Cloning and characterization of cobalt tolerance genes by metatranscriptomics approach

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By
VINITA
(ROLL NO. 601304016)

Under the esteemed guidance of
Dr. M.S. REDDY
(Professor)

DEPARTMENT OF BIOTECHNOLOGY
THAPAR UNIVERSITY
PATIALA, PUNJAB-147004
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Declaration

I, the undersigned, hereby declare that the research work presented in the M. Tech project entitled "**Cloning and characterization of cobalt tolerance genes by metatranscriptomics approach**" has been carried out by me under the supervision and guidance of Prof. M.S. Reddy, Department of Biotechnology, Thapar University, Patiala. Further, I declare that no part of this dissertation has been submitted for a degree or any other qualification of any other university or examining body in India/elsewhere.

Vinita

Place: Patiala Vinita
Date: 15-7-15 601304016

Certificate

This is to certify that thesis entitled, "**Cloning and characterization of cobalt tolerance genes by metatranscriptomics approach**" submitted by Ms. Vinita in partial fulfillment of the requirements for the award of Masters in Technology in Department of Biotechnology at Thapar University, Patiala is an authentic work carried out by her under our supervision and guidance. To the best of our knowledge, the matter embodied in this thesis has not been submitted to any other university/ institute for award of any Degree or Diploma.

Dr. M.S. Reddy
Professor
Department of Biotechnolgy

Dr. Dinesh Goyal
Head of Department
Department of Biotechnology

Dr. S.S. Bhatia
Dean
Academic Affairs
Thapar University, Patiala

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Vinita

Vinita

Abstract

Heavy metal contamination in soil is posing serious threat to environment. These are potentially toxic, depending on their bio available concentrations and sensitivity of the organism that is exposed. 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. In the present study, functional metatranscriptomics approach was adopted to identify cobalt tolerance genes expressed by eukaryotic species. Size fractionated libraries were constructed in the modified pFL61 yeast-*E. coli* shuttle vector. The fractionated library was screened for the presence of cobalt tolerance genes by functional complementation of *Saccharomyces cerevisae* mutant. The mutant strain, cobalt sensitive *COT1* was used in this study. Yeast transformants exhibiting cobalt tolerance were identified by plating on medium supplemented with cobalt further characterized by studying their growth kinetics in the presence of cobalt.

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Chapter 1

Introduction

Soil covers almost all of the terrestrial area on Earth and has an essential ecological function in the global cycles of carbon, nitrogen and sulfur. Due to their physico-chemical complication with many micro-niches, they abound with bio-diversity, both phylogenetically and functionally. Soil microbial biomass, huge microorganisms in soil has proved useful for modeling nutrient and energy cycling (Stockdale *et al*., 2006). A single gram of soil has been estimated to contain thousands to millions of different bacterial, archaeal and eukaryotic species (Torsvik *et al*., 2002; Gans *et al*., 2005) interwoven in extremely complex food webs. Soil microbial communities carry out a large number of small-scale processes that underlie many environmentally important functions. Microbial diversity provides infinite but largely untapped resources for the development of novel genes, enzymes, natural products, bioactive compounds, and bioprocesses that may significantly impact industrial and biotechnological applications.

However, the clear functional and ecological roles of individual taxa remain uncertain because it's not possible to cultivate most of them in laboratory condition (Prosser, 2007; Fierer *et al*., 2007). Such uncultivable eukaryotic microorganisms cannot be easily isolated from these complex environmental matrices and cannot be grown *in* vitro. With various metagenomic techniques, new scope in the characterization of complex microbial communities has been reached. Large scale shotgun sequencing approaches permit the discovery of many novel genes found in the environments independent of cultivation efforts (Martin-Cuadrado *et al*., 2007; Rusch *et al*., 2007; Tringe *et al*., 2005; Venter *et al*., 2004).

The term metagenomics, the genomic analysis of a population of microorganisms, was coined by Handelsman *et al*. (1998) with a concept to analyze a collection of similar but not identical items, as in the concept of meta-analysis (Handelsman *et al*., 1998). Metagenomics have been successfully developed to address the issue of analysis of uncultured microbes. Metagenomics refers to collective analysis of the overall microbial community genomes of a given environment, omitting the cultivation step. Other terms are used to describe this methodology, such as environmental genomics, ecogenomics, community genomics, metagenomics, and these are all identical. The power of metagenomics represents the majority of microbes on Earth, in order to discover novel genes and novel metabolic pathways.

Metagenomic approach is based on direct isolation of nucleic acids from environmental samples and has verified to be a powerful tool for comparing and for exploring the ecology and metabolic profile of composite environmental microbial communities (Tringe *et al*., 2005), as well as for identifying novel biomolecules by utilization of libraries constructed from isolated nucleic acids (Ferrer *et al*., 2009; Handelsman, 2004). Metagenomics is a valuable tool in the estimation of microbial-community responses to a variety of environmental conditions, including metal pollution.

1.1 Drawback of metagenomics

Metagenomics is not, conversely, the most appropriate approach to study eukaryotic microorganisms. Firstly, assessment 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 ratio of clones of eukaryotic origin. Secondly, due to repeated 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 doubtful to be expressed in a bacterial host. But, as metagenomic DNA-based analysis cannot differentiate between expressed and non-expressed genes, it fails to reflect the valid metabolic activity (Sorek and Cossart, 2010).

However, in almost all of the metagenomic studies, a separate complementary molecular typing method-usually based on PCR-amplified 16S rRNA genes-is desired to distinguish the gene discoveries in the perspective of the microbial phylogenetic diversity of the sample (Tringe *et al*., 2005; Venter *et al*., 2004; Gill *et al*., 2006).

These PCR-based typing methods-although very powerful, particular when combined with the novel pyro-sequencing technology (Huber *et al*., 2007; Sogin *et al*., 2006) have some well-known drawbacks:

(1) bias is introduced by primers and/or exponential amplification; (2) immediate quantitative estimation of all three domains of life is impossible and(3) determination of free DNA can bias the measurement of community responses to environmental changes.

Moreover, DNA-based metagenomic and diversity studies do not allow us to describe conclusions on the expression state of the genes and hence the functional role of genes or organisms in the investigated environment remains doubtful. In resemblance to post genomic studies of cultivated organisms, a next step in the metagenomic area therefore includes meta-transcriptomic technology.

1.2 Metatranscriptomics

To avoid these problems, a new approach, called metatranscriptomics is being used. 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 pulling out of environmental RNA instead of DNA. A novel environmental genomic approach, the 'Functional Metatranscriptomics', allows the detailed characterization of genes expressed by different eukaryotic microorganisms (e.g. Fungi, Protists) directly in the environmental samples.

One such dispute is that messenger RNAs (mRNAs) account for only ~ 5% of total cellular RNA. Various rRNA depletion methods have been developed to improve samples for mRNA. Simultaneous application of multiple methods (e.g., mRNA-ONLY™, MICROB *E*xpress™ and Message Amp™) can remove more of the rRNA in some instances, but efficacy remains partial, particularly when working with partially degraded rRNA(He *et al*., 2010).

1.3 Advantage of metatranscriptomics

Discovery of novel genes of interest for the bioindustry, bioremediation and biomarkers this approach has a strong potential in biotechnology. This approach will also allow us to distinguish genes concerned in adaptation to stressful atmosphere or involved in organic matter degradation expressed in stressful environments.

It is continuously recognized that a huge number of natural products exists in non-culturable microbes with chemical, biological, and functional activities for potential uses in diverse industrial and biomedical applications (Handelsman *et al*., 2004).Metatranscriptomics applied to environmental transcripts provides distinctive opportunities to reveal microbial activity in the environment and to discover novel enzymes of potential use in biotechnological applications (Kellner *et al*., 2011).

1.4 Heavy metals: their source and properties

1.4.1 Heavy metals

Any metal with a specific gravity of 5.0 or greater, particularly one that is toxic to organisms, as lead, mercury, cobalt and cadmium. Soil microorganisms are hindered by the heterogeneity of the soil composition, they are present in huge numbers (c. 10^9 individual cells) and diversity (>10^6 distinct taxa) in each gram of soil (Curtis and Sloan, 2005; Gans *et al*., 2005), and the lack of knowledge about the majority of the micro biota. Only a minority of soil bacteria, usually estimated at around 1% of the total number of cells observed by direct counting can be cultured routinely in laboratory media (Skinner *et al*., 1952).

1.4.2 Sources of metals in soils

Mineral rock weathering and anthropogenic sources supply two 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, cobalt, 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)

It has observed that around 17 types of heavy metals may be available for living cells based on their solubility under physiological condition and their significance for organism and ecosystems (Weast, 1984). Fe, Mo and Mn are important as micronutrients among these metals. Some metals, are essential, such as calcium, cobalt, chromium, copper, potassium, magnesium, sodium, nickel and zinc, and serve as micronutrients. And used in 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 but they are potentially toxic to microorganisms such as silver, aluminum, cadmium, gold, lead and mercury, are nonessential (Bruins *et al.,* 2000).

Microbes in metal-contaminated soils are classified as metal resistant/adapted to this stressed environment. Heavy metal resistance can be achieved by avoidance and/or tolerance. Avoiders are microbe that are able to protect themselves by preventing metal ions from entering their cellular cytoplasm, while tolerant microbe are able to detoxify metal ions that have crossed the plasma membrane or internal organelle bio membranes (Millaleo *et al*., 2010).

Trace metals play a key role as coenzymes or cofactors in many cellular processes. Several transition metals, including manganese, iron, nickel, cobalt, and copper, as well as zinc are required as catalysts in a range of enzymatic reactions (Hay, 1984). As these metals are normally found at relatively low concentrations in the environment, cells must accumulate them by using active transport processes (Hughes *et al*., 1981).Concentrations of trace metals in excess from normal physiological levels can be toxic. Toxicity results from the nonspecific combination of metal ions with cellular components and the disruption of the normal metabolism of other metals (Gadd *et al*., 1989). A lot of molecular mechanisms that are responsible for the transport and detoxification of a number of trace metals in prokaryotes are now known (Hughes *et al.*, 1989, Silver *et al*., 1989). Ion transporters, reductases and small proteins and compounds concerned in metal sequestration enable bacteria to grow in environments containing high levels of toxic metals (Silver *et al*., 1989). In eukaryotes, work has centered on the function of the glutathione related cadystins and the cysteine-rich metallothioneins proteins in the detoxification and storage of metals (Mehra *et al*., 1991).

1.4.3 Cobalt and its uses and application

Cobalt is an important magnetic element that has a widespread use in several industrial applications for example, production and the refining of alloys, jet engines, gas turbines, electrochemical materials and permanent magnets (Stadler and Schweyen, 2002). As well, cobalt is used in varnishes, paints, catalysts, inks, pigments, ceramics, and surgical implants

(Beyersmann and Hartwig, 1992). Biologically, cobalt is used as a cofactor of vitamin B12 and other enzymes in yeast, animals, bacteria, archaea and plants.

1.4.4 Cobalt as a threatening effect

Cobalt can be toxic for living systems when present at high concentrations, but the precise mechanism of this toxicity is still poorly understood. Transcriptomic and toxicogenomic studies have been carried out in *Escherichia coli* and human lung cells to identify potential signature to cobalt exposure. Overexposure to cobalt often occurs in several industrial occupations and in humans it can cause several diseases such as contact dermatitis, asthma, and pneumonia and lung cancer.

While in bacteria, excess cobalt cause inactivation of some Fe–S proteins and activates iron uptake as a probable compensatory mechanism (Ranquet *et al*., 2007), in mammalian cells, sharp exposure to cobalt induces up regulation of cobalt carriers and stress-responsive genes (Malard *et al*., 2007).

