Cloning and characterization of cobalt and zinc tolerance genes by using metatranscriptomics approach

DISSERTATION

Submitted by

Karamjeet Kaur

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In partial fulfilment for the award of the degree of

Master of Science in Biotechnology

Under the Guidance of

Prof. M.S Reddy

Department of Biotechnology

Thapar University, Patiala

JULY, 2017

CERTIFICATE

Certified that the thesis entitled '**Cloning and characterization of cobalt and zinc tolerance genes by using metatranscriptomics approach**' submitted by Ms. **Karamjeet Kaur** (301501007) in partial fulfilment of the requirement for the award of the degree of **Master's of Science** in Biotechnology in the Department of Biotechnology, Thapar University, Patiala, Punjab is the record of candidate's own independent and original research work carried out by her under my supervision and guidance. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for award of any degree.

Dr. M.S Reddy
(Supervisor)
Professor
Department of Biotechnology
Thapar University
Patiala, Punjab

DECLARATION

I hereby declare that the work which is being presented in this thesis **"Cloning and characterization of cobalt and zinc tolerance genes by using metatranscriptomics approach"** submitted by me for the award of the degree of **Masters in Science** in the Department of Biotechnology, Thapar University, Patiala, is true and original record of my own independent and original research work carried out under the supervision of Dr. M.S. Reddy, Professor, Department of Biotechnology, Thapar University, Patiala, Punjab, India. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree in India or Abroad.

Karamjeet Kaur.

(Karamjeet Kaur)

Place: Patiala

Date: July, 17, 2017

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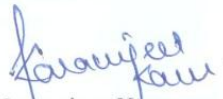
Date:

Place: Patiala



Karamjeet Kaur


Dedicated
To
My Mom
&
Dad

ABSTRACT

Heavy metals are probably toxic to the living beings depending upon their concentration and sensitivity in the ecosystem. These living beings starting from unicellular to multicellular and prokaryotes to eukaryotes have developed various known and unknown mechanisms to adapt such stressed or adverse environment. Functional environmental transcriptomics plays a significant role in recognizing genes responsible or part of these unknown yet important pathways. Metatranscriptomics approach provides information about expressed subset of genes within a microbial community. In the present study, functional metatranscriptomics approach was adopted to identify cobalt tolerance genes expressed by eukaryotic species. Size fractionated libraries constructed in the modified pFL61 yeast- *E.coli* shuttle vector screened through yeast transformation for the presence of cobalt and zinc tolerant cDNAs followed by functional complementation assay for the resistant clones. In this study cobalt sensitive Δ*cot1* mutant strain and zinc tolerant Δ*zrc1*derived from wild strain BY4741 was used. Yeast transformants showing cobalt and zinc tolerance were identified by plating on medium supplemented with respective metal and characterized by studying their growth kinetics.

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List of Abbreviations

□C	Degree Centigrade
Δ	Delta
%	Percent
β	Beta
bp	Base pair
cd	Cadmium
cDNA	Complementary deoxyribonucleic acid
Co	Cobalt
$CoCl_2$	Cobalt chloride
cm	Centimeter
Cu	Copper
cys	Cystein
DNA	Deoxyribonucleic acid
dNTPs	2'-deoxynucleoside-5'-triphosphate
EDTA	Ethylenediamine-tetraacetic acid
eg.	Example
Fig.	Figure
g	gram
g/L	gram/litre
Kb	Kilo base
Kg	Kilogram

LB	Luria-Bertani broth
m	Molar
mg	milligram
mg/ml	miligram/mililitre
ml	mililitre
mM	milimolar
mRNA	messenger RNA
Ni	Nickel
nm	nanometer
pb	Lead
PCR	Polymerase chain reaction
RNA	Reverse transcription
SD-Ura	Synthetic derived media without uracil
Tris	Tris- (Hydroxymethyl)-aminomethane
U.V	Ultraviolet
w/v	Weight by volume
Zn	Zinc
µg	microgram
µl	microlitre
µM	micromolar

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INTRODUCTION

Soil is a key component for the human survival and also hotspot of microbial diversity with thousands of different species. Soil is a complex environment in which numerous microbes are present such as bacteria, fungi, algae and yeast. Due to the different type of physical activities such as fire, volcanoes may release different pollutants in the ecosystem which causes adverse effect on living organisms. These problems occur by various anthropogenic activities like industrialization, urbanization which leads to the release of heavy metals into the environment (Reddy *et al*., 2016). Heavy metals refers to a metal that has relatively density 5g/cm^3 (Duruibe *et al*., 2007).There are two types of heavy metals essential such as Sodium (Na), Potassium (K), Copper (Cu), Zinc (Zn), Cobalt (Co), Magnesium (Mg), and Iron (Fe) and non-essential metals such as Cesium (Cs), Aluminum(Al), Cadmium(Cd), Lead (Pb), Mercury (Hg) (Violante *et al.,* 2007). Heavy metals are involved directly and indirectly in the growth, differentiation, metabolism of the microbes. The essential elements play critical role in the formation of structure of enzyme and proteins (Emanverdian *et al.,* 2015*).* Non-essential metals have least role as compared than essential metal. Small quantities of both essential and non-essential metals in plants are beneficial for their growth, metabolism and development. But in excess amount can cause reduction and inhibition of plant growth (Emanverdian *et al.,* 2015). Higher concentration of heavy metals in the soil causing effect on crops yield result into food insecurity (Chibuike *et al.,* 2014). The presence of heavy metals causes adverse effect on food chain, crops and on the health of human beings. Heavy metals cause badly effect in environment due to certain changes in pH, organic matter and cation exchange of the soil (Shah *et al.,* 2010). It was also observed that elevated concentration of heavy metals may activate the formation of free radicals and reactive oxygen species (Dietz *et al.,* 1999). Heavy metal contamination is accumulating in all over the world. Agricultural soil is contamined by heavy metals. It causing affects on industrial and defense related sites (Zafar *et al.,* 2007). This polluted soil also affect on microflora and micofauna. The toxicity of metals can have serious effect on the population of microbes (Giller *et al.,* 2009) and development of tolerance among different species of microbes against heavy metals (Bailly *et al.,* 2007). For studying such type of species, different approaches from the fields of biotechnology are used (Bashiardes *et al.*, 2016). Various culture independent approaches are used, as some species are not easy to isolate from the complex environment and not easy to culture in general lab conditions which is consortium of microflora. So, with the help

of these approaches the functional diversity and activities of microbial communities would be possible to study (Carvalhais *et al.,* 2012).

"Meta" approaches have come into practice such as metagenomics, metatranscriptomics, metaproteomics and metabolomics. Directly from environmental samples DNA, RNA, proteins and metabolites can be extracted and analyzed by using these approaches. Among these four "meta" approaches, metagenomics and metatranscriptomics are widely used. In complex microbial communities, metatranscriptomics approach provides best ability to identify and investigate the gene regulation of baseline gene expression for specific studies as compared with other approaches (Moran, 2009). As culture independent technique, metagenomics analysis, as it reveals genetic content of bacterial population, but metatranscriptomics proclaim genetic content as well as details about the population that are transcriptionally active (Bashiardes *et al.,* 2016). This approach elevates our knowledge of microbial responses and functionality towards adverse environment (Carvalhais *et al.,* 2012). Therefore, in environmental sample screening for eukaryotic functions focus only on metatranscriptomic approach to identify genes present in the genome (Damon *et al.,* 2011). This approach referred to as functional metatranscriptomics, is based on from environmental samples extraction of the polyadenylated mRNA and its conversion into complementary DNA. In yeast expression vector, cDNAs are cloned. The diversity of the genes in the ecosystem expressed by different eukaryotic species are represent by environmental cDNAs (Damon *et al.,* 2011).This genes plays an important role in adaptation to stressful conditions or involved in organic matter degradation.

Although heavy metals show a general toxicity towards most living organisms, some metal tolerant/ resistant species can tolerate relatively high concentration of these compounds. As a result, most "moderately-polluted" soils host several species of metal-resistant species which carry out basic biological processes necessary to maintain soil fertility in adverse condition or stress. One current challenge in environmental science is to understand the diversity of mechanisms leading to heavy metal resistance. Though number of these mechanisms such as chelation, excretion, cellular compartmentation has been described in a number of model species, through general lab cultivation they may be representative of the diversity of mechanisms developed in the numerous metal resistant species. This study based on functional metatranscriptomics allows characterization of the genes which are implicated in adaptation to heavy metal resistance. Here we report, cDNAs constructed by using the Mint-2 cDNA synthesis

kit from metal contaminated soil through yeast transformation screened for cobalt and zinc tolerance by sensitive yeast mutants. Selected clones were identified and sequenced. Characterizations of some of these genes were carried out to identify the genes having potential for metal tolerance.

Objective

- Cloning and characterization of cobalt and zinc resistance genes by yeast complementation assay

REVIEW OF LITERATURE

Soil contains a tremendous diversity of microflora and microfauna present in a state of dynamic equilibrium (Gans *et al.,* 2005; Roesch *et al.,* 2007). The entire world of soil microorganisms contest with each other for survival in their habitat. Due to the change in environmental conditions such as food supply, temperature, moisture, oxygen supply etc, can result in changes which cause one or many types of soil microbes to become temporarily dominant over the others. Microbial diversity at a particular soil ecosystem changes drastically with the alteration in the moisture content (Zhou *et al*., 2002), 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), pH, nutrients (Broughton and Gross, 2000), temperature (Ward *et al*., 1998), redox potential, and other variables such as the architecture of their habitats (Sessitsch *et al*., 2001). Soil is a significant component of rural and urban environment which is why, the management of soil is necessary. They are a major sink for heavy metals released into the environment by anthropogenic activities (Nagajyoti *et al.,* 2010) (Fig. 2.1). Unlike organic contaminants which are oxidized to carbon (IV) oxide by microbial action most metals do not undergo microbial or chemical degradation and their total concentration in soil strive for a long time (Wuana, 2011).

2.1 Toxicity in relation to heavy metal accumulation in soil

Contamination in soil may pose risks and hazards to humans and the ecosystem through direct ingestion or contact with contaminated soil, the food chain, drinking of contaminated ground water, reduction in food quality via phytotoxicity, reduction in land usability for agricultural production causing food insecurity, and land tenure problems (Wuana, 2011). Metal contamination becomes a major problem in all over the world. Heavy metal is the group of chemical elements, which are characterized with the high atomic weight and density. Atomic weight of heavy metals are more than 20 (Giller *et al.,* 1998*)* and mass density greater than 5 g/cm^3 (Emamverdian *et al.,* 2015). They have a tendency to release electrons in chemical reactions and form simple cations. Their deficiency and excess affects soil, plants, and microbial communities the most. From different observations it had been shown that heavy metals are inhibitors or stimulating factors for life processes (Szyczewski *et al.,* 2009). There are

two kinds of metals, essential micronutrients (Fe, Mn, Zn, Cu, Mg, Mo, and Ni) for normal plant growth and nonessential elements (Cd, Sb, Cr, Pb, As, Co, Ag, Se, and Hg) with physiological function (Emamverdian *et al.,* 2015). Essential metals are required by the plants for their growth, metabolism and development (Emamverdian *et al.,* 2015). These essential metals play key role in the metabolic processes but deficiencies of these essential metals causing diminish the body's capacities for detoxification and formation of free radicals. Essential elements are also affected by the toxicity of the heavy metals. Few metals replace the essential elements like Hg, Cd replace the Cu, Zn from metallothionein (Quig, 1998). The non essential metals are harmful for both plants and animals. The toxic level of the heavy metals depends on their concentration (Szyczewski *et al.,* 2009). Metals present in element and compound form with other soil components. Soil is affected by metals due to certain factors such as pH, the density, type of soil colloids, the degree of complexation with ligands, and the soil's relative surface area. Metals have capability to alter the soil biological properties (Chibuike *et al.,* 2014). These heavy metals are great impact on human health by ingestion (drinking or eating) or inhalation (breathing).

