tyrosine	300 mg/L
L-Uracil	200 mg/L
L-Valine	1500 mg/L

To make one liter of 10X –Ura Droupout Solution, combined all amino acids except Uracil.

S.O.C. medium

Tryptone	20 g/ L
Yeast extract	5 g /L
10mM NaCl	0.584 g/ L
2.5mM KCl	0.186 g/ L
20mM glucose	3.603 g/ L

10mM $MgCl_2$ (anhydrous 0.952 g) or 10mM $MgSO_4$