Various yeast studies have shown that cobalt ions are transported into the vacuole or mitochondrion by a cobalt transporter encoded by *COT1*, and over expression of *COT1* confers increased tolerance to cobalt and to rhodium (Conklin *et al*., 1992). Another gene, *COT2*, was also found to be concerned in cobalt resistance as well as to other divalent cations including Zn^{2+}, Mn^{2+}, and Ni^{2+}.

1.5 Gaps in study

Several human activities, either industrial (mining and ore processing) or agricultural (use of Phosphate fertilizers), Waste disposal lead to a long-term contamination of soils by different heavy metals. The consequent 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 sustain 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.

1.7 Objectives

Identification of gene responsible for cobalt tolerance by using metatranscriptomics approach

Chapter 2

Review of Literature

Soil microbial communities are concerned in significant ecosystem functions for example; decomposition and geochemical cycling (Carney and Matson, 2005; Nielsen *et al*., 2011) and strongly influence soil physical attributes (Feeney *et al*., 2006; Rillig and Mummey, 2006) as well as plant health and nutrition (Dennis *et al*., 2010). Soils are composite and provide enormous diversity of habitats that result from structural aspects such as the size, shape and connectivity of pore networks, as well as other factors including the complexity, resources, physicochemical conditions and biological interactions.

A range of environmental parameters, influence microbial community construction such as; temperature (Ward *et al*., 1998), pH (Dennis *et al*., 2009), substrate accessibility and complexity (Dennis *et al*., 2012), moisture content (Zhou *et al*., 2002), nutrient status (Broughton and Gross, 2000), contamination with pollutants (Muller *et al*., 2001), exposure to the roots of different plant species (Kuske *et al*., 2002), salinity (Nubel *et al*., 2000), predation (Jurgens and Matz, 2002), and other variables such as the architecture of their habitats (Sessitsch *et al*., 2001). Therefore, environmental heterogeneity contribute to the preservation of soil microbial communities that typically signify the largest fraction of below-ground biomass (Hassink *et al*., 1993) and are probable to constitute somewhere in the order of tens of thousands of microbial 'species' per gram of soil (Gans *et al*., 2005; Roesch *et al*., 2007). However, the relative influence of these parameters on microbial activities is poorly implicit.

The classic microbiological approach to examine the diversity and functioning of soil microbial communities were hindered for a long time by inability of the huge majority of microorganisms to grow in standard culture media. It has been seen that only ~1% of microbes are readily available by cultivation (Pace, 1997). Including eukaryotic, a majority of microbial species are uncultivable under laboratory conditions (Torsvik and Ovreas, 2002; Porter, 2008). In functioning of the soil ecosystem eukaryotic microbes play essential roles. Huge no. of technique used to examine microorganisms containing bioluminescent and other reporter genes in soil. For labeling specific cells with RNA or DNA probes in soil Fluorescent *In Situ* Hybridization (FISH) has been applied to soil with limited success.

Therefore, DNA microarray is used as a powerful tool for studying gene expression in many diverse organisms. A functional gene array, termed Geo Chip (He *et al*., 2007), is a precious tool in the measurement of microbial-community responses to a variety of environmental conditions, including metal pollution. It has been successfully applied to the simultaneous expression of several thousands of genes and to large-scale gene innovation, and polymorphism screening and mapping of genomic DNA clones. It is a high throughput, highly parallel RNA expression assay skill that permits quantitative investigation of RNAs transcribed from both known and unknown genes. In diagnostic fingerprints, this technique provides gene expression patterns in normal and pathological cells by comparing them, and because it can simultaneously track expression levels of many genes, it provides a basis of operational context for inference and predication about complex cell control systems. However, Geo chip also have many technical, experimental, and data analysis challenges that need to improve the sensitivity and quantitative accuracy of the arrays.

2.2 Emerging fields of culture-independent approaches

The development of culture independent approaches has extensively increased our understanding of soil microbial ecology for prokaryotic species and eukaryotic microbes which cannot be simply isolated from complex environmental matrices and/or cannot be grown *in vitro* (Bailly *et al*., 2007). But now DNA, RNA, proteins and metabolites can be extracted directly from environmental samples easily and analysed via:

- Metagenomics
- Metatranscriptomics
- Metaproteomics
- Metabolomics

The arrival of high-throughput sequencing technologies used in metagenomics and metatranscriptomics has made it possible to attain datasets that are suitable to the complexity of these microbial communities.

2.2.1 Metagenomics

Metagenomics describes the methodological approach of accessing the total genomic DNA and hence the metabolic potential enclosed within a microbial community. It is an intensifying field surrounding culture-independent studies of the structures and functions of microbial communities and their interactions with the habitats they occupy. Metagenomics offers novel approach into the functional prospective of microbial communities and provides reference genes and genomes for advance study (Shi *et al*., 2011). Metagenomics includes (1) shotgun sequencing of microbial DNA isolated directly from a given environment, (2) high-throughput screening of expression libraries, constructed from cloned community DNA, to identify specific functions such as antibiotic resistance (functional metagenomics), (3) profiling of RNAs and proteins produced by a microbiome, and (4) identification of a community's metabolic network .

This approach can be achieved in two different ways: (a) sequence-based and (b) function-based. Both approaches are based on construction of metagenomic clone libraries, which involves the extraction of environmental DNA, its shearing and consequent ligation into suitable cloning vectors. Sequence-based approaches engage screening of clones for the highly conserved 16S rRNA genes for identification purposes and then complete sequencing of clones containing phylogenetic anchors that specify the taxonomic group that is feasible source of the DNA fragment (Riesenfeld *et al*., 2004; Hoff *et al*., 2008). Sequence analysis guided by the recognition of phylogenetic markers is a potent approach first proposed by the De Long group, which created the first genomic sequence linked to a 16S rRNA gene of an uncultured anchor (Stein *et al*., 1996). Alternatively, random sequencing can be conducted, and once a gene of interest is recognized, phylogenetic anchors can be sought in the flanking DNA to provide a link of phylogeny with the functional gene followed by bio informatics analysis.

These studies focused on the understanding of microbial diversity and ecology. Although, the discovery of novel biocatalysts is feasible using sequence-based metagenomics (Warnecke *et al*., 2007) recognition of enzymes depends purely on bioinformatics tool. Despite the absence of native chaperones in expression hosts that are currently used for functional screening, cloning of metagenomic clone libraries has been used successfully for the discovery of novel biocatalysts. Function-based metagenomics is the constraint of a simple screening approach (e.g. color change or clearing zones around colonies), in order to enable the screening of several thousands of clones on a single agar plate for the activity of interest. Functional metagenomics, involves screening of metagenomic libraries for a particular phenotype, e.g. salt tolerance, enzyme activity or, antibiotic production and then identifying the phylogenetic basis of the cloned DNA (Dinsdale *et al*., 2008).This is based on the direct removal of whole DNA from environmental samples and it's direct cloning in appropriate vectors (e.g. plasmids) and then propagated in cultivated bacterial cells (*E. coli*) (Dinsdale *et al*., 2008). In 1991, first such cloning in a phage vector was reported (Schmidt *et al*., 1991) and idea of cloning DNA directly from ecological samples was first proposed by Pace *et al*. (1997). These environmental DNA libraries contain consequently fragments of the genomes of all microbial species, present in the original ecological sample. Screening of these libraries by using different skills is now becoming a standard approach for discovering novel genes of biotech potential, from which are 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 illuminating the global gene content of the microbial community and adaptation of these communities to definite environmental conditions (Gilbert *et al*., 2008).This approach has been used successfully to discover numerous novel enzymes such as alcohol dehydrogenases, esterases, and lipases (Ferrer *et al*., 2009).

However, function-based metagenomics is more cost efficient and more targeted than random shotgun DNA sequencing. But, the respective activities might not be detected when screening small insert metagenomic clone libraries. The main advantage of function-based activity screens compared to the sequence-based strategy is that they allow the discovery of completely novel biocatalysts which would most likely be missed by purely bioinformatics analysis.

2.2.2 Metatranscriptomics

Metatranscriptomics facilitates the potential expression of genes at the time of the sampling. Functional metatranscriptomics first requires the extraction of total RNA from environmental samples. Eukaryotic 3′ polyadenylated (poly-A) messenger RNAs are then be purified from total RNA by affinity chromatography to eliminate the ribosomal RNA, other non-coding RNAs as well as the bacterial mRNAs that largely organize environmental metatranscriptomes (Bailly *et al*., 2007; Damon *et al*., 2011; Lehembre *et al*., 2013). Poly-A mRNAs are then converted into cDNAs which are cloned in an suitable expression vector. Such eukaryotic-specific environmental cDNA libraries, first described by Grant *et al*. (Grant S *et al*., 2006), thus include protein coding genes expressed by the diverse eukaryotic microorganisms present in the original environmental sample (Bailly J *et al*., 2007; Grant S *et al*., 2006). Genes of interest can be screened by expressing them in a suitable eukaryotic organism such as the yeast *Saccharomyces cerevisiae* (Bailly *et al*., 2007; Kellner *et al*., 2011; Damon *et al*., 2011; Lehembre *et al*., 2013). By using reverse transcribed poly-A mRNA, expression cloning of eukaryotic genes is a basic technology in molecular biology. Conversely, obtaining libraries enriched in long cDNAs remains challenging for the production of functional proteins. The first step is the reverse transcription of mRNAs into cDNAs. In yeast, environmental cDNA expressed from soil, was first implemented in *Pinus* forest soil and allowed for the isolation of histidine biosynthetic genes by complementation of a histidine auxotrophic yeast mutant (Bailly *et al*., 2007).

2.2.3 Metaproteomics

Metaproteomics is the description of the protein complement of a microbial community under a given set of conditions at a specific point (Su *et al*., 2012). In Metaproteomics, proteins are extracted from a mixed microbial community, followed by fractionation, separation using liquid chromatography and detection with tandem mass spectrometry (MS/MS) (Langley *et al*., 2013). In order to describe the proteins detected, the amino acid sequences need to be known and thus a complete dataset of proteins or metagenomic data is necessary (Hettich *et al*., 2007) Although metatranscriptomics provides insight into gene expression and activity, supplementary levels of cellular localization and regulation occur at the protein level and therefore the signal from the transcriptome and the proteome can be substantially different (Langley *et al*., 2013).

2.3 Approaches used in study gene expression

For studying gene expression of diverse organism present in environment, a no. of molecular approaches has been used in earlier period. Some important approaches and tools used in these gene expression studies are as follow:

Xiang *et al*., (2000) this study revealed that the cDNA microarray is the most potent tool for studying gene expression in many different organisms It has been successfully applied to the simultaneous expression of many thousands of genes and its application to gene discovery and to diagnostics for certain diseases.

Urich *et al.,* (2008) apply an RNA-centered meta-transcriptomic approach is applied to concurrently obtain information on both structure and function of a soil community and this approach could be extensively applied in microbial ecology by proficiently linking community structure and function in a single experiment while avoiding biases inherent in other methods.

Cakar *et al*., (2009) this study resistant the fact that evolutionary engineering is useful in selecting strains with very specific phenotypes by isolating cobalt hyper-resistant mutants of *Saccharomyces cerevisiae* by *in vivo* evolutionary engineering approach and further illustrated the importance of the strategy chosen to isolate the best evolved strain.

Ramesh *et al*., (2009) in this study two MT genes were characterized, Hc*MT1* and Hc*MT2,* from the ectomycorrhizal fungus *Hebeloma cylindrosporum* These results show that ectomycorrhizal fungi encode different MTs and that each of them has a particular pattern of expression, suggesting that they play specific roles in improving the survival and growth of ectomycorrhizal trees in ecosystems contaminated by heavy metals.

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

Sangha *et al*., (2010) reveled the efficient isolation of high quality and quantity of total RNA from plants is highly desirable for the construction of a good-quality cDNA library, so they identified an improved method for RNA isolation and cDNA library construction from immature seeds of *Jatropha curcas*L.