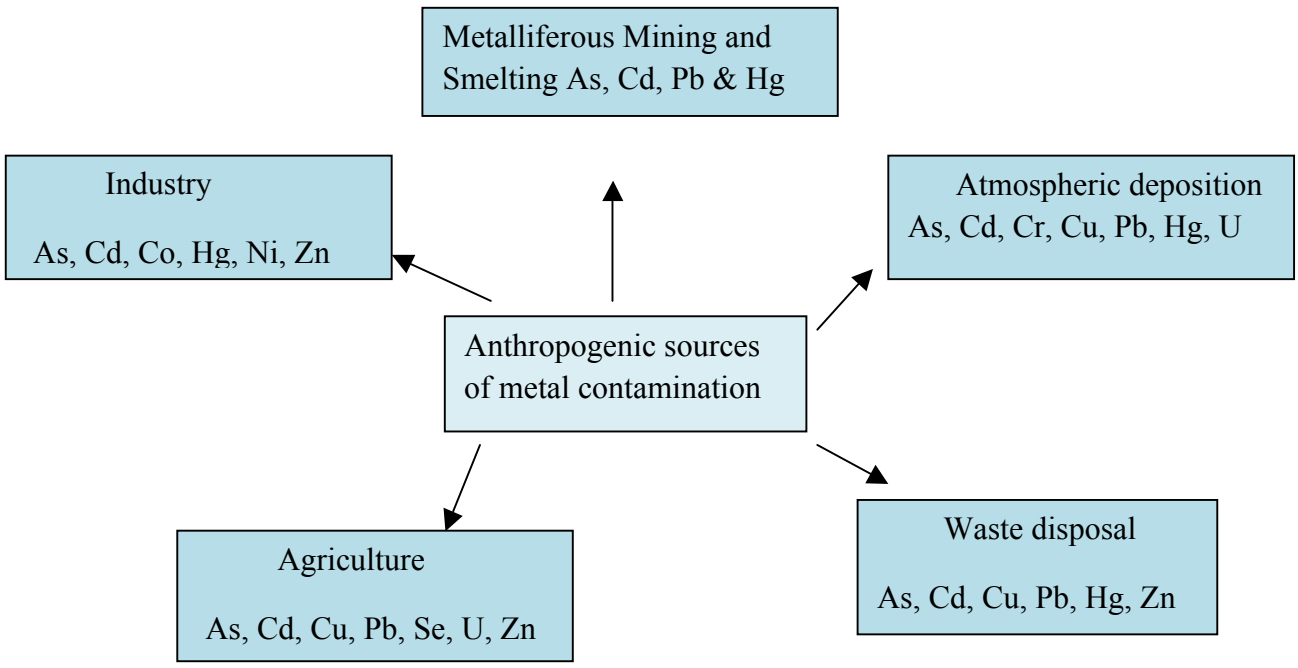

Fig. 2.1 Anthropogenic activities leading to the contamination of soils with heavy metals **(**Nagajyoti *et al.,* 2010

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and skin and may cause high blood pressure, cancerous changes in heart rhythm, abdominal cramps, nose ulcers and damage to these visceral organs (Martin *et al.,* 2009). Heavy metals contamination in the natural environment are related to various natural process as rock decay, volcanic eruptions, evaporation of oceans, forest fire and soil formation processes. Heavy metals are included into the environment from number of industrial sources such as the power industry, transport, municipal waste management, waste dumping sites, fertilizers etc. The anthropogenic activities disperse heavy metal in the environment and they contaminate soil, water and air. Directly and indirectly, through plants it enters into human and animal bodies (Bellion *et al*., 2006; Szyczewski *et al*., 2009).

2.2 Tackling the harmful manifestations of environment heavy metals

Heavy metals, such as copper, lead, chromium and mercury are major environmental pollutants. Plants growing in metal polluted soil show changes in metabolic processes in a variety of ways. Heavy metals hinder the formation of protein structures as they bind with sulfhydryl groups (Nagajyoti *et al.,* 2010), changing the essential element from the specific binding site thus causing dysfunction (Farid et al., 2013; Ali *et al.,* 2013) Also, increased level of redox heavy metals (Cr, Cu, Mn and Fe) in plants, generate oxidative injury according to haber weiss and fenton reaction. This leads to the production of reactive oxygen species (ROS), such as O^{2-} (Superoxide free radicals), OH^{-} (Hydroxyl free radicals) (Schutzendubel *et al.,* 2002 ; Zengin *et al*., 2005; Hossain *et al*., 2012; Sytar *et al*., 2013; Flora *et al*., 2009) causing of multiple disorders such as damage to photosynthetic pigments, ion leakage, oxidative DNA attack, redox imbalance, denature of cell structure and membrane etc. which may resulting the activation of programmed cell death pathways (Hatata *et al*., 2008; Sharma *et al*., 2012) (Fig 2.2). For example: - cobalt contamination in tomato plant shows reduction in plant nutrient content. As well cobalt contamination in radish shows reduction in root, shoot length, and decrease the plant sugar, amino acids, antioxidant enzyme activity (Jayakumar *et al.,* 2007). Furthermore, contamination of zinc metal in soil resulted into changes in structure of chloroplast, reduction in photosystem II activity and reduced plant growth in pea plant (Doncheva *et al.,* 2001).

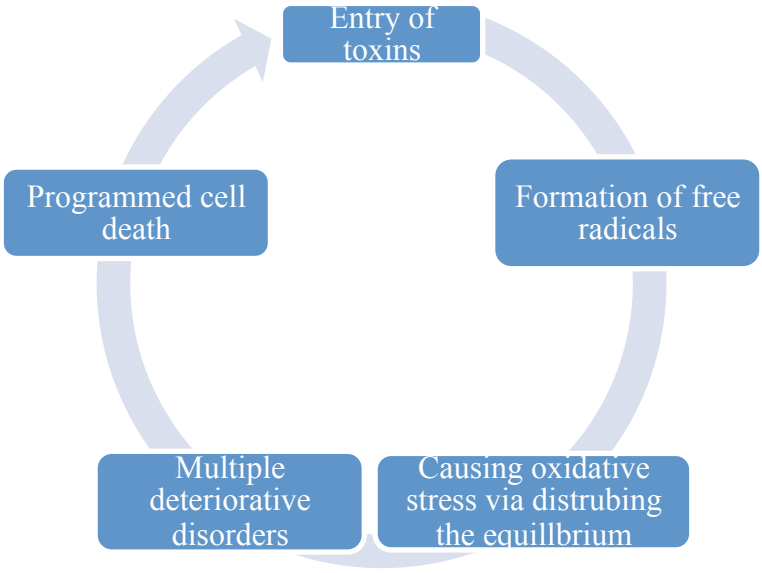


Fig. 2.2 Damage caused by heavy metal toxicity (Emamverdian *et al.,* 2015)

As a first step towards removal of metal toxicity, plant achieved different mechanisms such as immobilization of metals, metal sequestration, biosorption to cell wall, crystallization, transformation of metals (Dalvi *et al*., 2013; Patra *et al*., 2004; Zafar *et al*., 2007). For metal sequestration two main ligands peptides are used: - metallothioneins (MTs) (Hamer, 1985; Kagi, 1993, phytochelatins (PCs) (Murasugi *et al.,* 1981; Emamverdian *et al.,* 2015). Plants also use various molecules such as nicotianamine, spermine, organic acids, glutathione etc as metallochaperones to avoid or tolerate heavy metals (Sharma *et al*., 2006). Different biological molecules such as- superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), alkaloids are also used for metal detoxification (Michalak *et al*., 2006; Rastgoo *et al*., 2011).

Micro-organisms are more sensitive to heavy metal stress than animals and plants growing on the same soil. Heavy metals can enter inside microorganisms by binding to the cell surface. Studies on heavy metal toxicity to soil microorganisms have concentrated on effects where loss of microbial function can be observed and also effects on biodiversity within microbial populations and communities (Sobolev *et al.,* 2008) (Fig. 2.3) For example: cobalt may enter in the yeast *Saccharomyces cerevisiae* cell by using different transporter such as: the low affinity iron transporter, Fet4, via the phosphate transporter Pho84, via the manganese transporter Smf2 (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). 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.

2.3 Approaches for studying heavy metal tolerance: Culture dependant and independent techniques

Various prokaryotes and eukaryotes have a variety of natural capacities to remove toxic heavy metal ions (Kapoor and Viraaraghavan, 1997). These microorganisms use various influx and efflux mechanisms for the removal of heavy metals (Mirlahiji *et al*., 2014; Mohsenzadeh *et al.,* 2012). Prokaryotic and eukaryotic species cannot be easily isolated from complex environmental samples and cannot be grown *in vitro* (Bashiardes *et al*., 2016). Different culture independent approaches such as metagenomics (Shi *et al.,* 2011; Hoff *et al*., 2008), metatranscriptomics and metaproteomics (Su *et al.,* 2012; Langley *et al.,* 2012), are being used to study complex environmental matrices. Metagenomics approach in a culture independent manner allows us to sequence genomes as a single unit from a complex collection of microbes (Riesenfeld *et al*., 2004; Moran, 2009). The functional basis of screening is considered better when compared to sequence based because function based selection allows the discovery of novel biocatalyst which would be missed by purely bioinformatics analysis (Warnecke *et al.,* 2009).

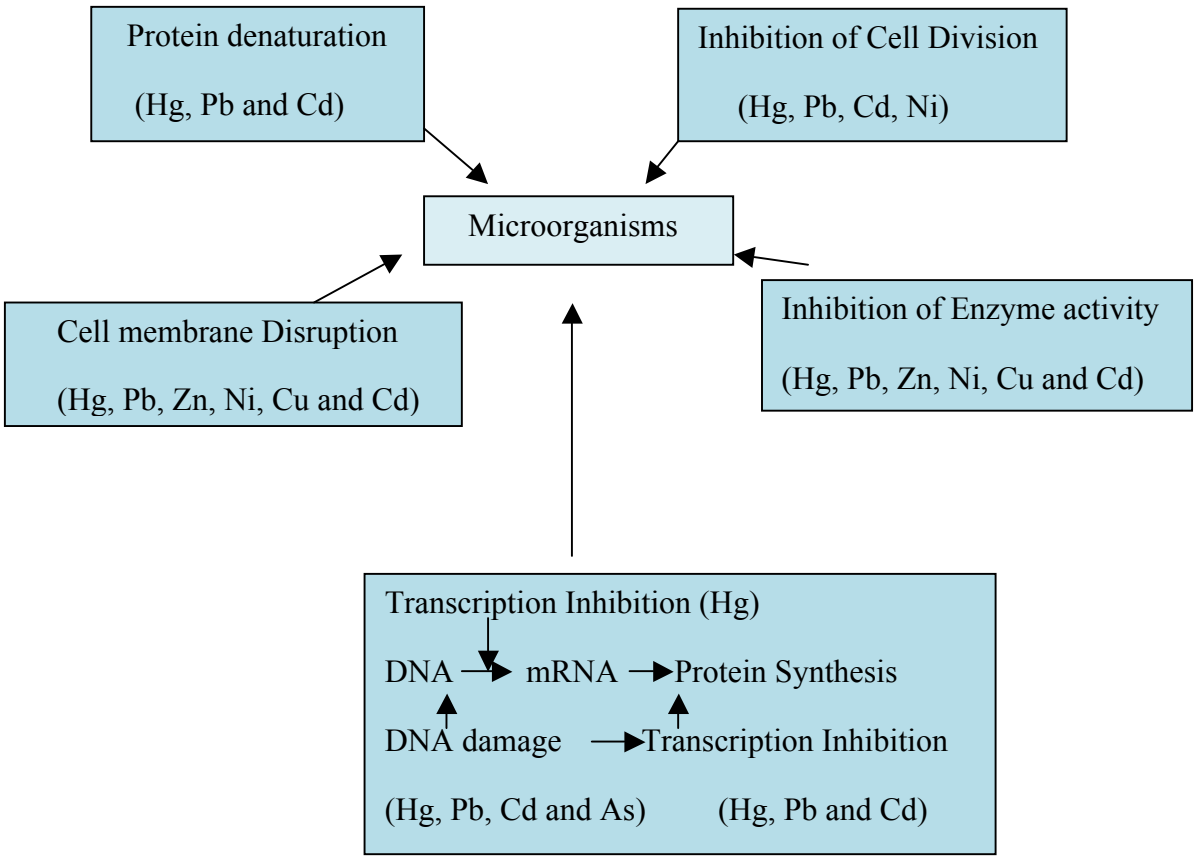


Fig. 2.3 Heavy metal toxicity effect on microorganisms (Sobolev *et al.,* 2008)

Metaproteomics, on the other hand, provides the image of entire protein complement of the microbial community (Hettich *et al.,* 2013; Moran, 2009). However, metatranscriptomics approach provides information about expressed subset of genes within a microbial community at a specific point of time (Warnecke *et al.,* 2009). Metatranscriptomic study is hence preferred because in the case of metagenomics, it analyses the environment sample but it does not distinguish whether the isolated genomic DNA comes from viable or non-viable source. Metagenomics approach also does not provided clear information about the predicted genes such as at what condition genes were actually expressed (Bashiardes *et al.,* 2016). While in metaproteomics approach, for protein extraction, separation, and identification many technical issues arises (Moran, 2009).