Hirsch *et al*., (2010) Initiation of nucleic acid-based molecular methods, particularly in the polymerase chain reaction (PCR), has revolutionized the study of soil microbial ecology and avoiding problems associated with laboratory culture conditions.

Kellner *et al*., (2011) this study tells us about metatranscriptomics application to environmental transcripts and provides unique opportunities to expose microbial activity in the environment to find out novel enzymes of potential use in biotechnological applications.

Carvalhais *et al*., (2012) Due to progress in sequencing technologies, an increasing number of metagenomics studies are being conducted on samples from diverse environments including soils. This information has not only increased our consciousness of the functional potential of soil microbial communities, but also constitutes potent reference material for soil metatranscriptomics studies. Metatranscriptomics provides a snapshot of transcriptional profiles that correspond to distinct populations within a microbial community at the time of sampling. This information can indicate the potential activities of complex microbial communities and the mechanisms that regulate them. So, metatranscriptomics applied to soil environments and approaches for gaining biologically meaningful insight.

Bashir *et al*., (2014) Metagenomics provide us new molecules and novel enzymes with diverse functions and enhanced features compared to the enzymes from the culturable microorganisms. Additionally the application of metagenomics for unlocking novel biocatalysts from nature, it also has found applications in fields as miscellaneous as bioremediation, personalized medicine, xenobiotic metabolism, and so forth.

Tsementzi *et al*., (2014) Evaluation of metatranscriptomics protocols and application to the study of freshwater microbial communities and advance understanding of the activity and ecology of abundant and rare members of temperate freshwater microbial communities.

Yadav *et al*., (2014) It has been reported that construction of high quality cDNA libraries from the usually low amounts of eukaryotic mRNA, extracted from environmental samples, and this is a simple and cost effective tool. This technique facilitate expression cloning of environmental eukaryotic genes and contribute to a better understanding of basic biological and/or ecological processes carried out by eukaryotic microbial communities.

2.1 Mechanism of metal tolerance

Heavy metal contamination in soil caused by human activities, such as mining and industrial activities, is a serious problem all over the world. Heavy metals are potentially toxic, depending on their bioavailable concentrations and sensitivity of the organism that is exposed. 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. 2.1).

2.4.1 Extracellular chelation and cell-wall binding

2.4.1.1 Chelation by organic acids

Metals that get past the plasma membrane need to be chelated by diverse ligands in order to diminish their undesired interaction with cellular compounds. Such ligands can be oligopeptides, organic acids, amino acids, or proteins. Metal-ligands complexation is an significant part of the molecular mechanism of metal homeostasis. Some eukaryotes can emit organic acids into the rhizosphere such as Mycorrhizal fungi and plants. The benefits of organic acid emission might be to liberate base cations from soil minerals (Landeweert *et al.,* 2001) 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 compatible organic substrates, making the dynamics of these acids in the rhizosphere complex (Jones, 1998). Their affinities for particular metal ions are also important in their cycling and these properties will also be pH dependent. Oxalic acid secretion into the rhizosphere was higher in mycorrhizal Scots pine compared to non-mycorrhizal controls when no toxic metals present for a variety of fungal isolates from contaminated sites (Ahonen-Jonnarth *et al.,* 2000). Mycorrhizal species/strains also directed in their organic acid secretion in symbiosis in the absence of metal exposure. To chelate metal ions, different organic molecules particularly di- and tricarboxylic acids that do not go to the matrix of the cell wall are excreted by fungal cells, among other functions. Citrate has been exposed to be the most significant Al3+ complex former in soil solution from podzolized forest soils (Landeweert *et al.*, 2001; Van Hees *et al.,* 2001). The stimulation of oxalic acid efflux correlated closely with Cu tolerance in brown rot fungi (Green and Clausen, 2003), and over secretion of oxalic acid possibly contributed to the metal tolerance exhibited by *Beauveria caledonica* (Fomina *et al.*, 2005). A like, fungi also often respond to metal exposure by increased oxalate exudation (Ahonen-Jonnarth *et al.,* 2000; Cumming *et al.,* 2001). Exudation of organic acids may supply a source of protons for metal solubilization from metal-containing minerals, often resulting in soil acidification (Devevre *et al.,* 1996; Fomina *et al.,* 2005). Metal-tolerant ectomycorrhizal fungi grew and solubilized metal-containing minerals better than non tolerant 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 improved resistance to copper and decreased free copper in solution to a greater extent. It seems that this enhanced precipitation is a tolerance mechanism (Adriaensen *et al.,* 2006).

2.4.1.2 Heavy metal binding in the extramatrical mycelium

Fungi can successfully bind metals to cell walls or extracellular polysaccharides may have been recommended as a tolerance mechanism in fungi (Ross, 1994; Gadd, 1993). Similar mechanisms have been recommended to protect mycorrhizal roots from metal toxicity. It was recommended that sorption of metals to fungal tissues or intracellular uptake and detoxification in fungal vacuoles consequently reduced metal uptake into the host plant (Brown and Wilkins 1985, Jones and Hutchinson 1986). Marschner *et al*. (1998) measured Pb sorption on the mycelium of the ectomycorrhizal fungi *Paxillus inolutus* and *Laccaria bicolor*. They found that Pb was most likely bound to cation exchange sites, even though dependent on the fungal species, some Pb may also be immobilized by precipitation. The ECM fungal infection increased the metal tolerance of *Betula papyrifera*, but sensitivity varies widely between fungal species. They accomplished that the protection against metals in successful symbiosis was an ability to produce a densely infected and large root system.

2.4.1.3 Heavy metal binding by cell wall components

The fungal cell wall has been suggested as the main barrier protecting fungal hyphae against uptake of potentially toxic metal species. Its composition implies glucan-chitinand galactosamine-containing polymers, and a minor amount of proteins. Thus a large number of potential-binding sites are exhibited by free carboxyl, amino, hydroxyl, phosphate and mercapto groups (Strandberg *et al.,* 1981). Binding to the wall, also called biosorption (Gadd, 1993) is a mechanism not depending on the metabolic activity of the fungus, whereas precipitation with excreted substances relies on the activity of the cells. Biosorption of heavy metals to fungal structures may reduce the intracellular accumulation of metals and their effect on cytoplasmic processes (Brown and Wilkins 1985). Melanins obtained from different fungi were also shown to be efficient biosorbants for heavy metals (Senesi *et al.,* 1987; Gadd and De Rome, 1988). These heterogeneous compounds, which are located in or exterior to the cell walls are important fungal pigments, which enhance the survival of many species in response to environmental stress (Gadd, 1993).

2.4.1.4 Transport mechanisms involved in metal tolerance

The plasma and vacuolar membranes are the main transport membranes of the fungi. There has been little work on the transport of metals in ectomycorrhizal fungi. 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 (Williams *et al.,* 2000; 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*.

The yeast cadmium factor (Ycf1) gene encodes Mg ATP 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). This hypothesis was further supported by X-ray microanalysis, which revealed that the accumulation of Cd correlated tightly with the accumulation of sulphur in electron-dense bodies in the vacuolar compartment (Ott *et al.,* 2002). However, the chemical nature of these sulphur components involved in Cd complexation (Fig.2.1) was not confirmed in this study. 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

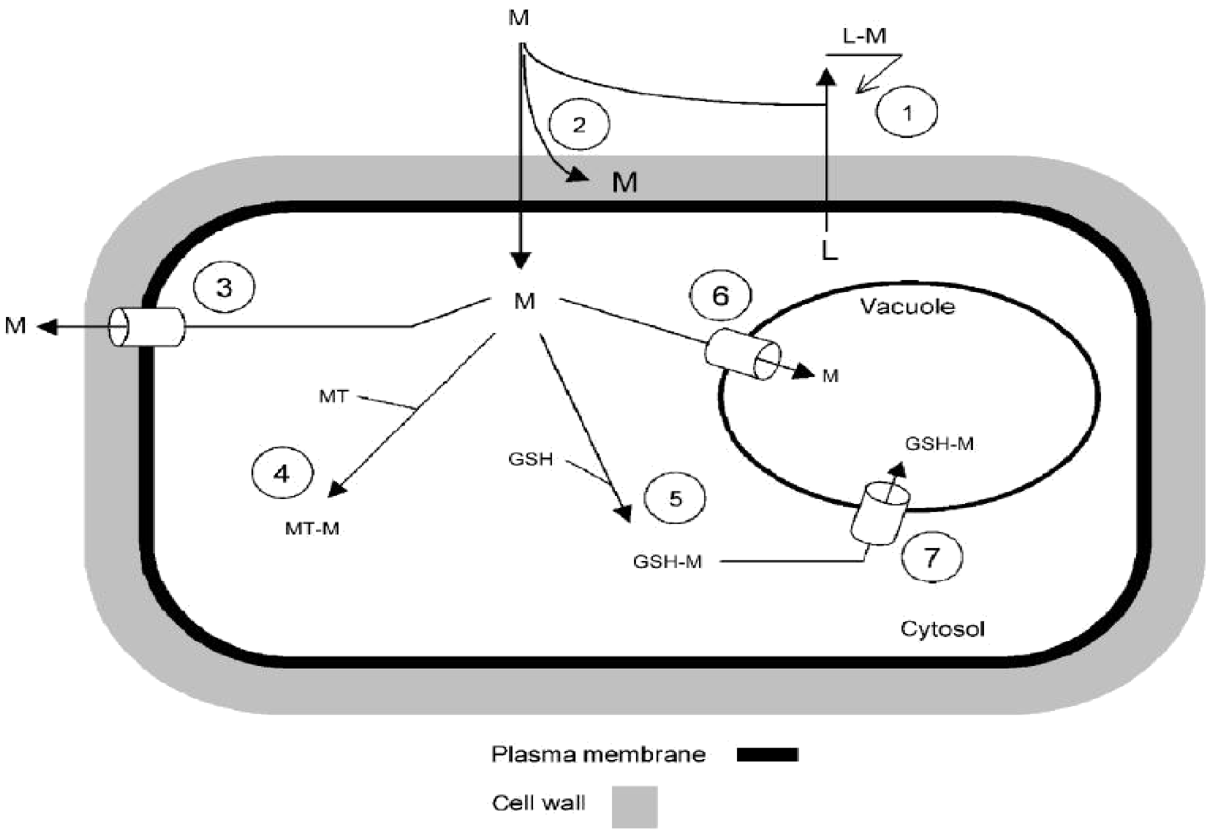


Figure 2.1 Schematic representation of cellular mechanisms potentially involved in metal tolerance in ectomycorrhizal fungi (Adapted from Bellion *et al*., 2006)

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).

2.4.2 Intracellular complexation by peptides

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).

2.4.2.1 Metallothioneins

The discovery of a cadmium (Cd) binding, cysteine-rich protein from horse kidney by Margoshes and Vallee (1957) was the influential finding that manifests the birth of a field of research focused on the study of a low-molecular-weight polypeptide super family, the metallothioneins (MTs). 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. Aromatic amino acids are usually absent. All Cysteines occur in the reduced form and are coordinated to the metal ions through mercaptide bonds, giving rise to spectroscopic features characteristic of metal-thiolate clusters (Hamer, 1986; Kagi, 1993).

2.4.2.2 Classification

MTs have been found in a vast range of taxonomic groups includes animal kingdom, higher plants, eukaryotic microorganisms, and in many prokaryotes (Hamer, 1986; Kagi, 1993).

2.4.2.3 Function

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 metallothioneins 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).