2.4 Metatranscriptomics: Holistic approach to understand microbial functions

To better understand the functioning of the microbial community metatranscriptomics approach is used. The new field of metatranscriptomics, which studies sequences of microbial genes expressed within intact natural communities, allows us to understand microbial gene expression patterns (Fig. 2.4). It involves the extraction and analysis of mRNA (the metatranscriptome) which provides information on the regulation and expression profiles of complex communities. It is based upon extraction of total environmental RNA instead of DNA followed by purification by affinity chromatography of the eukaryote specific polyadenylated messenger RNA from the total environmental RNA mixture. These poly-A mRNAs, when converted into cDNA, could be cloned in appropriate expression vectors such as pFL61 which also acts as a shuttle vector between yeast and bacteria as a result allow expression of the cloned genes in the eukaryotic yeast *Saccharomyces cerevisiae*. These environmental cDNA libraries are therefore representative of the diversity of genes expressed by the different eukaryotic microorganisms present in the original soil sample. This protocol, from soil to an environmental cDNA expressed in yeast, was first implemented by Bailly *et al* (2007) for a *Pinus* forest soil to isolate of histidine biosynthetic genes by complementation of a histidine auxotrophic yeast mutant (Bailly *et al*., 2007).

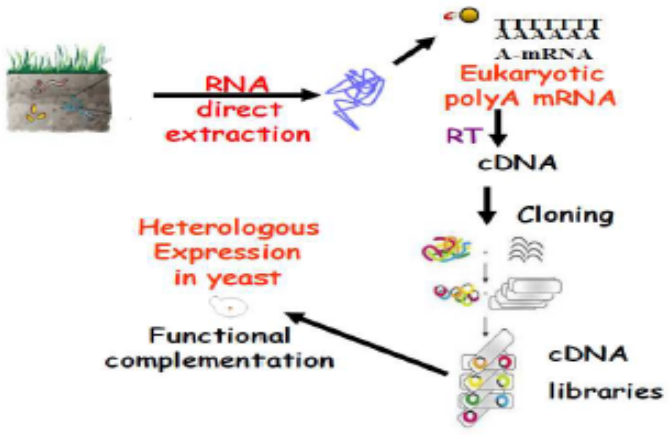


Fig. 2.4 Functional metatranscriptomics approach (Bailly *et al*., 2007).

There is much evidence which shows, that metatranscriptomics approach is used to study the diversity of microbial community. In 2011, two novel environmental oligopeptide transporters,

of fungal origin, were identified in soil through functional metatranscriptomics approach by studying their expression in yeast mutant (Damon *et al.,* 2011). These identified PTR2 and DAL5 transporters could transport more than 60-80 dipeptide (Damon *et al.,* 2011; Homann *et al*., 2005; Cai *et al*., 2007). Metatranscriptomics approach also concluded that in forest soil, the plant cell wall degrading enzymes were active on major polymers like cellulose, hemicelluloses, pectin, lignin while in case of organic matter such as extracellular proteases, lipases, a phytase, P450 monooxygenases coding enzymes played important role in degradation (Damon *et al.,* 2012). Lejzerowicz *et al*., (2013) studied the diversity of the deep-sea benthic foraminifera in the marine ecosystem via metatranscriptomics approach and reported that calcareous species are the most active component of foraminiferal community. Culture independent approaches were used to determine the mechanisms adapted by genes in acid mine drainage area under various environmental stress conditions such as heavy metals, low pH and oxidative stress (Chen *et al.,* 2015). For studying about the diversity of protists, metatranscriptomics approach was used. Using this approach, 12 different mineral and organic soil samples with broad diversity of protists such as alveolata in peat soil, rhizaria and amoeboza dominating in forest and grassland soils were detected (Geisen *et al.,* 2015).

Lehembre *et al* (2013) studied metal tolerance intensity in yeast species by metatranscriptomics approach. High degrees of diversity and novelty among the recovered sequences were obtained (Lehembre *et al*., 2013). cDNAs library was constructed from *R. atropurpurea* and obtained genes involved in metal tolerance (Leonhardt *et al.,* 2014). Metatranscriptomics approach also used to study diversity in water microbial community. In 2016, it was revealed that three samples which were collected from the Marcellus Shale shows that water microbial communities are able to form biofilm and also capable of sulfide production. These communities followed different stress management mechanisms to survive adverse condition (Vikram *et al*., 2016). In 2013, by using metatranscriptomics approach different known and unknown genes were identified which are highly responsive to extracellular electron transport (EET) stimuli. From this study, it was observed that only two microbial groups, *Desulfobulbaceae* and *Desulfuromonadales* showed the most significant gene expression responses to applied EET stimuli and also based on this study, it was estimated that how these microbes adapt to specific ecological stimuli (Ishii *et al.,* 2013).

2.5 Metranscriptomics and its advantages: A direct method to study gene expression

Metatranscriptomics approach is a potent tool to consider the timing, regulation, as well as microbial dexterity of complex microbial communities hence harbors a vast scope in the field of biotechnology. It acts as a tailor-made platform for relative studies and involves random sequencing of microbial community mRNA without the use of primer and to predict important genes in advance.

In microbial community, metatranscriptomics helps in identifying and investigating the genes and its regulation from baseline expression level suitable for comparative studies (Moran, 2009). Metatranscriptomics is an efficient tool for sequence-based detection of novel biocatalyst (Wecknecke *et al.,* 2009). Similarly, diverse individualized treatment plans and novel therapeutic approaches were identified by using metatranscriptomics approach (Lim *et al.,* 2013). This approach is a powerful tool for characterizing novel heavy metal resistance genes by functionally complementing mutant yeast (Lehembre *et al*., 2013). Combinations of "omics" tools and new bioinformatics approaches will allow us to appreciate integrated activity patterns between plants and microbes, and determine how this metaorganism can be modified themselves for survive at the heavy metal contaminated soil (Bell *et al*., 2014). Furthermore, metatranscriptomic approach is not only useful for predicting the functions of abundant community members, but are also useful for predicting the functions of 0.05% of the total cell population (Helbling *et al*., 2012).

Metatranscriptomics shed light on microbial metabolism *in situ* and provide critical clues for directing the culturing of uncultured microorganisms. By choosing a condition under which the desired organism is rapidly proliferating and focusing on highly expressed genes encoding hydrolytic enzymes, binding proteins, and transporters, one can identify an organism's nutritional preferences and design a culture medium (Bomar *et al*., 2011).

Material and Methods

The metatranscriptomic library, A (0.1 kb-0.5 kb) and B (0.5 kb-1 kb) synthesized from soil sample collected from France by Yadav *et al.,* (2014) were screened through yeast transformation by using Li-Ac method. Isolation of total RNA by RNA PowerSoil® Total RNA Isolation Kit (Mo Bio laboratories, Carlsbad, CA), synthesis of cDNAs by Mint-2 cDNA synthesis kit, size fractionation of cDNAs libraries followed by cloning into Sfi sites of modified pFL61 followed by elctrotransformation. In the end the transformants were pooled in and library plasmid was extracted by Zyppy™ Plasmid Maxiprep Kit for further screening experiments.

3.1 Screening of cDNA library

After the preparations of cDNAs library A (insert size 0.1-0.5 kb) and B (insert size 0.5-1 kb), screening of these libraries were performed through yeast transformation by standard Li-Ac method (Gietz *et al*., 1998).

3.1.1 Yeast and culture conditions

The *Saccharomyces cerevisae* strains, cobalt- sensitive *Δ cot1* and zinc- sensitive *ΔZrc1* derived from the wild type strain BY4741 (*MATα his3D1 leu2D0 met15D0 ura3D0*) were used in this study. YPD agar medium (Appendix I) was used for maintain these yeast strains. For yeast transformants and mutant strain selective media SD-Ura (Appendix I) and SD-Ura+ metal Co (2 mM) was used.

3.1.2 Transformation of yeast cells by Li-Ac method

To screen the libraries A and B, yeast transformation through the standard lithium acetate method was performed. Under this method, cobalt sensitive strain *Δcot1* mutant and zinc sensitive *ΔZrc1* mutant strain of *S. cerevisiae* were used for transformation of specific cDNA containing plasmids. These plasmids were isolated from constructed library A (insert size 0.1-0.5 kb) and library B (insert size 0.5-1 kb). Similarly wild strain BY4741 and mutant strain *Δcot1* also transformed with empty pFL61 vector cells to use them as control in further experiments.

Procedure

1. In 20 ml YPD medium a single colony of the yeast strain was inoculated and incubated at 200 rpm, 30ºC overnight.
2. Fresh 50 ml YPD media was taken and its O.D.600 nm was set at 1.00 by inoculating from overnight grown culture. It was then incubated at same conditions till the O.D. 600 reaches 2.00.
3. The culture was added in 50 ml falcon tube and centrifuged at 3000 rpm for 10 minutes.
4. Discard the supernatant and pellet was resuspended with 25 ml autoclaved distilled water followed by centrifugation for 10 minutes at 3000 rpm.
5. After centrifugation, discard the supernatant and resuspended the pellet with 1 ml autoclaved distilled water. Centrifuged the 1 ml cell suspension in a mini centrifuge for 30 sec, discarded the supernatant and make up the total volume 1ml with distilled water.
6. 100 µl of yeast suspension was added into 1.5 ml eppendorf tube and centrifuged for 30 seconds with the bench centrifuge. The supernatant was discarded.
7. The pellet was resuspended by adding the following mixture.

Transformation mixture

Components	Concentration (µl)
Autoclaved Distilled water	74
PEG 3350	240
Li-AC	36
SS DNA	10
Template	1

9. 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.
10. The pellet was resuspended with 1 ml autoclaved distilled water.
11. The 100 µl yeast cells were spread on SD-Ura solidified media and remaining cell suspension on SD-Ura + metal incubated for 2-5 days at 30ºC.

3.2 Colony PCR

This technique can be used to determine insert size and orientation in the vector. Isolated plasmids were used for PCR reaction by insert specific primers to know the plasmid DNA contained the insert.

Procedure

A pin-point amount of yeast cell was added in 10 microliters of 10 mM NaOH, mixed and incubated at 98ºC for 30 minutes. The tubes were then quick chilled in ice and vortex for 5 seconds. Then they were quick centrifuged.

1. 1µl of supernatant was transferred in fresh tube. PCR mastermix was added in the tube as follows:

PCR Reaction mixture

Components	Concentration
MQ Water	11.2µl
10X Buffer	2µl
dNTPs	1.5µl
NF	1µl
NR	1µl
Taq polymerase	0.3µl

2. The PCR amplification was carried out with primers pFL61 NF (5'-CTTCTAACCAAGGGGTGGTTTAGTTTAG-3') and pFL61 NR (5'-CTGCATAAAGGCATTAAAAAGAGGAGCG-3'). The PCR program for amplification was carried out as following:

PCR Programme

Stage	Temperature	Time	
Initial denaturation	94˚C	5 minutes	
Final denaturation	94˚C	30 seconds	25- 35 cycles
Annealing temperature	55˚C	1 minute	
Initial extraction	72˚C	1minute	
Final extraction	72˚C	10 minutes	
Extending	4˚C	∞	

3. Amplified products were visualized on a 0.8% (w/v) agarose gel (Appendix I) prepared in 0.5 X TBE, pH 8.0 (Appendix I) using 6X loading dye at 70 volts for 45-60 minutes. Ethidium Bromide (0.5 µg/ml) was added to stain the gel prior to pouring visualised on a U.V. transilluminator (Hoefer, U.S).