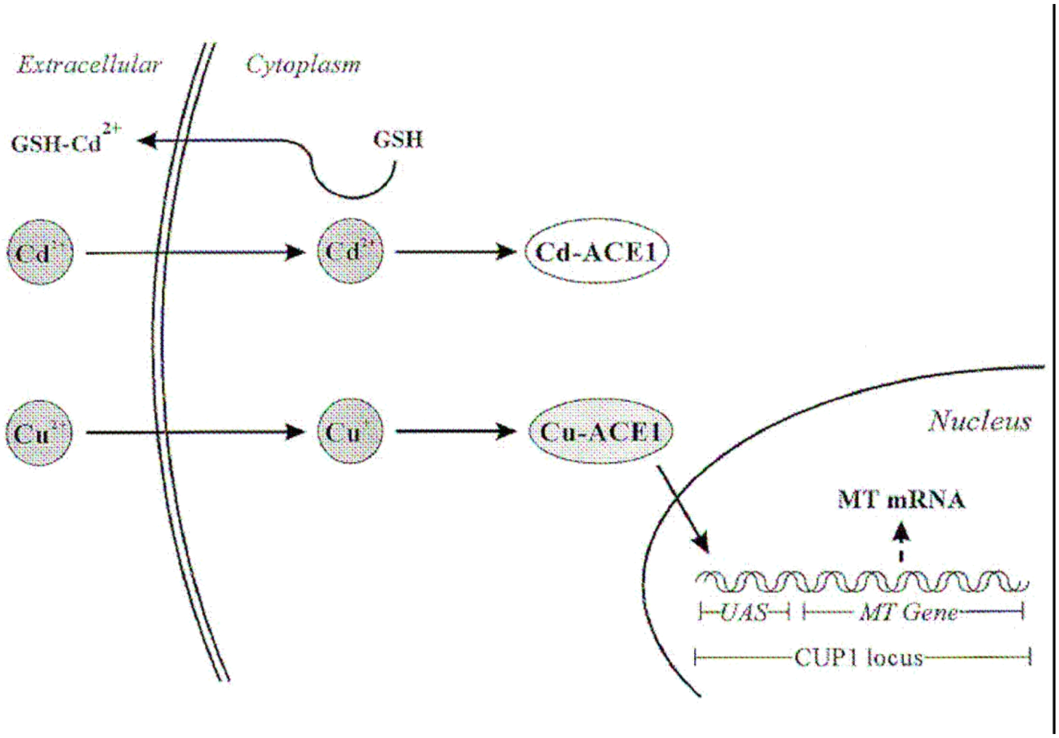


Figure 2.2 Metal detoxification pathways in yeast. *Saccharomyces cerevisiae* detoxify copper by sequestering it with the metal-binding protein metallothioneins (MT). The synthesis of MT in *S. cerevisiae* is transcriptionally regulated via the binding of Cu^+, respectively, to the transcription factors 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 metallothioneins gene, leading to increased synthesis of metallothioneins (Dameron *et al.,* 1993). ACE1 has little affinity for the UAS when Cd^{2+} ions are bound (Dameron *et al.*, 1993) (Fig. 2.2). The metallothioneins induction in *S. cerevisiae* is thus specific for Cu^{2+} ions. Although the *S. cerevisiae* metallothioneins 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.

2.4.2.4 Phytochelatins

In addition to the cysteine rich peptides of the metallothioneins family, another group of metal binding molecules synthesized by fungi are short, cysteine containing γ-glutamyl peptide, encompassed by the trivalent name 'phytochelatins' (Rauser, 1990). These are mainly synthesized by plants and algae as well as in a range of filamentous fungi and yeasts (Grill *et al.,* 1985). Phytochelatin (PC) was first discovered in 1981 in fission yeast, and was named cadystin (Murasugi *et al.,* 1981). It was then found in higher plants in 1985 and was named phytochelatin.

By far the most detailed characterization of the pathway of PC biosynthesis has come from studies in the fission yeast (*Schizosaccharomyces pombe)* and in *Arabidopsis*. They are found in plants, fungi, nematodes and all groups of algae including cyanobacteria. 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. Phytochelatin seems to be transported into the vacuoles, so that the metal ions it carries restored safely away from the proteins of the cytosol.

2.4.2.5 Antioxidative mechanism

The formation of free radical species, which can be initiated directly or indirectly by metals, can cause strict 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 catalyzed by redox-active metals e.g. Cu, Fe, Cr, V (Halliwell and Gutteridge, 1999). Cobalt toxicity arises not only from the capability of cobalt ions to generate reactive oxygen species (ROS) via Fenton reactions, but also from its competition with other essential metals such as iron, calcium and zinc, for the binding to macromolecules probably resulting in the inhibition of their proper function (Simonsen *et al*., 2012). In addition, the chemical affinity of cobalt for sulfur atoms has also been invoked as a possible mechanism of cobalt toxicity, due to the likely dysfunction of proteins whose activity depends on thiol groups (Barceloux *et al.*, 1999).

In the yeast *Saccharomyces cerevisiae*, cobalt may enter the cell through the low affinity iron transporter, Fet4, via the manganese transporter Smf2, or via the phosphate transporter Pho84 (Dix *et al*., 1994; Liu *et al*.1997; Jensen *et al*., 2003). In line with the mechanisms of cobalt-induced toxicity, the cellular response to cobalt excess includes the activation of the major iron responsive transcription factor, Aft1 (Stadler *et al*., 2002; Philpott *et al*., 2008). One target of Aft1 is the *COT1* gene that codes for a vacuolar transporter (Stadler *et al*., 2002; Conklin *et al*., 1992). *COT1* presumably renders protection against the damaging effects of cobalt through cobalt sequestration in the vacuole (Conklin *et al*., 1992; Li, J., *et al.*, 1998). However, over expression of *COT1* gene does not confer increased cobalt tolerance to cells deficient in high affinity iron transporter system, which met their iron requirements by increasing the expression of low affinity iron transporters. In a second line of defense against cobalt surplus, yeast also increases the expression of genes involved in oxidative stress response (Stadler *et al*., 2002). The yeast transcription factor Yap1, is an essential regulator of the cellular response to oxidative stress.

Chapter 3

Material and Methods

The work emphasizes on screening of the fractionated cDNA library of size 0.1-0.5kb from cadmium polluted soil by protocol adopted by Yadav *et al*., (2014). For screening library, mutant yeast has genetically transformed with plasmids of the library, having appropriate marker genes and was functionally analyzed by performing functional complimented assay.

3.1 Yeast Transformation

3.1.1 Yeast strains and culture conditions

The mutant *S. cerevisae* strains, cobalt- sensitive *COT1* strain derived from the wild type strain BY4741 (*MATα his3D1 leu2D0 met15D0 ura3D0*) was used in this study along with wild strain BY4741. These yeast strains were maintained on YPD agar medium (Appendix 3). For complementation studies, transformed yeast cells were selected on basis of selection marker of pFL61 i.e. Ura 3 gene which enable it to grow in complete synthetic medium (SD, Appendix 4).

3.1.2 Transformation

Yeast transformation was performed with constructed library A (0.1-0.5kb) for mutant strain *COT1*. Wild type strain BY4741 and mutant *COT1* transformed with empty pFL61 was used as control for further experiment by using the standard lithium acetate method as described below (Ronald Agatep *et al*., 1998).

1. 20 ml of freshly prepared YPD medium was inoculated with fresh colony of yeast strain incubated at 200 rpm, 30ºC for overnight. Preheating of media was also done by incubating 40 ml YPD media flask in similar incubation conditions.
2. Preheated YPD media (40 ml) was taken and its O.D.600nm was set at 1.0 by inoculating from previous overnight grown culture. Final volume 50 ml was then incubated at same conditions till the O.D.$_{600}$ reaches 2.0

3. Centrifuged the above 50 ml of secondary culture at 3000 rpm for 5 mins and resuspended the pellet in 25 ml autoclaved distilled water for washing of the cells. Then, again centrifuge at 3000 rpm for 5 mins.
4. Pellet was resuspended with autoclaved distilled water and cell suspension was transferred in a 1.5 ml eppendorf tube to make up the total volume 1ml.
5. Centrifuged for 30 seconds with the bench centrifuge and supernatant was discarded. Resuspended the pellet with 1 ml autoclaved distilled water.
6. Cell suspension was distributed in volume of 100 µl in 1.5 ml eppendorf tubes, centrifuged for 30 seconds with the bench centrifuge and supernatant was discarded.
7. The following mixture was added in each tube.

Reagents	**Volume (µl)**
PEG 3350 50% w/v	240
Lithium acetate 1M	36
Salmon sperm ssDNA 10mg/ml	10
Template DNA	1
dH_2O	74
Total	360

8. The eppendorf tube of PEG/LiAc/ssDNA/Template/yeast cells mixture was incubated in a water bath for 1 hour at 42ºC and then centrifuged for 30 seconds on a bench centrifuge and discarded the supernatant.
9. The pellet was resuspended with autoclaved distilled water to make up the final volume 1ml and cell suspension was spread on selective media SD-Ura with or without cobalt and incubated for 2-3 days at 30ºC.

3.2 Yeast functional complementation assay

3.2.1 Drop Assay

To check maximum tolerance of yeast transformants, drop test was performed at various concentration of metal from 1.5 mM $CoCl_2$ to 12 mM $CoCl_2$.

1. For drop test, transformed mutant yeast strain *COT1*pFL61 was used as negative control and BY4741as positive control and cDNA yeast transformants as test samples.
2. OD_{600} for BY4741, *COT1*pFL61 and yeast transformants was adjusted to 1.0.
3. Serial dilutions of cultures was prepared from10^{-1} to 10^{-4} and then 5 µl of each serial dilutions was placed on SD –Ura agar plates and SD –Ura agar supplemented with different cobalt concentration i.e. from 1.5 mM $CoCl_2$ to12 mM $CoCl_2$.
4. Plates were incubated for two days at 30ºC and further results were observed.

3.2.2 5-Fluoroorotic Acid Test (FOA)

To confirm whether cDNA inserts were responsible for metal tolerance or not FOA test was performed.

5-Fluoroorotic Acid (5-fluorouracil-6-carboxylic acid monohydrate; 5-FOA), is used in yeast molecular genetics to detect expression of the URA3 gene, which encodes orotine-5'-monophosphate (OMP) di carboxylase. Yeasts with an active URA3 gene (Ura+) convert 5-FOA to fluorodeoxyuridine, which is toxic to cells. Yeast strains carrying a mutation in the URA3 gene grow in the presence of 5-FOA if the media is supplemented with uracil. To confirm whether tolerance of transformants toward cobalt was due to cDNA Lib A FOA test was performed.

1. Prepared SD-Ura +FOA (1gm/liter of media) agar plates.
2. All the transformants were streaked on these plates and incubated for 24-48 hours at 30ºC and results were observed.

3.2.3 Drop test after FOA

cDNA yeast transformants which had grown on SD+Ura+FOA were selected and further tested for metal tolerance by drop assay and these transformants with knocked out the plasmid or insert and *COT1*pFL61, BY4741 (after FOA) were further used for performing drop out assay.

1. Yeast strain *COT1* pFL61 (after FOA) was used as negative control and BY4741 (after FOA) as positive control and Lib A yeast transformants as test samples.
2. OD_{600} for BY4741,*COT1*pFL61 and yeast transformants (after FOA) was adjusted to 1.

3. Serial dilutions of cultures was done up to 10^{-4} and put the drop of 5 µl volume of each serial dilution on SD +Ura media plates and SD +Ura media supplemented with metal concentrations.
4. Plates were incubated for two days at 30ºC and further results were observed.

3.3 Colony PCR

Colony PCR was performed to check the successful transformation of Lib A in mutant yeast by visualizing the amplified band of required or particular size with appropriate primers.