3.3 Yeast functional complementation assay

Yeast functional complementation assay was performed for screening the cDNAs libraries. This assay was carried out by using drop out assay.

3.3.1 Drop out assay

This assay was performed for checking the tolerance shown by yeast transformants on SD-Ura +cobalt (2 mM, 3 mM and 4 mM concentrations) and similar, on SD-Ura + 10 mM zinc plates by using different serial dilutions. From serial dilutions different level such as highly, moderately, least and none resistance showing numerous of transformants were obtained.

Procedure

1. Cultures of *Δ cot1* yeast cells carrying respective plasmids from library A (insert size 0.1-0.5 kb) and B (insert size 0.5-1 kb) were grown in 10 ml of selective medium and incubated at 30 ºC and 200 rpm.
2. Similar, step 1 was performed for *ΔZrc1* yeast cells carrying respective plasmids from library B (insert size 0.5 kb -1 kb).
3. Also, wild strain BY4741 and mutant strain transformed with pFL61 were also grown at same conditions. In this assay, these wild and mutant strains were used as a control.
4. Yeast cultures were adjusted to OD_{600} =1.0 and different range of serial dilutions 10^0 to 10^{-4} were prepared. Then from each different dilution, 5 µl samples were spotted on SD plates and on SD supplemented with 2 mM to 4 mM $CoCl_2$ plates.

5. Then plates were incubated at 30ºC for 2-3 days and results were observed.

3.4 FOA (5-Fluoroorotic acid) Test

Using 5-Fluoroorotic Acid (5-FOA) is a common screening method for the counter-selection of yeast. Under this method 5-FOA is converted to the toxic form i.e. 5-flurouracil. In the presence of FOA toxicity, yeast transformants will not be grown as they tend to lose their ura + plasmid carrying specific cDNA and hence respective transformants carrying metal resistant gene will be considered for further study. FOA test depicts the role of cDNA transformed into specific mutant yeast cells towards specific metal as yeast cells in the presence of FOA after flushing out plasmid carrying metal resistance gene will not be grown on SD+ura+metal plates.

Procedure:

1. SD+Ura agar media was prepared and autoclaved at 121 ºC for 15 minutes.
2. After cooling it, FOA was added into the media with the specific concentration of FOA (1 g/l) + Co (2 mM) and FOA (1 g/l) + Zn (10 mM) plates were poured.
3. Similarly control plates were prepared with SD+Ura media without metal.
4. After solidifying the plates, transformants were streaked on control plate i.e. SD+ Ura + FOA and SD+Ura+ FOA+ metal plates.
5. Then plates were incubated at 30 ºC for 2 days and results were observed.

3.5 Liquid growth assay

Growth assay was performed for those transformants which have shown resistance against cobalt on plates. Growth assay of transformants carried out in SD- Ura broth supplemented with different concentrations of respective metal.

Procedure

1. Flask containing 20 ml of fresh SD-Ura media were inoculated with mid-log precultures of wild strain BY4741, *Δcot1* containing pFL61, transformants carrying respective plasmids from library A (insert size 0.1-0.5 kb) and B (insert size 0.5-1 kb) and incubated at 30ºC and 200 rpm to attain a starting optical density of 0.02 at 600 nm.
2. Similar step 1 was performed for the liquid growth assay for Zn resistance transformants.
3. Wild strain BY4741, *Δcot1* and *Δzrc1* containing pFL61 were used as control.
4. After inoculation for 5 hours, 2 mM to 4 mM range $CoCl_2$ were added into each flasks.

5. In case of Zn, after inoculation for 5 hours 10 mM $ZnSO_4$ was added into each flasks.
6. Then optical densities of these cultures were measured at 600 nm after 48 hours.

3.6 Plasmid isolation of resistant clones of $CoCl_2$

The plasmid DNAs of the recombinant yeast cells and bacterial cells were isolated to confirm the presence of the plasmid in the cells.

3.6.1 Yeast plasmid isolation and elctrotransformation

To identify gene and mechanisms behind the tolerance of various above screened transformants we will sequence the genes by isolating plasmid carrying gene from yeast followed by transform into E.coli DH10β.

Procedure

1. Take 2 ml of culture in the eppendorf (1.5 ml).
2. Then centrifuged it at 600 rcf for 2 minute and discard the supernatant.
3. Then added 200 µl of solution 1 (Appendix II) and 3 µl of Zymolyase to each tube.
4. Resuspend the pellet by flicking with finger or mild vortexing.
5. Incubate at 37 ºC for 15-60 minutes.
6. Added 200 µl solution 2 (Appendix II) to each tube and mix well.
7. Added 400 µl solution 3(Appendix II) to each tube and mix well.
8. Centrifuge at maximum speed for 3 minute.
9. Transfer the supernatant to the Zymo spin -1 column.
10. Spin the Zymo spin -1 column for 30 seconds.
11. Discard the flow through in the collection tube and added 550 µl of wash buffer onto the column with the collection tube and spin for 1-2 minutes. Discard the wash buffer .Place column into a new 1.5 ml microfuge tube.
12. Added 10 µl of water or TE and spin for 30 seconds to 1 minute to elute plasmid off the column into a new 1.5 ml microfuge tube.
13. 3-4 µl of isolated yeast plasmid was eletrotransformed with electro competent *E.coli* DH10β cell. Spreading on LA+ amp (100 µg/ ml).
14. The presence and size of insert was determined by bacterial PCR colony with specific NF and NR primers. The program for bacterial PCR was same as in 3.2.

3.6.2 Bacterial plasmid isolation

The plasmid isolation from the bacterial transformants was done by using QIAprep® Spin Miniprep Kit.

1. Taken 1-5 ml bacterial overnight culture. The culture was centrifuged at >8000 rpm for 3minutes at room temperature (15-25˚C).
2. In 250µl of Buffer P1 (Appendix I), pellet was resuspended and transferred it into microcentrifuge tube.
3. Added 250µl of Buffer P2 (Appendix II) and mix thoroughly by inverting 4-6times the microcentrifuge. Clear solution
4. After this, 350µl Buffer N3 (Appendix II) was added and immediately mixed it by inverting the tube.
5. Then centrifuged the lysate at 13,000 rpm for 10 minutes in a table-top microcentrifuge.
6. Taken 800 µl of supernatant from step 5, added into the QIAprep 2.0 spin column by pipetting. Then centrifuged the solution at 13,000 for 30-60 seconds and discard the flow-through.
7. Washing was done by adding 0.5 ml of Buffer PB (Appendix II) into QIAprep 2.0 spin column. For proper washing centrifuged for 30-60 seconds and discard the flow- through.
8. Again wash by using 0.75 ml Buffer PE (Appendix II). Centrifuged for 30-60 seconds and discard the flow-through. Transferred the QIAprep 2.0 spin column to the collection tube.
9. To remove residual wash buffer, centrifuged for 1 minute.
10. Then placed the QIAprep 2.0 column in a clean 1.5 ml microcentrifuge tube. Added 50µl Buffer EB to elute DNA. Let column stand for 1 minute and centrifuge for 1 minute.
11. Amplification of isolated plasmid was done by PCR using a reaction mixture of 20µl containing 10X Taq Buffer (2µl), 2mM dNTP (1.5µl), Forward primer pFL61 NF (5'-CTTCTAACCAAGGGGTGGTTTAGTTTAG-3'), Reverse primer pFL61 NR (5'-CTGCATAAAGGCATTAAAAAGAGGAGCG-3'), Taq polymerase (0.3µl), and water final volume upto 20µl.
12. The PCR program was carried out as follows: initial denaturation at 94ºC for 5 minutes, followed by 35 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.

13. Amplified products were visualized on a 0.8% (w/v) agarose gel (Appendix I) prepared in 0.5 X TBE, pH 8.0 (Appendix I) using 6X loading dye at 70 volts for 45-60 minutes. Ethidium Bromide (0.5 µg/ml) was added to stain the gel prior to pouring visualised on a U.V. transilluminator (Hoefer, U.S).
14. Isolated plasmids, after confirmation of presence of insert through PCR amplification were sent for sequencing.

3.7 Bioinformatics analysis

3.7.1 Sequencing and Sequence analysis

The inserts in the plasmids were sequenced by using an Applied Biosystems automatic sequencer (DNA sequencing facility, Department of biochemistry, South campus, Delhi university, New Delhi, India).

Comparison of clone sequences was performed using the BLAST program (Altschul *et al.,* 1997) to reported nucleotide and protein sequences in the database Gen Bank accessible through NCBI (National Centre for Biotechnology Information – http://www.ncbi.nlm.nih.gov). For the translation of DNA into protein, ExPSAY tool was used.

RESULT AND DISCUSSION

Soil metatranscriptomic libraries A (0.1 kb -0.5 kb), B (0.5 kb-1 kb) were synthesize by Yadav *et al* (2014). Out of these two libraries, we report screening of part of library A and B in this work through yeast transformation by standard Li-Ac method. Under this screening process, cDNA libraries were screened based on their capacity to confer tolerance or resistance against the cobalt.

4.1 Screening of cDNA library A and B for cobalt and zinc

Yeast transformations were performed for screening of library A and B of cobalt resistant genes on selective media supplemented with 2 mM cobalt. Similarly yeast transformation was performed to screen library B for zinc resistant genes at 10 mM concentration. These different transformations were done to identify the cDNAs with a potential of tolerance or resistance against this metal.

A total of 9060 clones were screened from both the libraries for cobalt metal resistance. While comparing the transformation results of two libraries, it was observed that out of total 3000 clones of library A screened for metal resistance, none of them showed resistance unlike library B where total 6060 clones were obtained on SD-Ura plates as well as 120 clones were obtained on SD-Ura + cobalt (2 mM). These results depict that out of 6060 clones screened only 120 clones had shown tolerance against cobalt (2 mM). Similarly, it was observed that total 580 clones were screened from library B (0.5 kb-1 kb) for zinc metal resistance and only 5 were able to grow on SD + Ura + Zn. These results showed that the synthesized metatranscriptomic library was a consortium of genes having capability to resist or tolerate two heavy metals i.e. Co and Zn at various concentrations.

In similar study about 15,000 cobalt resistant yeast transformants were obtained by transforming Yep24-based library containing COT1 gene into a yeast CYX 118-5C and observed that over-expression of COT1 gene confers tolerance towards cobalt metal while deletion of the same lead to hypersensitivity towards the cobalt (Conklin *et al*., 1992). Bailly *et al* (2007) used similar metatranscriptomic approach to study eukaryotic functional diversity of soil environment through sequencing of 18S rDNA or reversed transcribed RNA. They observed that seventy

percent of the sequences belong to fungi, protists and metazoa (Bailly *et al.,* 2007). In 2011, a novel fungal family of oligopeptide transporters expressed by fungi was screened through yeast transformation of soil metatranscriptomic library and it was observed that these oligopeptide transporters had broad specificity and could relocate 60-80 dipeptides. These observations reported earlier concluded that metatranscriptomics unveil various genes, mechanisms and phenomena responsible for numerous biological activities in an environment.

4.2 Functional complementation assay for cobalt and zinc resistance clones

After screening metal resistant clones carrying cDNAs through yeast transformation, drop out assay of these metal resistant transformants was performed for respective metals i.e. Co and Zn. The wild strain BY4741 and mutant strain *Δcot1* and *Δzrc1* were used as control.

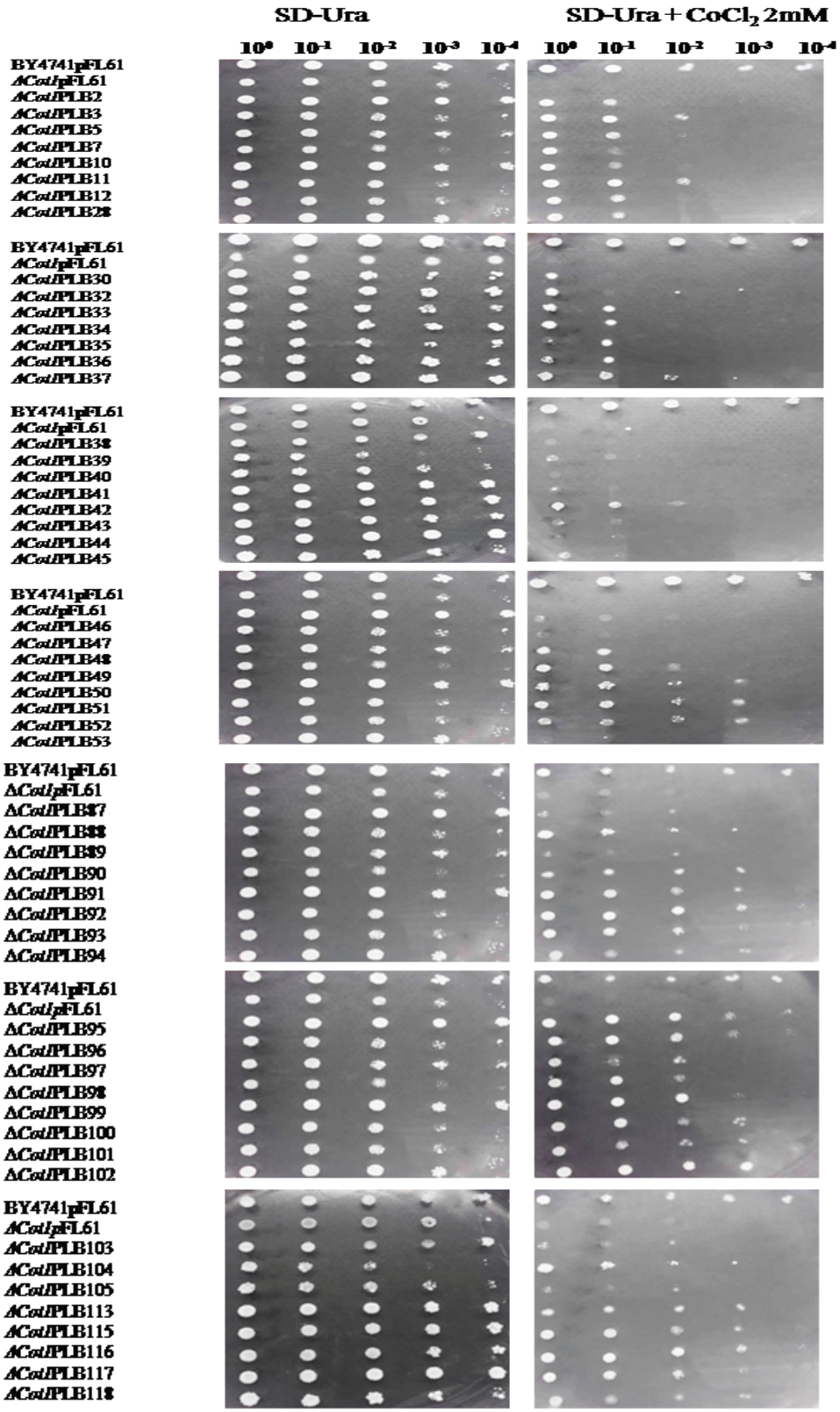
4.2.1 Drop assay of Co resistant transformants at 2 mM $CoCl_2$

It was observed that at a concentration of 2 mM Co, the transformants exhibited extreme tolerance. As screening of transformants was done on 2 mM $CoCl_2$ hence drop test was performed to confirm extent of tolerance at this concentration (Fig. 4.2.1).

In 2 mM concentration of cobalt metal, out of 120 screened clones, a total of 65 clones showed resistance out of which 29 were highly resistant, 14 were moderately resistant and 22 showed low level of resistance (Fig. 4.2.1). However, remaining clones were not able to grow in broth. This parameter of high, moderate and low resistance was in comparison to wild strain BY4741 with empty pFL61 dropped on metal amended media. The complementation studies of *Δcot1* on cobalt containing medium showed that the cobalt sensitive phenotype of the *Δcot1* carrying resistant genes were able to fully complement 65 clones.

Table 4.1 Yeast transformants were shown different level of resistance towards $CoCl_2$ at 2 mM concentration

Resistance level	**Transformants showing resistance towards $CoCl_2$ at 2mM conc.**
Highly resistance	*ΔCot1*PLB (32, 37, 42, 45, 50, 52, 54, 55, 61, 67, 80, 88, 90, 91, 92, 93, 94, 95, 96, 99, 100, 101, 102, 103, 107, 112, 114, 116 and 117)
Moderately resistance	*ΔCot1*PLB (3, 11,48, 49, 83, 84, 87, 97, 98, 106, 111, 113, 115 and 118)
Least resistance	*ΔCot1*PLB (7, 24, 27, 28, 33, 34, 35, 36, 43, 46, 53, 56, 57, 58, 59, 60, 62, 65, 78, 79, 85 and 86)


SD-Ura
SD-Ura + CoCl2 2mM
10⁰ 10⁻¹ 10⁻² 10⁻³ 10⁻⁴
BY4741pFL61
ΔCot1pFL61
ΔCot1PLB2
ΔCot1PLB3
ΔCot1PLB5
ΔCot1PLB7
ΔCot1PLB10
ΔCot1PLB11
ΔCot1PLB12
ΔCot1PLB28
BY4741pFL61
ΔCot1pFL61
ΔCot1PLB30
ΔCot1PLB32
ΔCot1PLB33
ΔCot1PLB34
ΔCot1PLB35
ΔCot1PLB36
ΔCot1PLB37
BY4741pFL61
ΔCot1pFL61
ΔCot1PLB38
ΔCot1PLB39
ΔCot1PLB40
ΔCot1PLB41
ΔCot1PLB42
ΔCot1PLB43
ΔCot1PLB44
ΔCot1PLB45
BY4741pFL61
ΔCot1pFL61
ΔCot1PLB46
ΔCot1PLB47
ΔCot1PLB48
ΔCot1PLB49
ΔCot1PLB50
ΔCot1PLB51
ΔCot1PLB52
ΔCot1PLB53
BY4741pFL61
ΔCot1pFL61
ΔCot1PLB87
ΔCot1PLB88
ΔCot1PLB89
ΔCot1PLB90
ΔCot1PLB91
ΔCot1PLB92
ΔCot1PLB93
ΔCot1PLB94
BY4741pFL61
ΔCot1pFL61
ΔCot1PLB95
ΔCot1PLB96
ΔCot1PLB97
ΔCot1PLB98
ΔCot1PLB99
ΔCot1PLB100
ΔCot1PLB101
ΔCot1PLB102
BY4741pFL61
ΔCot1pFL61
ΔCot1PLB103
ΔCot1PLB104
ΔCot1PLB105
ΔCot1PLB113
ΔCot1PLB115
ΔCot1PLB116
ΔCot1PLB117
ΔCot1PLB118

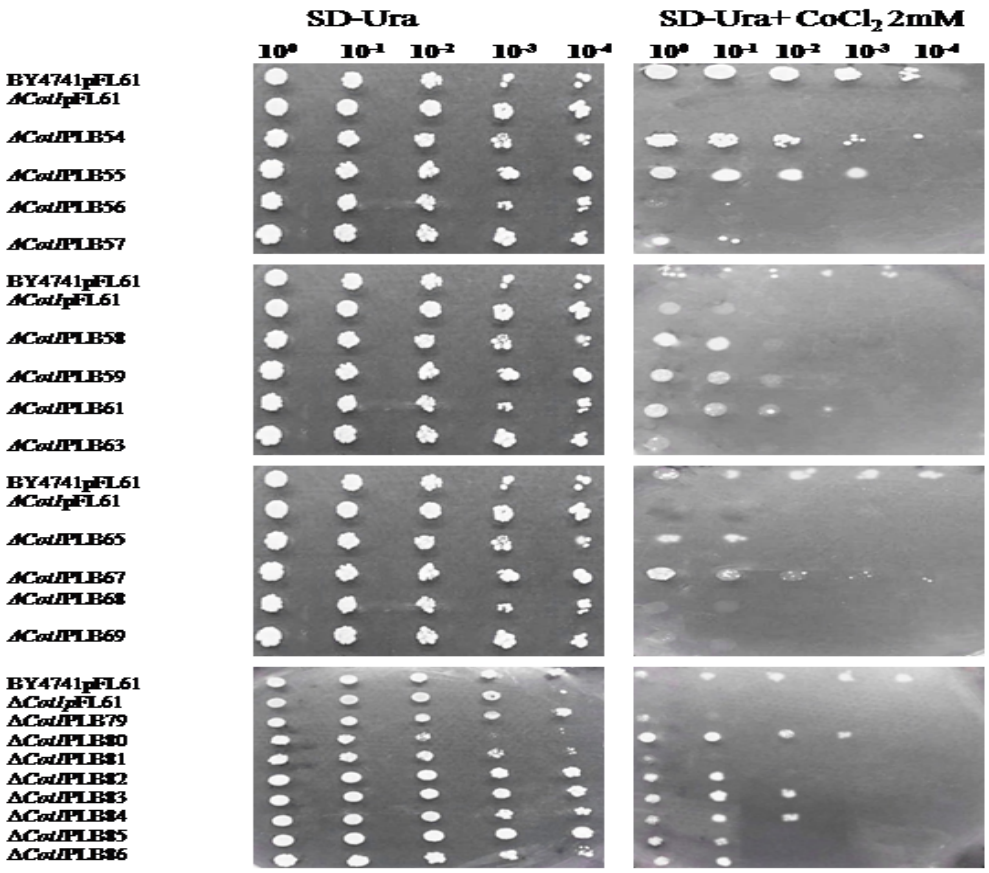


Fig. 4.2.1 Functional complementation of yeast transformants on SD-Ura medium amended with 2 mM cobalt where Δ*Cot1* cells with empty pFL61 acts as negative control and wild strain BY4741pFL61 acts as positive control

4.3 Drop test for Zn resistant transformants at 10 mM $ZnSO_4$

Drop test for Zn resistant transformants was performed at 10 mM conc. It was observed that out of 5 transformants only 3 had shown resistance. All the three clones showed high resistance at 10 mM $ZnSO_4$ conc. in comparison to BY4741pFL61.

Table 4.2 Yeast transformants were shown different level of resistance towards $ZnSO_4$ at 10 mM concentration

Resistance level	**Transformants showing resistance towards $ZnSO_4$ at 10 mM conc.**
Highly resistance	ΔZrc1PLB (1, 2 and 3)
Moderately resistance	No transformants were shown moderately resistance
Least resistance	No transformants were shown moderately resistance

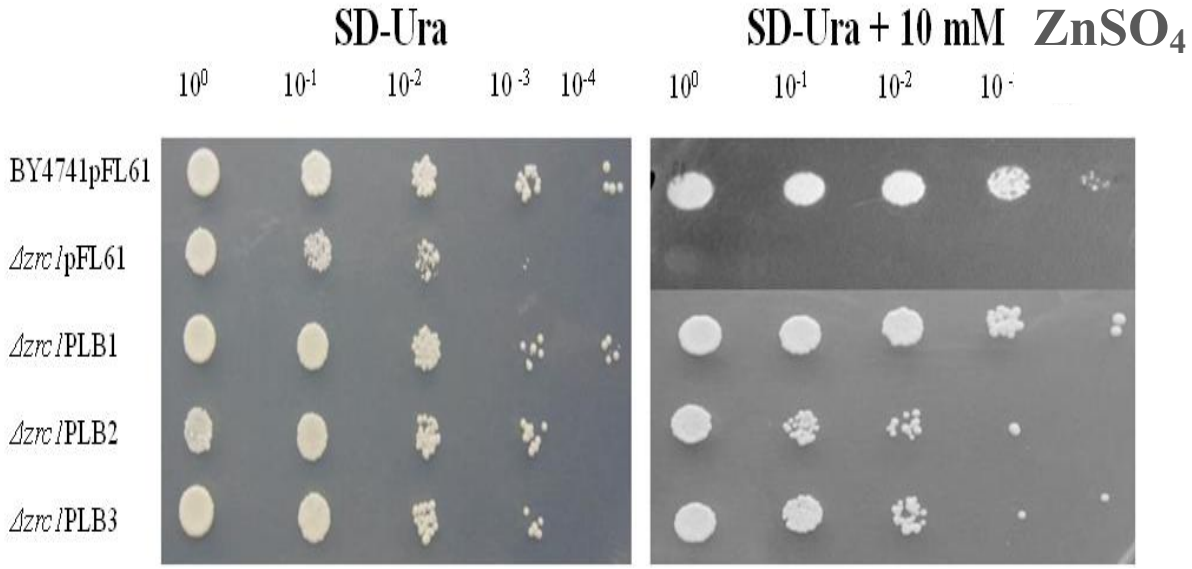


Fig. 4.3.1 Functional complementation of yeast transformants on SD-Ura medium amended with 10 mM $ZnSO_4$ where Δ*zrc1* cells with empty pFL61 acts as negative control and wild strain BY4741pFL61 acts as positive control

4.4 FOA (5-Fluoroorotic acid) Test

To authenticate the metal resistant phenotype of cloned cDNA, 65 cobalt resistant clones and 3 zinc resistant clones were further grown on FOA amended media. 5-Fluoroorotic acid is an analog of uracil. Presence of FOA in the medium causes toxicity to the yeast cells carrying URA3 gene, hence yeast cells either die or survive by losing the plasmid carrying ura3 gene (Boeke *et al.,* 1987). This property of FOA has been used in metatranscriptomics to reveal the significance of cDNAs transformed into a eukaryotic host.