1. A pin-point amount of yeast cells was added in 10µl of 20 mM NaOH solution mixed and incubated at 98ºC (in PCR) for 30 minutes.
2. The tubes were then quick chilled in ice and centrifuge for 30 seconds and 1µl of supernatant was transferred in fresh PCR tube with 19 µL of reaction mixture.
3. PCR master mix was added in the tube. The PCR was performed with primers pFL61NF (5'-CTTCTAACCAAGGGGTGGTTTAGTTTAG-3') and pFL61 NR (5'-CTGCATAAAGGCATTAAAAAGAGGAGCG-3').
4. The PCR program was carried out as: initial denaturation at 95ºC for 2 mins, followed by 35 cycles at 95ºC for 30 seconds, 55ºC for 30 seconds annealing temperature, 72ºC for 2mins and final extension at 72ºC for 5 minutes.
5. Amplified products were visualized on 1.8% (w/v) agarose gel (Appendix 2) prepared in 0.5 X TBE, pH 8.0 (Appendix 1) using 6X loading dye at 3 volts/cm for 45-60 minutes. Ethidium Bromide (0.5 µg/ml) was added to stain the gel prior to pouring visualized on a U.V. transilluminator (Hoefer, U.S).

3.4 Growth kinetics

Growth kinetics was performed to study the growth profile of transformants with SD-Ura and SD-Ura supplemented with cobalt. In liquid broth media cobalt would be available to all the cells uniformly so this experiment will give assured and definite result for about resistance of transformants towards cobalt. *COT1*pFL61 grown on SD-Ura and SD-Ura+cobalt as a control. Growth kinetics test was performed for all well grown transformants on that concentration on the concentration at which they were showing maximum resistance.

1. Initial O.D was maintained same for each transformants culture before doing inoculation.
2. Each transformants were inoculated in 20 ml of fresh SD-Ura media and also inoculated mid-log cultures of mutant yeast strain *COT1*pFL61 in two different flasks of 20 ml fresh SD-Ura media and cells were allowed to grow at 30ºC and 200 rpm for 5 hours to attain a starting optical density of 1.0 at 600nm.
3. After 5 hours incubation metal was added in one flask of mutant yeast strain *COT1*pFL61 and in all other flasks of yeast transformants in concentration of 12 mM $CoCl_2$.
4. The optical densities of the cultures were measured at 3 hours interval for 48 hours.

Chapter 4

Result and Discussion

Yeast transformation was performed to screen the environmental cDNAs Lib A (0.1-0.5 Kb) for tolerance toward cobalt metal. A total of 36 transformants were screened and various test were performed to study their tolerance toward cobalt i.e. colony PCR, Drop out tests, FOA test, after FOA test. Transformation of lib A of size 0.1-0.5 kb for cobalt metal tolerance was performed in mutant *cot 1* yeast strains by standard lithium acetate method (Ronald Agatep *et al*., 1998).

4.1 Yeast transformation

Yeast transformation results showed 36 cobalt tolerant transformants. Transformation with empty pFL61 was also performed as a control for which colonies grown only on SD-Ura media plates which ensure that pFL61 empty does not carry any metal tolerance itself. Further these transformed cell suspension was also spread on SD-Ura plates to screen all metal tolerance and all 36 transformants were streaked on SD-Ura plates supplemented with 1.5 mM $CoCl_2$ to further confirm their growth. Empty pFL61 transformed *COT1* cells were unable to grow at 1.5 mM $CoCl_2$ supplemented media but transformation was found with mutant *COT1*yeast strains with pFL61(Lib A).

4.2 Drop assay

1. Drop assay of all these 36 transformants were performed on various range of metal concentration starting from minimum tolerance 1.5 mM $CoCl_2$ to maximum of 12 mM $CoCl_2$ Lib A yeast transformants as test samples were inoculated in SD-Ura medium and incubated at 30 ºC and 200 rpm. Transformed mutant yeast strain *COT1* pFL61 was used as negative control and BY4741as positive control OD_{600} for BY4741, *COT1*pFL61 and yeast transformants was adjusted to 1.0. 5 µl serial dilutions were spotted on SD−Ura media plates and on SD−Ura media supplemented with 1.5 mM $CoCl_2$. Plates were incubated for 24-48 hours at 30ºC.

4.2.1 Drop assay at 1.5 mM $CoCl_2$

It was observed that at this concentration of cobalt, the transformants showed extreme tolerance toward cobalt. As screening of transformants was done on 1.5 mM $CoCl_2$ so we performed drop test on this concentration (Fig. 4.2.1) and rich growth was observed for each transformants up to last dilution i.e. $10^{-4.}$

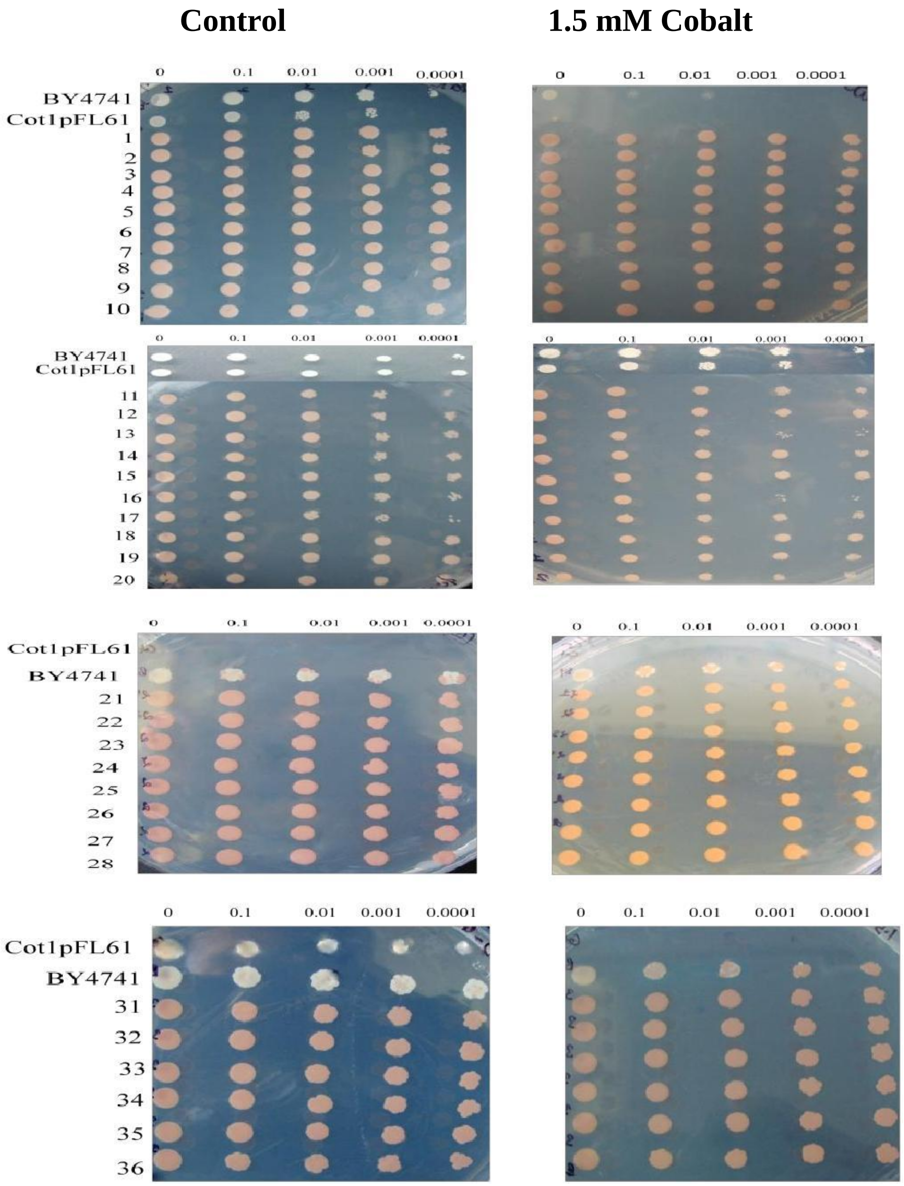


Fig 4.2.1 Functional complementation of yeast mutants on SD-Ura medium supplemented (or not) with 1.5 mM Cobalt. *COT1* cells harbouring the control vector pFL61 were unable to grow on Cobalt containing media, where as all 36 transformants and wild type strain BY4741 cells were grown well on Cobalt medium

4.2.2 Drop assay at 6 mM $CoCl_2$

It was observed that at 1.5mM $CoCl_2$ concentration of metal all transformants were showing extreme metal tolerance up to last dilution i.e. 10^{-4} so drop out assay was further carried out at higher range of metal concentration i.e. from 6 mM $CoCl_2$ (Fig. 4.2.2).

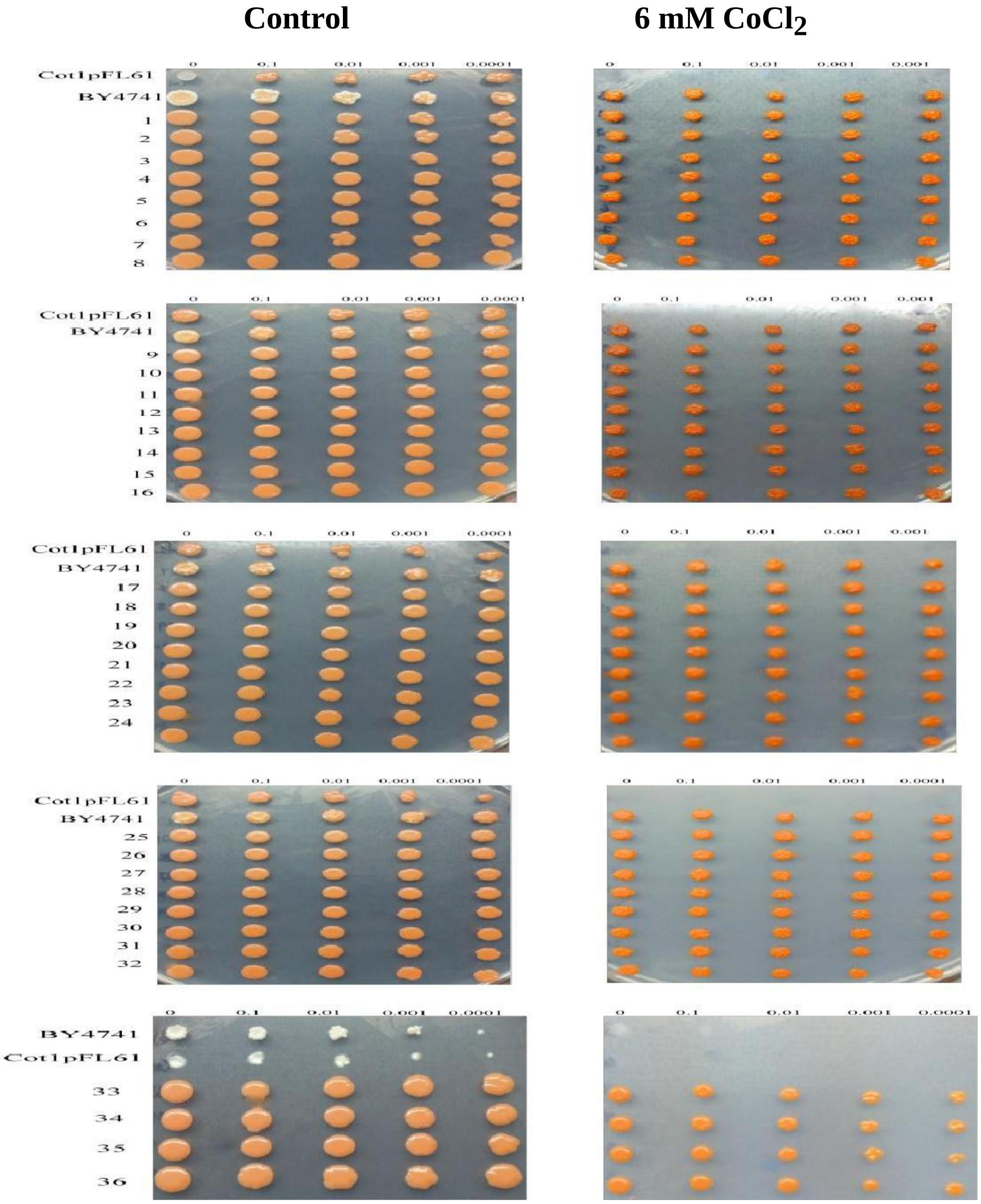


Fig 4.2.2 Functional complementation of yeast mutants on SD-Ura medium supplemented (or not) with 6 mM Cobalt. *COT1* cells harbouring the control vector pFL61 were unable to grow on Cobalt containing media, where as all 36 transformants and wild type strain BY4741 cells were grown well on Cobalt medium

4.2.3 Drop assay at 10 mM $CoCl_2$

It was observed that at 6 mM $CoCl_2$ concentration of metal all transformants were showing extreme metal tolerance up to last dilution i.e. 10^{-4} so drop out assay was further carried out at higher range of metal concentration i.e. from 10 mM $CoCl_2$ (Fig. 4.2.3).