Confirmation of true plasmid-borne transformants out of 65 resistant clones was done by FOA test. Those clones survived on FOA amended media were further grown on SD + Ura + Co (2 mM) media. Out of these clones, those which grew on metal amended media even after losing plasmids were discarded as they might have mutated to develop a mechanism or genetically reverted to their wild type to resist metal. Therefore under this situation only those clones were

selected for further work which were able to grow on FOA plates but not on metal amended SD + Ura plates after FOA (Boeke *et al.,* 1987).

Table 4.3 50 Co resistant transformants and 3 Zn resistant showing positive results in FOA test. Red color indicates highly resistant clones + FOA positive clones while grey color indicate moderate resistant FOA positive transformants.

Cobalt resistant transformants with positive FOA test				
Δ*Cot1*PLB3	Δ*Cot1*PLB16	Δ*Cot1*PLB51	Δ*Cot1*PLB81	Δ*Cot1*PLB103
Δ*Cot1*PLB6	Δ*Cot1*PLB17	Δ*Cot1*PLB52	Δ*Cot1*PLB82	Δ*Cot1*PLB104
Δ*Cot1*PLB7	Δ*Cot1*PLB28	Δ*Cot1*PLB53	Δ*Cot1*PLB88	Δ*Cot1*PLB108
Δ*Cot1*PLB8	Δ*Cot1*PLB29	Δ*Cot1*PLB54	Δ*Cot1*PLB91	Δ*Cot1*PLB109
Δ*Cot1*PLB9	Δ*Cot1*PLB32	Δ*Cot1*PLB55	Δ*Cot1*PLB94	Δ*Cot1*PLB111
Δ*Cot1*PLB11	Δ*Cot1*PLB37	Δ*Cot1*PLB61	Δ*Cot1*PLB95	Δ*Cot1*PLB113
Δ*Cot1*PLB12	Δ*Cot1*PLB42	Δ*Cot1*PLB65	Δ*Cot1*PLB98	Δ*Cot1*PLB115
Δ*Cot1*PLB13	Δ*Cot1*PLB44	Δ*Cot1*PLB67	Δ*Cot1*PLB99	Δ*Cot1*PLB116
Δ*Cot1*PLB14	Δ*Cot1*PLB49	Δ*Cot1*PLB70	Δ*Cot1*PLB101	Δ*Cot1*PLB117
Δ*Cot1*PLB15	Δ*Cot1*PLB50	Δ*Cot1*PLB77	Δ*Cot1*PLB102	Δ*Cot1*PLB118
Zinc resistant transformants with positive FOA test				
Δ*Zrc1*PLB1	Δ*Zrc1*PLB2	Δ*Zrc1*PLB3		

On performing FOA test, it was observed that for cobalt resistant clones, out of 65 cobalt resistant clones and 3 zinc resistant clones, only 50 cobalt resistant and 3 zinc resistant clones were positive for FOA and showed no growth on (SD +Ura+ 2 mM $CoCl_2$/ 10mM $ZnSO_4$ + FOA) plates. The remaining yeast clones which had shown resistance towards metal after FOA treatment were discarded. On comparing results of both drop assay (2 mM $CoCl_2$ and 10 mM $ZnSO_4$) and FOA test, for Co only 22 transformants (15 highly resistant and 7 moderately resistant) out of 50 FOA positive clones were further used in this study. In case of Zn, all the three positive clones were selected (Table 4.3).

4.5 Yeast colony PCR

Drop test and FOA test proved that the metal tolerant phenotype was due to presence of metal tolerant insert transformed into metal sensitive mutant. To assure this point, colony PCR of 22 cobalt tolerant transformants was performed by primers NF and NR. Similarly, colony PCR of 3 zinc tolerant transformants was performed. The presence of insert was confirmed by visualizing the bands of amplified product on 1.0% agarose gel.

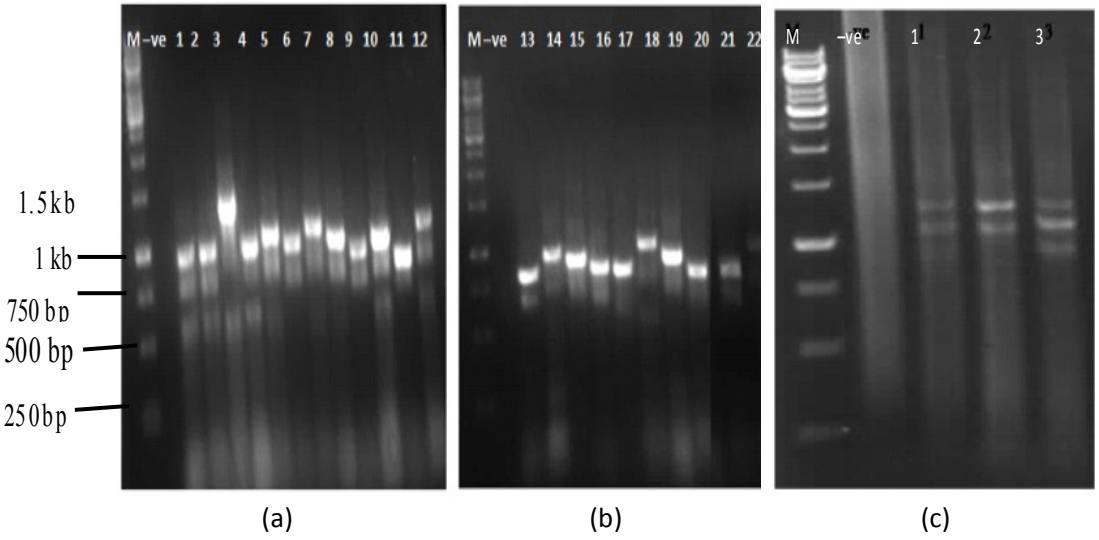


Fig. 4.5.1 (a) Colony PCR of screened yeast transformants, Lane M: l kb ladder, -ve: negative control, Lane 1-12 cobalt tolerance transformants, (b) Lane M: 1kb ladder, -ve: negative control, Lane 13-22 yeast cobalt tolerance screened transformants and (c) Colony PCR of zinc tolerant transformants, Lane M: 1kb ladder, -ve: negative control Lane 1, 2 and 3 zinc tolerance transformants

4.6 Drop assay at 3 mM $CoCl_2$

It was observed that at 2 mM $CoCl_2$ concentration, 15 transformants were showing extreme metal tolerance so drop out assay at higher range of Co concentration (3 mM) was carried out (Fig. 4.6.1).

Table 4.4 Yeast transformants were shown different level of resistance towards $CoCl_2$ at 3 mM concentration.

Resistance level	Yeast transformants resistant towards $CoCl_2$ at 3 mM conc.
Highly resistance	*ΔCot1*PLB (42, 50, 55 and102)
Moderately resistance	*ΔCot1*PLB (95 and 99)
Least resistance	*ΔCot1*PLB (101, 116, 117 and 52)

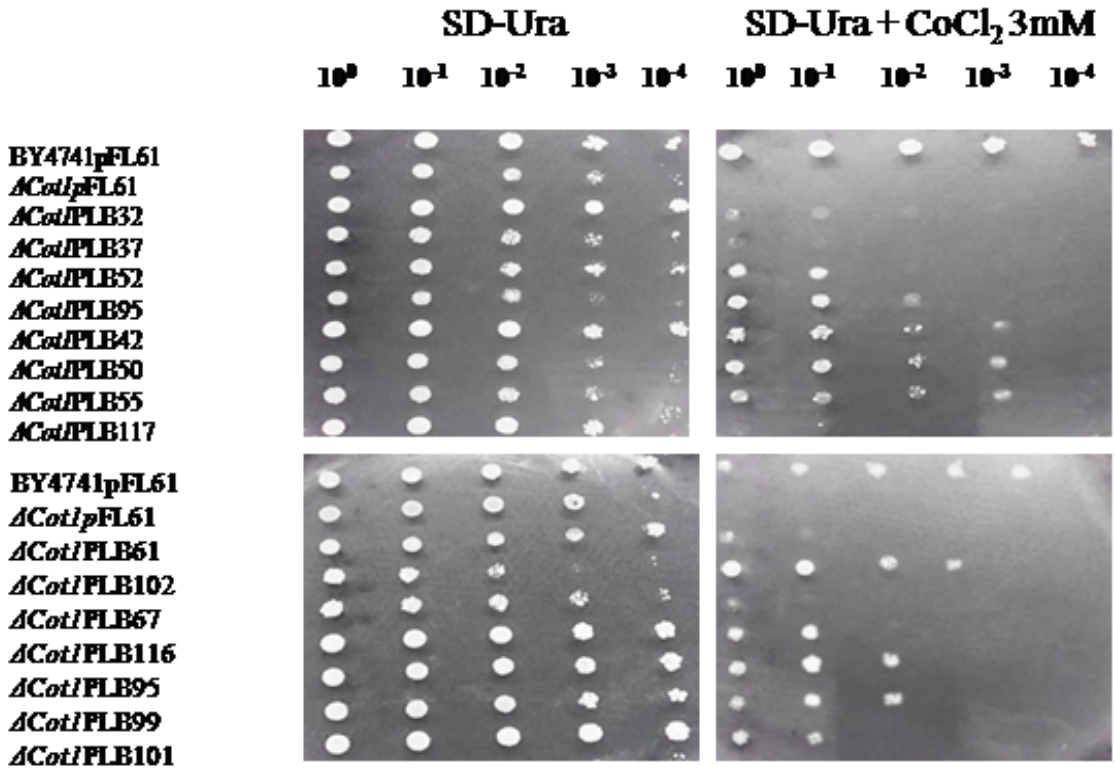


Fig. 4.6.1 Functional complementation of yeast transformants on SD-Ura medium supplemented with 3 mM cobalt where Δ*Cot1* cells with empty pFL61 acts as negative control and wild strain BY4741pFL61 acts as positive control

Out of 15 resistant yeast transformants only 10 clones had shown resistance at 3 mM cobalt concentration, in which 4 were highly resistant; 3 were moderately resistant and 3 transformants showed low resistance (Fig. 4.6.1).

4.7 Drop assay at 4 mM $CoCl_2$

To measure the potential and range of metal tolerance by resistant clones, drop assay of highly resistant clones (3 mM) was further carried out at 4 mM $CoCl_2$ (Fig. 4.7.1).

Table 4.5 Yeast transformants were shown different level of resistance towards $CoCl_2$ at 4 mM concentration

Resistance Level	**Yeast transformants resistant at 4 mM $CoCl_2$ conc.**
Highly resistance	*ΔCot1*PLB (42 and 102
Moderately resistance	No transformants shown moderately resistance
Least resistance	*ΔCot1*PLB(50 and 55)

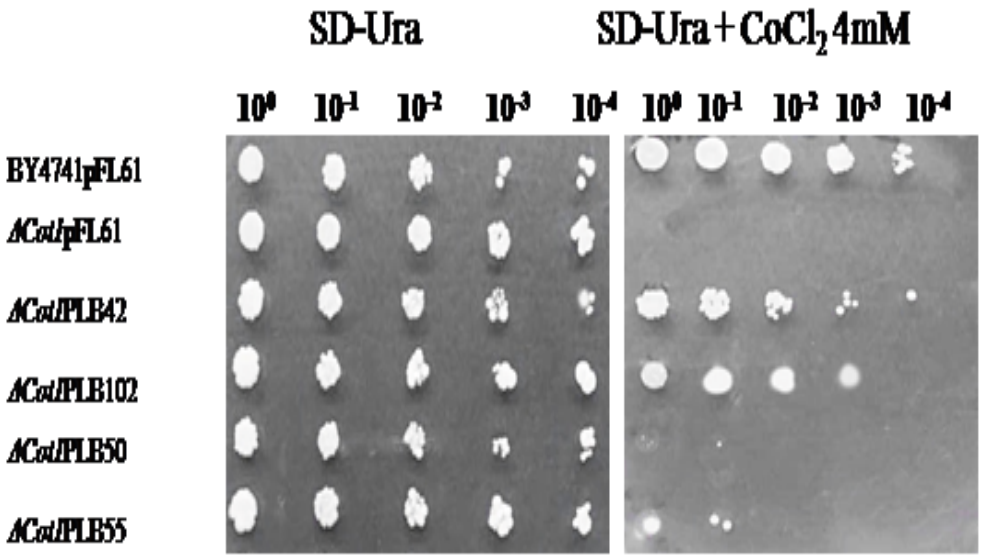


Fig. 4.7.1 Functional complementation of yeast transformants on SD-Ura medium supplemented with 4 mM cobalt where Δ*Cot1* cells with empty pFL61 acts as negative control and wild strain BY4741pFL61 acts as positive control

On performing drop test at 4 mM concentration, 2 transformants were able to resist this level of cobalt metal. After filtering out from 68 resistant clones, only 02 clones showed highly resistance at 4 mM (Fig. 4.7.1). Furthermore, it was concluded from the above results that *ΔCot1*PLB42 and *ΔCot1*PLB102 showing high tolerance at different range of cobalt conc. These two clones had shown higher resistance on all concentrations of cobalt i.e. 2 mM, 3 mM and 4 mM. Hence this study concluded that both the transformants carried cDNA possessing ability to resist heavy metal from a range of lower to higher concentration.

Khouja *et. al.,* (2013) by using functional complementation studies, observed that cDNAs of membrane transporters coding gene OmZnT1 and OmFET genes, isolated from *Oidiodendron maius*, conferred tolerance to the *Δzrc*1 yeast mutant. It was also reported that one of the cDNA i.e. OmZnT1 helps in restoring tolerance to cobalt sensitive phenotype of the *Saccharomyces cerevisae* i.e. *Δcot1* mutant against cobalt metal at 2 mM conc. (Khouja *et al.,* 2013). In a similar work, screening of cDNA library of *Russula atropurpurea* through metatranscriptomics using Cd sensitive yeast mutant *Δycf1* followed by functional complementation assay revealed that RaZBP1 and RaZBP2 cDNAs were helping this ectomycorhizal fungal species to sequester heavy metals like Cd and Zn metal (Leonhardt *et al.,* 2014).

4.8 Liquid growth Assay

The resistance potential of fifteen high tolerant yeast transformants screened through yeast transformation, drop test (2 mM) and FOA test was further confirmed by liquid medium growth assay in a range of 2 mM to 4 mM $CoCl_2$ for 48 hours. The growth assay had shown the similar results which were obtained in case of drop out assay.

Table 4.6 Growth assay of Co resistance yeast transformants (*ΔCot1*PLB32, *ΔCot1*PLB42, *ΔCot1*PLB50, *ΔCot1*PLB52) *at* different conc. of $CoCl_2$. BY4741pFL61 and *ΔCot1*pFL61 were used as control

Sample	Concentration of $CoCl_2$		
	2 mM	3 mM	4 mM
BY4741pFL61	0.515±0.001	0.285±0.013	0.172±0.012
*ΔCot1*pFL61	0.067±0.001	0.012±0.013	0.015±0.012
*ΔCot1*PLB32	1.244±0.098	0.303±0.034	0.197±0.010
*ΔCot1*PLB42	1.723±0.012	0.943±0.037	0.754±0.024
*ΔCot1*PLB50	1.444±0.109	0.771±0.031	0.652±0.022
*ΔCot1*PLB52	1.340±0.016	0.872±0.037	0.652±0.070

Values are represented as Mean±SE, n = 2

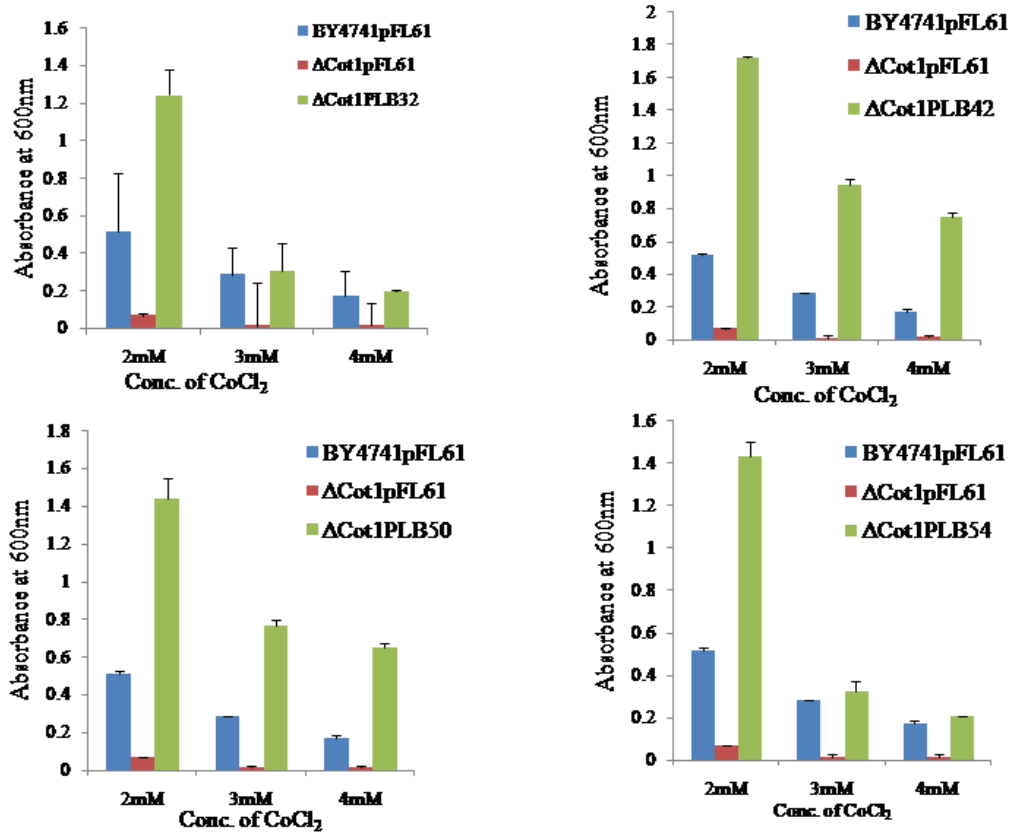


Fig. 4.8.1 Growth assay of Co resistance yeast transformants at different conc. of $CoCl_2$. BY4741pFL61 and *ΔCot1*pFL61 were used as control

Table 4.7 Growth assay of Co resistance yeast transformants (*ΔCot1*PLB54, *ΔCot1*PLB55, *ΔCot1*PLB61 and *ΔCot1*PLB67) at different conc. of $CoCl_2$. BY4741pFL61 and *ΔCot1*pFL61 were used as control

Sample	Concentration of $CoCl_2$		
	2 mM	**3 mM**	**4 mM**
BY4741pFL61	0.515±0.001	0.285±0.013	0.172±0.012
*ΔCot1*pFL61	0.067±0.001	0.012±0.013	0.015±0.012
*ΔCot1*PLB54	1.432±0.010	0.321±0.052	0.205±0.001
*ΔCot1*PLB55	1.640±0.026	0.763±0.016	0.560±0.060
*ΔCot1*PLB61	1.623±0.012	0.432±0.001	0.321±0.056
*ΔCot1*PLB67	1.061±0.010	0.213±0.010	0.106±0.012

Values are represented as Mean±SE, n = 2

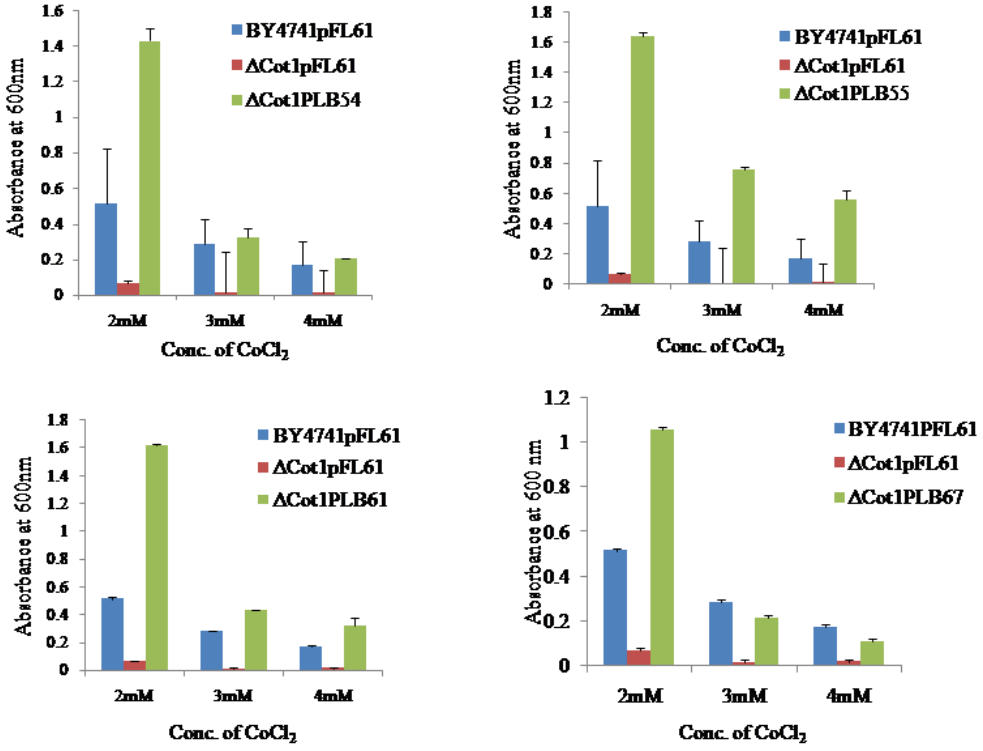


Fig. 4.8.2 Growth assay of Co resistance yeast transformants at different conc. of $CoCl_2$. BY4741pFL61 and *ΔCot1*pFL61 were used as control

Table 4.8 Growth assay of Co resistance yeast transformants (*ΔCot1*PLB95, *ΔCot1*PLB99, *ΔCot1*PLB101 and *ΔCot1*PLB102) at different conc. of $CoCl_2$. BY4741pFL61 and *ΔCot1*pFL61 were used as control

Sample	Concentration of $CoCl_2$		
	2 mM	3 mM	4 mM
BY4741pFL61	0.515±0.001	0.285±0.013	0.172±0.012
*ΔCot1*pFL61	0.067±0.001	0.012±0.013	0.015±0.012
*ΔCot1*PLB95	1.532±0.010	0.956±0.011	0.123±0.010
*ΔCot1*PLB99	1.550±0.010	0.955±0.012	0.141±0.012
*ΔCot1*PLB101	1.153±0.012	0.621±0.012	0.102±0.011
*ΔCot1*PLB102	1.821±0.011	1.331±0.011	0.753±0.011