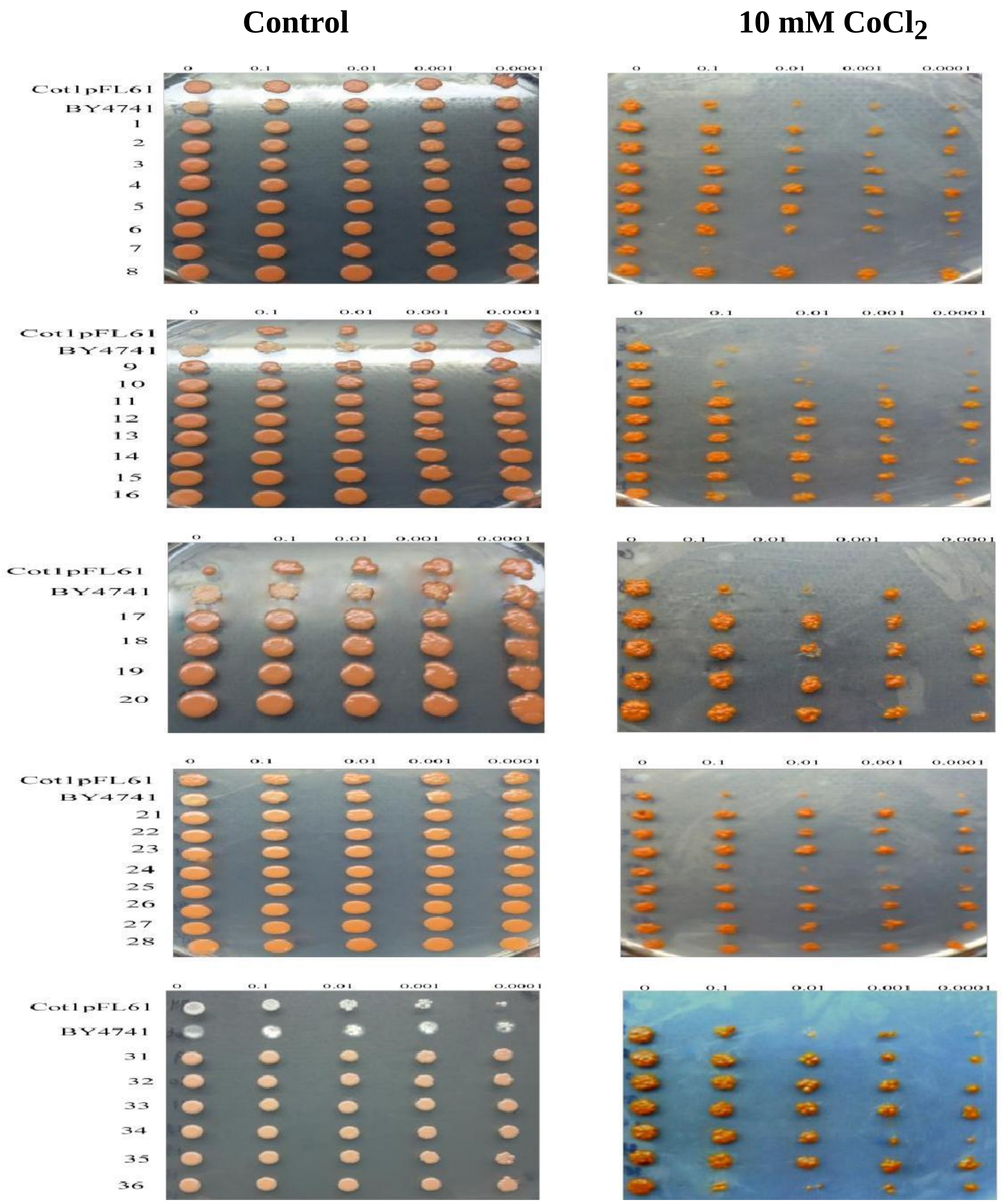


Fig 4.2.3 Functional complementation of yeast mutants on SD-Ura medium supplemented (or not) with 10 mM Cobalt. *COT1* cells harbouring the control vector pFL61 were unable to grow on Cobalt containing media, where as all 36 transformants and wild type strain BY4741 cells were grown well on Cobalt medium

4.2.4 Drop assay at 12 mM $CoCl_2$

It was observed that at 10 mM $CoCl_2$ concentration of metal a few transformants were showing metal tolerance up to last dilution i.e. 10^{-4} so reassure metal tolerance level of transformants drop assay was further carried out at higher range of metal concentration i.e. 12 mM $CoCl_2$ (Fig. 4.2.4). Find out that maximum tolerance of metal concentration by transformants at this level.

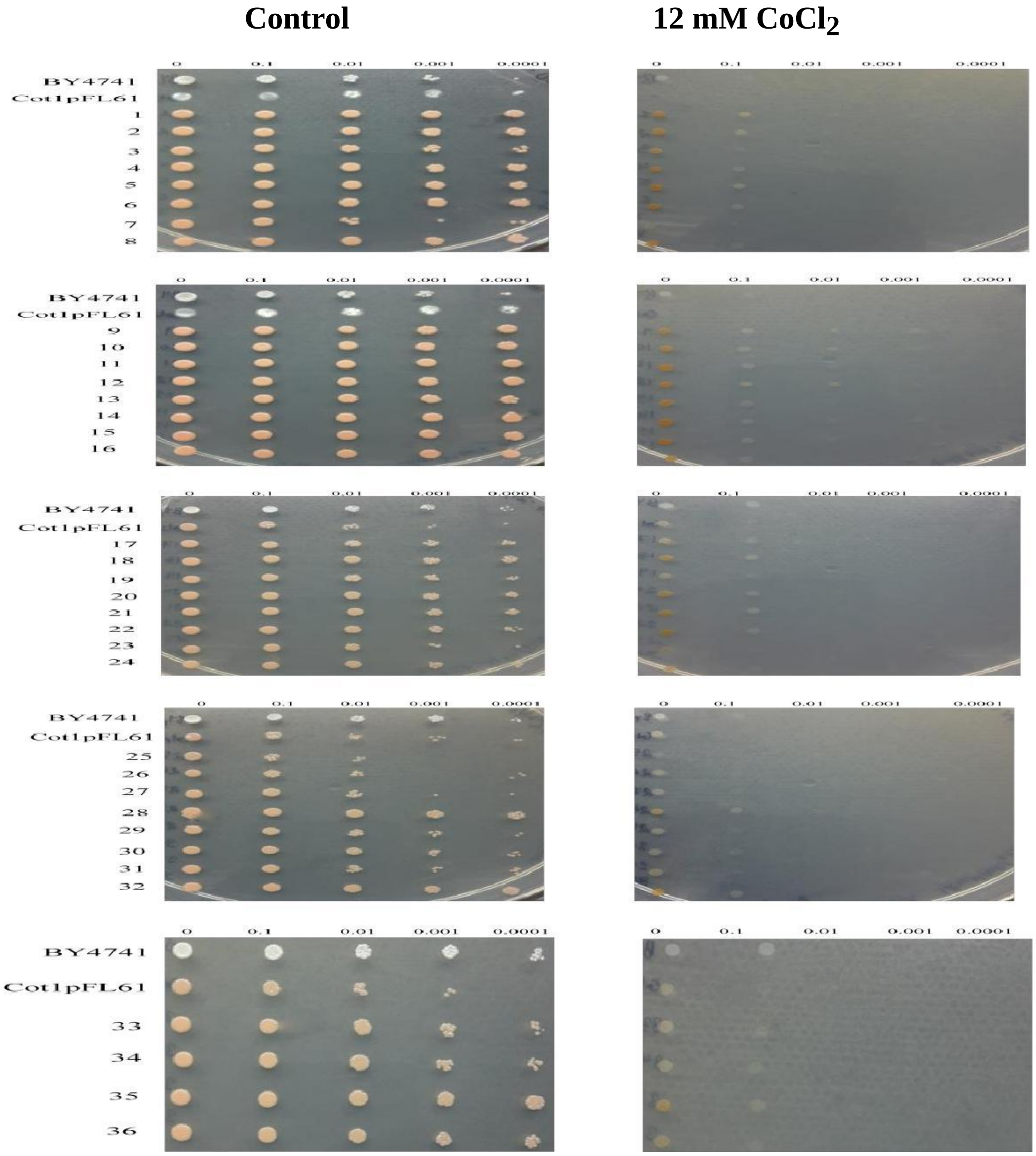
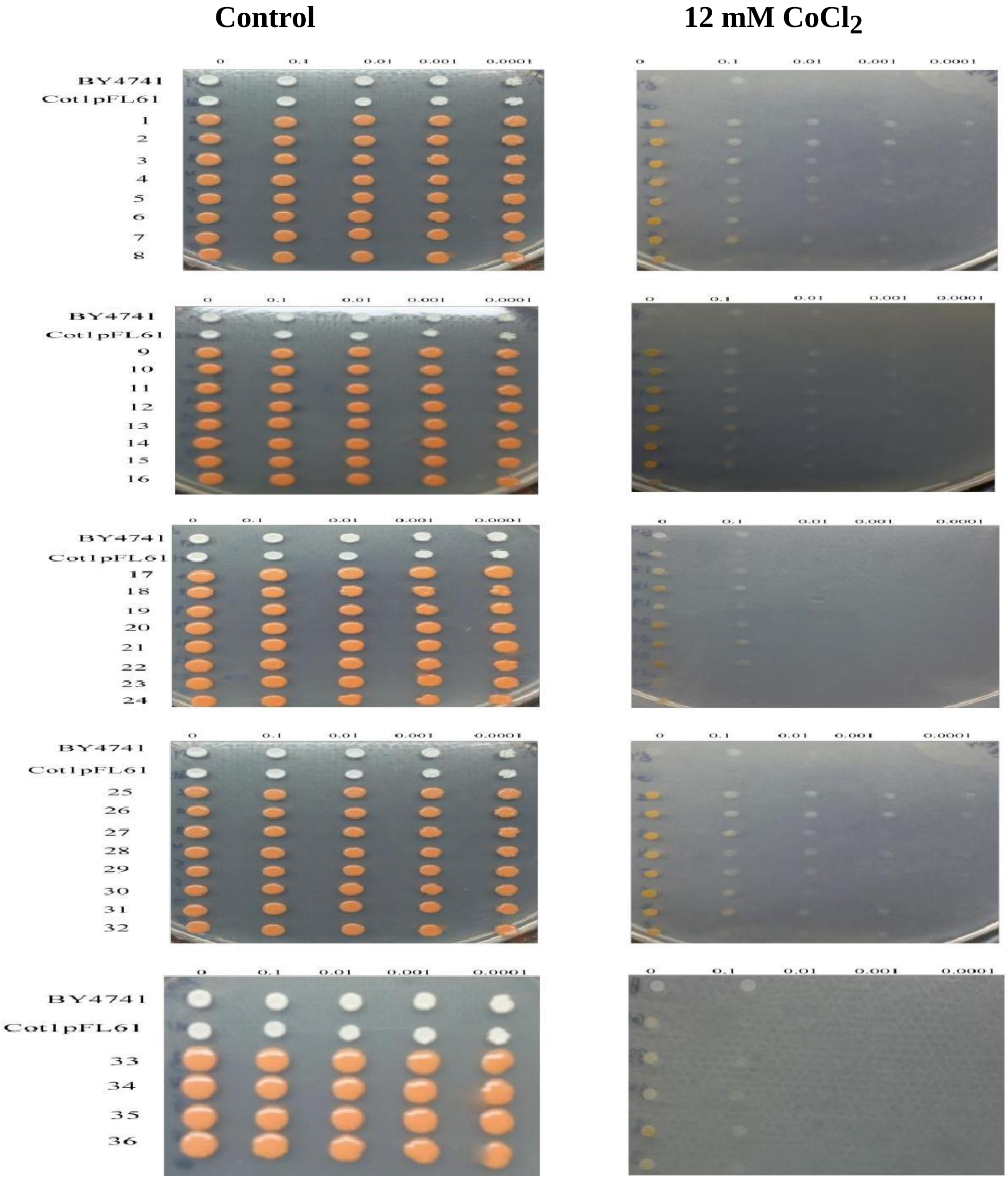


Fig 4.2.4 Functional complementation of yeast mutants on SD-Ura medium supplemented (or not) with 12 mM Cobalt. *COT1* cells harbouring the control vector pFL61 were unable to grow on Cobalt containing media, where as all 36 transformants and wild type strain BY4741 cells were grown well on Cobalt medium

4.2.5 Drop assay at 12 mM $CoCl_2$ (after FOA)

To assure whether cDNAs insert were responsible for metal tolerance at drop assay, further drop assay was performed for FOA treated transformants at 12 mM $CoCl_2$ concentration of metal and no tolerance was found by transformants at this level (Fig 4.2.5).


Control

12 mM $CoCl_2$

Fig 4.2.5 Functional complementation of yeast mutants on SD+Ura medium supplemented (or not) with 12 mM Cobalt. *COT1* cells harbouring the control vector pFL61(after FOA) were unable to grow on Cobalt containing media, where as all 36 transformants(after FOA) and wild type strain BY4741(after FOA)cells were grown well on Cobalt medium

4.3 Colony PCR

After the drop assay result, colony PCR was performed to check the successful transformation of cDNA insert in mutant yeast by visualizing the amplified band of required or particular size with appropriate primers i.e. pFL61NF and pFL61NR and observed that in first 10 transformants no amplification was found but in other transformants same size bands visualized (Fig 4.3.1, 4.3.2).

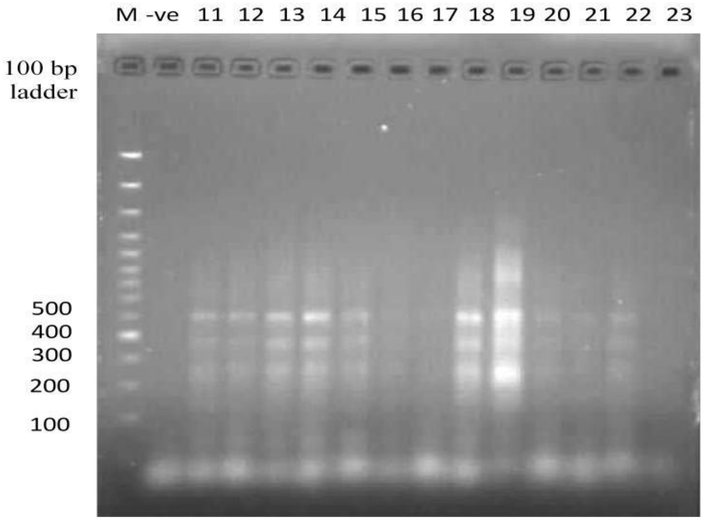


Figure 4.3.1 Colony PCR of cDNA inserts. Lane 1:100 bp ladder, Lane 2: -ve control, Lane 3-13: 11-23 Transformants of Lib A

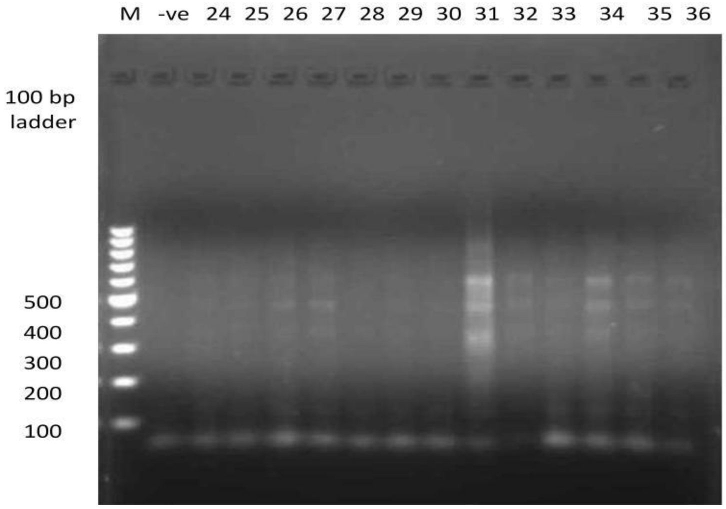


Figure 4.3.2 Colony PCR of cDNA inserts. Lane 1:100 bp ladder, Lane 2: -ve control, Lane 3-12: 24-36 Transformants of Lib A

4.4 Growth kinetics

The mutant yeast *COT1*containing empty pFL61, all the 36 transformants were tested for resistance to 12 mM of cobalt in a growth kinetics medium for 48 hours. The results showed that the *COT1* containing empty pFL61 cells were unable to grow on 12 mM cobalt containing media and their growth was completely inhibited, whereas in case of all transformants growth was increased significantly with increasing time of incubation (Table 4.1, 4.2, 4.3) and (Fig. 4.4.1, 4.4.2, 4.4.3) and the growth of mutant yeast *COT1*pFL61 without metal was also significantly increased with increasing of incubation time. The present results indicated that insert in the plasmid was responsible for cobalt tolerance.

Table 4.1 Growth curve of cobalt tolerant transformants (2, 4, 6, 8, 10, 13) and *COT1* cells transformed with the control vector pFL61 in SD−Ura medium supplemented (or not) with 12 mM Cobalt

Time (hours)	***COT1*PFL61**	***COT1*pFL61+ Co**	**2 ± Co**	**4 ± Co**	**6 ± Co**	**8 ± Co**	**10 ± Co**	**13 ± Co**
0	0.010± 0.010	0.01± 0.000	0.010± 0.000	0.010± 0.000	0.010± 0.00	0.010± 0.00	0.010± 0.00	0.010± 0.00
5	0.088± 0.016	0.102± 0.000	0.217± 0.037	0.262± 0.024	0.265± 0.071	0.337± 0.070	0.287± 0.038	0.271± 0.038
8	0.270± 0.022	0.118± 0.000	0.417± 0.071	0.485± 0.095	0.488± 0.098	0.522± 0.052	0.625± 0.002	0.571± 0.026
11	0.551± 0.000	0.123± 0.003	0.684± 0.009	0.709± 0.082	0.791± 0.073	0.740± 0.053	0.859± 0.020	0.815± 0.016
14	0.939± 0.012	0.123± 0.004	0.854± 0.022	0.994± 0.098	1.024± 0.041	1.026± 0.060	1.149± 0.021	1.067± 0.062
17	1.207± 0.031	0.121± 0.002	1.109± 0.028	1.285± 0.103	1.286± 0.038	1.274± 0.055	1.365± 0.055	1.275± 0.098
20	1.360± 0.036	0.125± 0.001	1.381± 0.012	1.478± 0.078	1.456± 0.050	1.469± 0.051	1.593± 0.036	1.491± 0.062
23	1.430± 0.012	0.136± 0.005	1.592± 0.088	1.641± 0.083	1.624± 0.109	1.626± 0.025	1.731± 0.026	1.680± 0.060
26	1.415± 0.007	0.127± 0.001	1.675± 0.026	1.697± 0.060	1.693± 0.081	1.706± 0.049	1.776± 0.022	1.736± 0.045
29	1.459± 0.007	0.129± 0.007	1.872± 0.025	1.834± 0.031	1.829± 0.002	1.836± 0.031	1.898± 0.005	1.859± 0.012
32	1.449± 0.021	0.353± 0.004	1.904± 0.011	1.855± 0.034	1.896± 0.000	1.871± 0.055	1.933± 0.019	1.906± 0.042
35	1.453± 0.024	0.339± 0.036	1.979± 0.002	1.917± 0.002	1.967± 0.008	1.965± 0.042	1.982± 0.000	2.019± 0.038
38	1.472± 0.007	0.156± 0.008	2.017± 0.007	1.965± 0.020	1.971± 0.008	1.965± 0.049	2.057± 0.016	2.004± 0.002
41	1.484± 0.033	0.125± 0.007	2.083± 0.023	2.011± 0.046	2.042± 0.036	2.040± 0.027	2.076± 0.038	2.021± 0.004
44	1.488± 0.025	0.129± 0.007	2.110± 0.000	2.039± 0.012	2.029± 0.016	2.047± 0.025	2.075± 0.004	2.037± 0.056

n=2, Mean ± SD

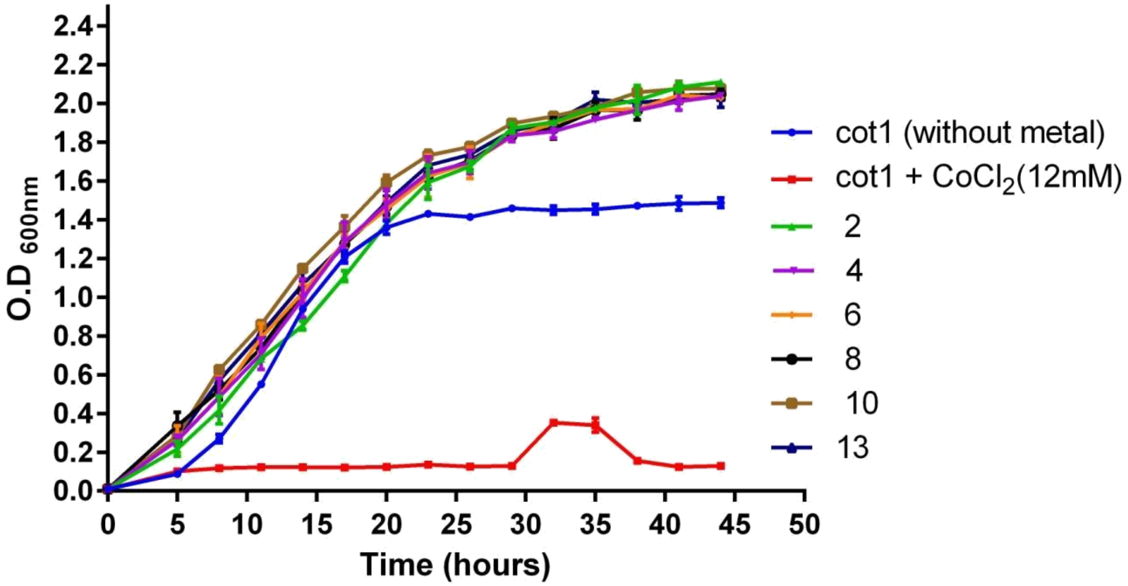


Figure 4.4.1 Growth curve of cobalt tolerant transformants (trans 2, 4, 6, 8, 10, 13) and *COT1* cells transformed with the control vector pFL61 in SD−Ura medium supplemented (or not) with 12 mM cobalt

Table 4.2 Growth curve of copper tolerant transformants (1 5, 1 6, 17) and *COT1* cells transformed with the control vector pFL61 in SD−Ura medium supplemented (or not) with 12 mM Cobalt

Time (hours)	***COT1*pFL61**	***COT1*pFL61+ Co**	**15+Co**	**16+Co**	**17+Co**
0	0.010±0.010	0.01±0.000	0.010±0.00	0.010±0.000	0.010±0.000
5	0.088±0.016	0.102±0.000	0.154±0.025	0.115±0.012	0.111±0.004
8	0.270±0.022	0.118±0.000	0.184±0.026	0.144±0.008	0.138±0.004
11	0.551±0.000	0.123±0.003	0.414±0.040	0.325±0.005	0.284±0.006
14	0.939±0.012	0.123±0.004	0.563±0.037	0.469±0.007	0.384±0.016
17	1.207±0.031	0.121±0.002	0.751±0.060	0.592±0.008	0.481±0.002
20	1.360±0.036	0.125±0.001	0.914±0.067	0.725±0.005	0.586±0.009
23	1.430±0.012	0.136±0.005	1.045±0.065	0.843±0.077	0.728±0.035
26	1.415±0.007	0.127±0.001	1.135±0.094	0.994±0.011	0.821±0.033
29	1.459±0.007	0.129±0.007	1.223±0.098	1.111±0.053	0.888±0.039
32	1.449±0.021	0.353±0.004	1.573±0.060	1.523±0.021	1.253±0.084
35	1.453±0.024	0.339±0.036	1.750±0.028	1.708±0.030	1.448±0.011
38	1.472±0.007	0.156±0.008	1.827±0.050	1.768±0.006	1.556±0.031
41	1.484±0.033	0.125±0.007	1.911±0.048	1.824±0.009	1.629±0.028
44	1.488±0.025	0.129±0.007	1.958±0.022	1.721±0.043	1.667±0.002

n=2, mean ± SD

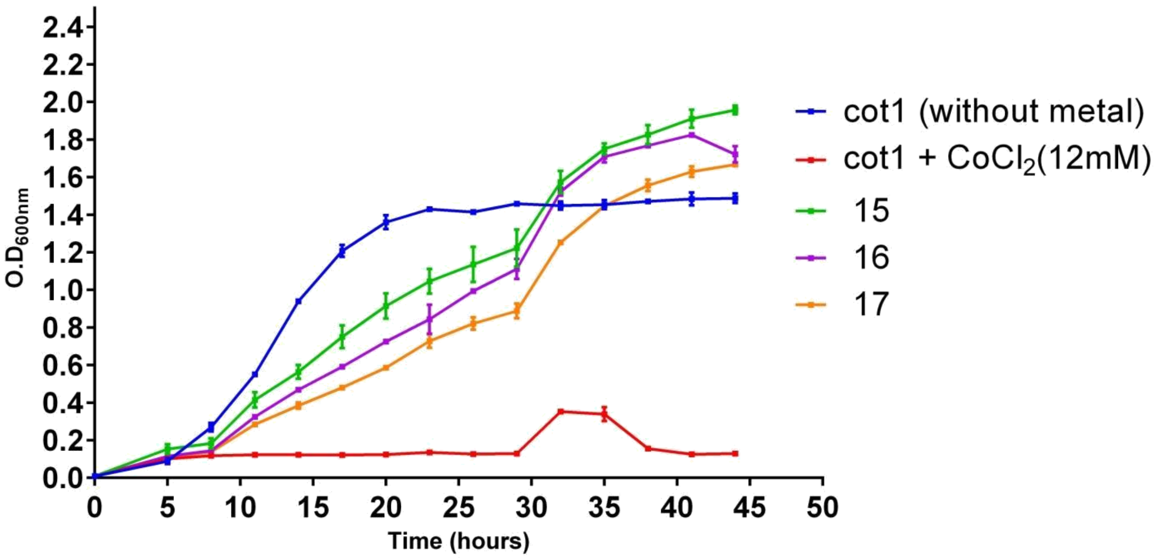


Figure 4.4.2 Growth curve of cobalt tolerant transformants (15, 16, 17) and *COT1* cells transformed with the control vector pFL61 in SD−Ura medium supplemented (or not) with 12 mM cobalt

Table 4.3 Growth curve of copper tolerant transformants (18, 24, 2 8, 33, 3 6) and *COT1* cells transformed with the control vector pFL61 in SD−Ura medium supplemented (or not) with 12 mM Cobalt

Time (hours)	***COT1*pFL61**	***COT1*pFL61+ Co**	**18+ Co**	**24+ Co**	**28+Co**	**33+Co**	**36+Co**
0	0.010± .010	0.01± 0.000	0.010±0.010	0.010±0.000	0.001±0.000	0.010±0.00	0.010±0.000
5	0.088±0.016	0.102± 0.000	0.129±0.023	0.154±0.009	0.144±0.004	0.142±0.018	0.130±0.008
8	0.270±0.022	0.118± 0.000	0.166±0.021	0.204±0.012	0.199±0.001	0.192±0.007	0.183±0.004
11	0.551±0.000	0.123± 0.003	0.351±0.006	0.452±0.021	0.415±0.002	0.416±0.003	0.362±0.003
14	0.939±0.012	0.123± 0.004	0.494±0.034	0.639±0.016	0.570±0.020	0.566±0.002	0.502±0.002
17	1.207±0.031	0.121± 0.002	0.583±0.019	0.784±0.060	0.729±0.008	0.716±0.001	0.604±0.006
20	1.360±0.036	0.125±0.001	0.717±0.019	0.975±0.102	0.899±0.030	0.942±0.014	0.731±0.004
23	1.430±0.012	0.136±0.005	0.860±0.059	1.189±0.007	1.075±0.002	1.083±0.007	0.917±0.003
26	1.415±0.007	0.127±0.001	1.011±0.012	1.261±0.002	1.187±0.011	1.174±0.041	1.042±0.002
29	1.459±0.007	0.129±0.007	1.083±0.026	1.334±0.014	1.232±0.019	1.285±0.060	1.111±0.002
32	1.449±0.021	0.353±0.004	1.494±0.022	1.659±0.008	1.560±0.031	1.596±0.024	1.454±0.005
35	1.453±0.024	0.339±0.036	1.661±0.014	1.79±0.0254	1.712±0.046	1.737±0.028	1.594±0.006
38	1.472±0.007	0.156±0.008	1.753±0.049	1.851±0.052	1.752±0.026	1.780±0.025	1.713±0.004
41	1.484±0.033	0.125±0.007	1.815±0.017	1.896±0.020	1.822±0.036	1.890±0.077	1.751±0.002
44	1.488±0.025	0.129±0.007	1.803±0.028	1.931±0.014	1.869±0.040	1.885±0.009	1.752±0.002

n= 2, Mean ± SD

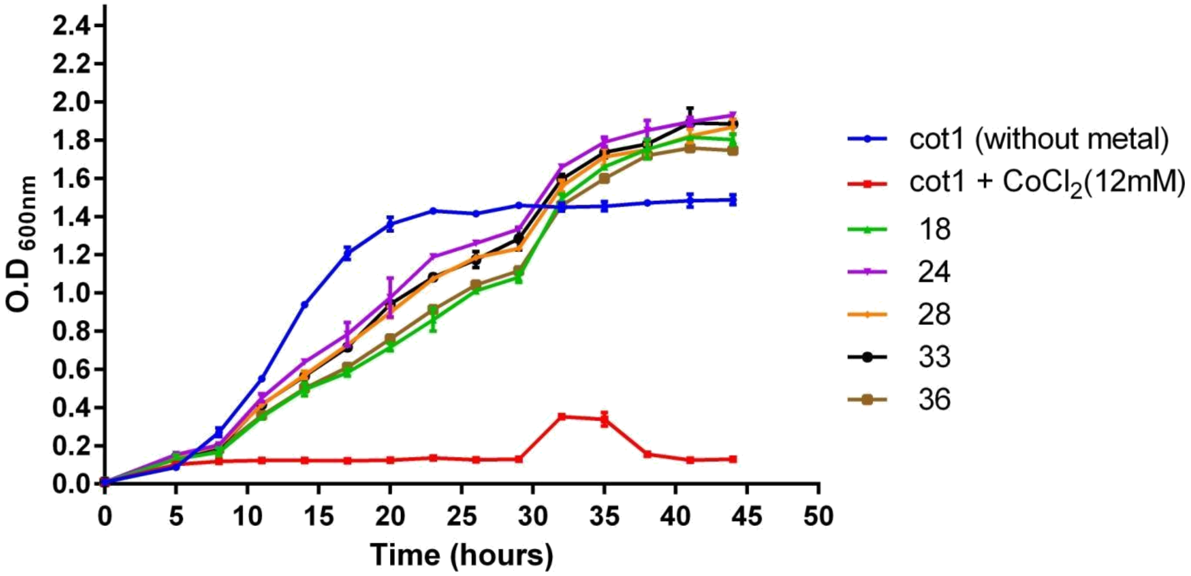


Figure 4.4.3: Growth curve of cobalt tolerant transformants (18, 24, 28, 33, 36) and *COT1* cells transformed with the control vector pFL61 in SD−Ura medium supplemented (or not) with 12 mM cobalt

In the present study, transformants were selected for their ability to rescue the metal (cobalt) sensitive phenotype of yeast mutants by functional complementation studies. The cDNA inserts in the plasmid which was transformed in mutant yeast cells may be responsible for providing this phenotype. Towards investigating this mechanism, the recent studies by following the same approach indicated that *Saccharomyces cerevisiae* cells resistant to high cobalt concentrations up to 8 mM, by employing different evolutionary engineering strategies: selection under constant or gradually increasing stress levels, and selection under continuous or pulse exposure to cobalt stress. Selection under continuous exposure to gradually increasing cobalt stress levels yielded the most resistant cell population to cobalt (Cakar *et al*., 2005). However, the resistance was highly heterogeneous within the mutant populations ranging from 3 to 3700 fold survival rate of isolated individuals to 8 mM $CoCl_2$ in the most resistant population. Moreover, cobalt-resistant individual colonies were associated with 2–4 times lower intracellular cobalt contents as compared to wild-type, and with cross-resistance to metals such as nickel, zinc, manganese, but not to copper and chromium ions (Cakar *et al.,* 2009*)*. Contrary to mutants evolved under continuous exposure to cobalt, those isolated by pulse exposure strategy also exhibited resistance to heat shock and hydrogen peroxide stress. Taken together, this study reinforced the fact that evolutionary engineering is useful in selecting strains with very specific phenotypes, and further illustrated the importance of the strategy chosen to isolate the best evolved strain.

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).

Conclusions

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 libraries. Cobalt tolerant transformants were obtained from the transformation with plasmids from library. 36 transformants were obtained by this transformation and further characterized by drop out assay and growth kinetics. 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 the genes conferring metal tolerance.

This study demonstrates the feasibility of the metatranscriptomics approach, from soil-extracted RNA to the recovery of functional cDNAs expressed in a eukaryotic 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

1. TBE Buffer (10x)

Tris-HCl 0.09 M (pH 8)
Boric acid 0.9 M
EDTA 0.02 M (pH 8)

2. Agarose gel loading dye (6X)

Bromophenol blue 0.25%
Xylene cyanol FF 0.25%
Glycerol in water 30.0%

3. YPD Medium

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.

4. SD medium

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

5. 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-M ethionine	200 mg/L
L-Phenylalanine	500 mg/L
L-Threonine	2000 mg/L
L-Tryptophan	200 mg/L
L-T yrosine	300 mg/L
L-U racil	200 mg/L
L-Valine	1500 mg/L

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