Values are represented as Mean±SE, n = 2

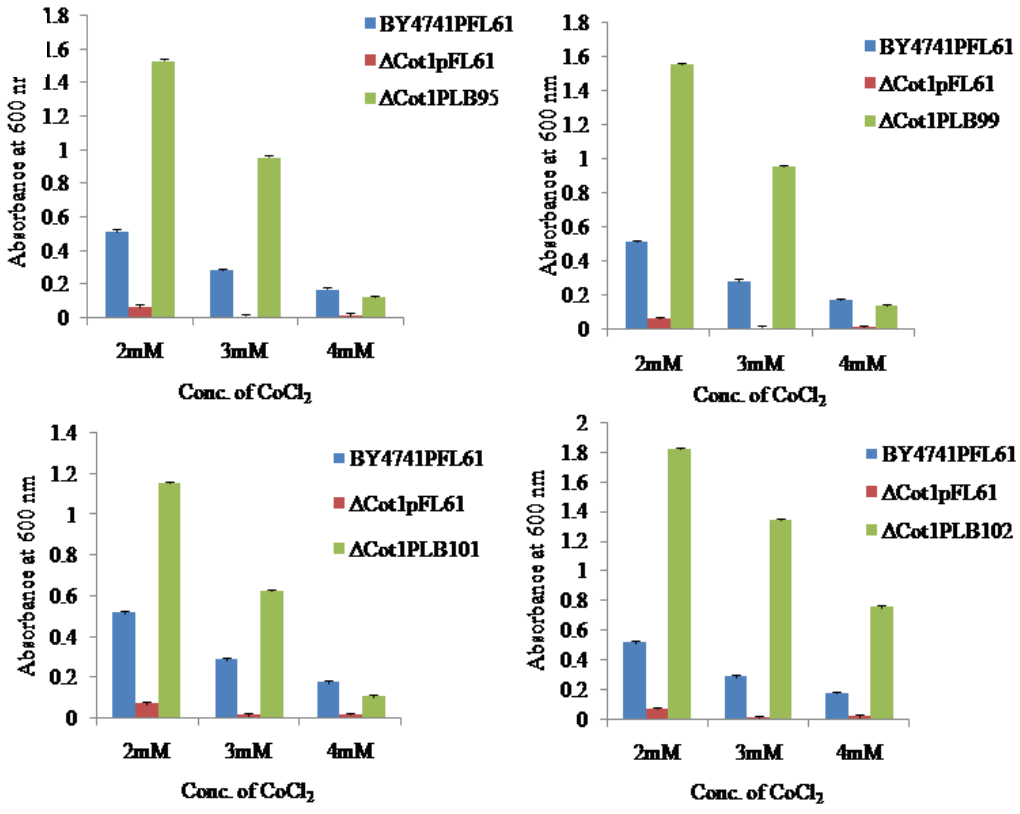


Fig. 4.8.3 Growth assay of Co resistance yeast transformants at different conc. of $CoCl_2$. BY4741pFL61 and *ΔCot1*pFL61 were used as control

Table 4.9 Growth assay of Co resistance yeast transformants (*ΔCot1*PLB104, *ΔCot1*PLB116, and *ΔCot1*PLB117) at different conc. of $CoCl_2$. BY4741pFL61 and *ΔCot1*pFL61 were used as control

Sample	Concentration of $CoCl_2$		
	2 mM	3 mM	4 mM
BY4741pFL61	0.515±0.001	0.285±0.013	0.172±0.012
*ΔCot1*pFL61	0.067±0.001	0.012±0.013	0.015±0.012
*ΔCot1*PLB104	1.632±0.011	0.752±0.011	0.102±0.011
*ΔCot1*PLB116	1.345±0.011	0.451±0.011	0.116±0.010
*ΔCot1*PLB117	1.491±0.010	0.325±0.010	0.124±0.010

Values are represented as Mean±SE, n = 2

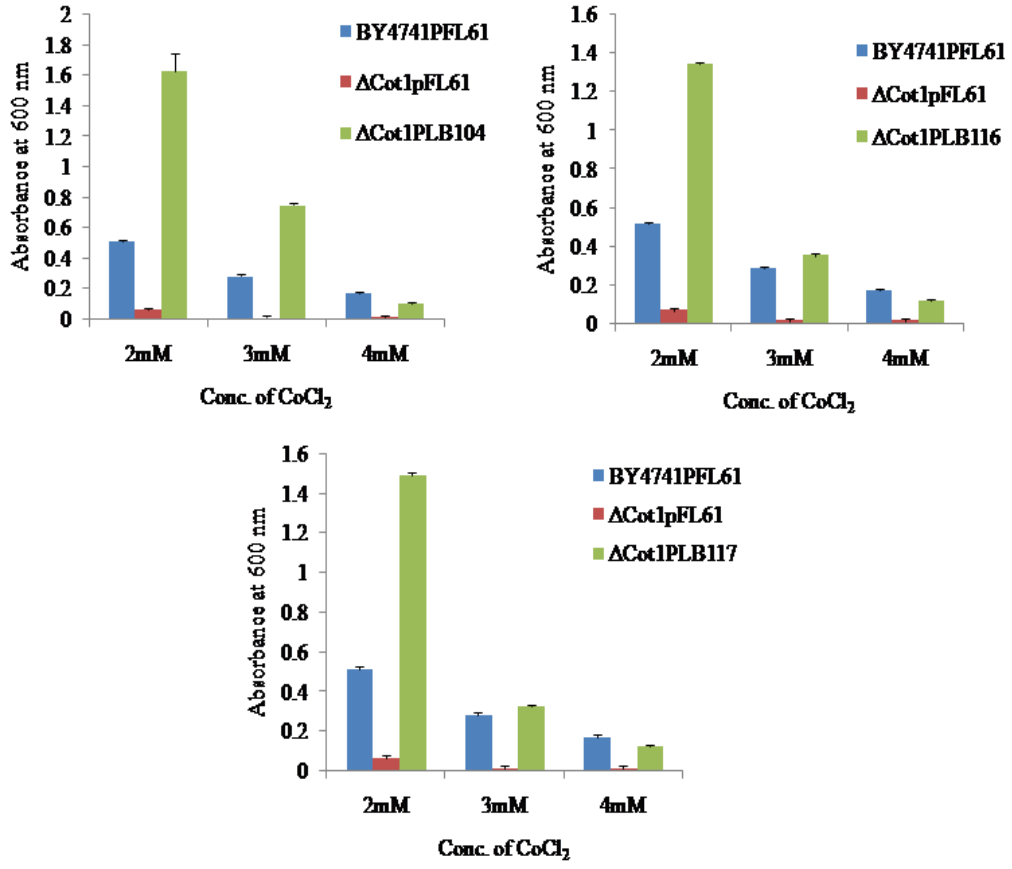


Fig 4.8.4 Growth assay of Co resistance yeast transformants at different conc. of $CoCl_2$. BY4741pFL61 and *ΔCot1*pFL61 were used as control

It was indicated that the transformants were shown higher tolerance against cobalt as compared to wild strain BY4741pFL61 and mutant strain *ΔCot1*pFL61. The *Δcot1* containing empty pFL61cells were unable to grow on 2 mM to 4 mM containing media and their growth was completely inhibited, whereas in case of metal resistant yeast transformants growth was increased significantly with increasing time of incubation. With further increase in concentration, growth was slightly decreased in the SD-Ura broth media. The transformants had shown higher resistance in case of 2 mM concentration as similar result shown in drop out assay. Therefore, from the results, it was concluded that some transformants shown higher resistance as compared with other transformants. Level of tolerance shown by yeast transformants in ascending order as follows: *ΔCot1*PLB102 > ΔCot1PLB42 > *ΔCot1*PLB55 > *ΔCot1*PLB104 > *ΔCot1*PLB61 > *ΔCot1*PLB99 > *ΔCot1*PLB95 > ΔCot1PLB117 > *ΔCot1*PLB50 > *ΔCot1*PLB54 > *ΔCot1*PLB116 > *ΔCot1*PLB52 >*ΔCot1*PLB32 >*ΔCot1*PLB101 > *ΔCot1*PLB67 in SD-Ura medium amended with $CoCl_2$ concentration (Fig. 4.8.1, Fig. 4.8.2, Fig. 4.8.3, Fig. 4.8.4)

4.9 Liquid growth assay of Zn resistance transformants

All the three zinc resistant transformants screened at 10 mM $ZnSO_4$ were showing high resistance even in liquid assay growth test at same concentration.

Table 5.0 Growth assay of Zn tolerance transformants at 10 mM conc. of $ZnSO_4$. BY4741pFL61 and *ΔZrc1*pFL61 used as control

Samples	**10 mM conc. of $ZnSO_4$**
BY4741pFL61	0.570±0.132
*ΔZrc1*pFL61	0.090±0.012
*ΔZrc1*PLB1	1.915±0.152
*ΔZrc1*PLB2	1.754±0.001
*ΔZrc1*PLB3	1.563±0.013

Values are represented as Mean±SE, n = 2

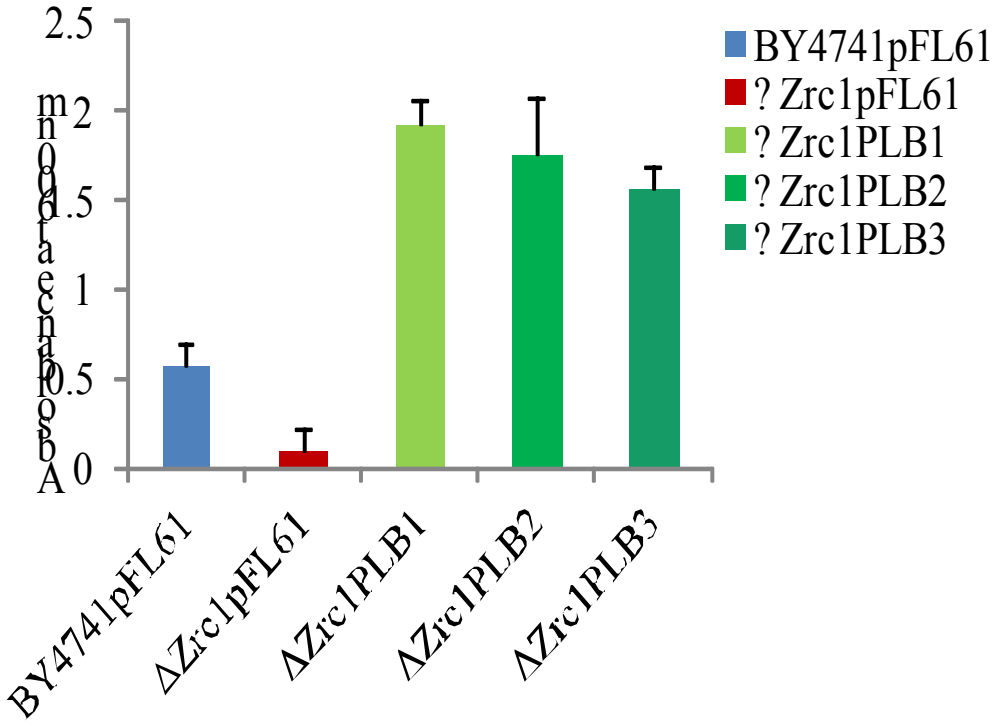


Fig. 4.9.1 Growth assay of Zn resistance transformants (*ΔZrc1*PLB1, *ΔZrc1*PLB2 and ΔZrc1PLB3 at 10 mM conc. BY4741pFL61 and *ΔZrc1*pFL61 used as control

5.0 Bioinformatics Analysis

Yeast plasmid isolation was done to determine the sequence of the cDNAs conferring resistance towards cobalt and zinc metals. The isolated yeast plasmids were transformed into electro-competent cells and sequenced. Sequence data thus obtained were analyzed by BLASTX. Out of 22 cobalt resistant clones and 3 zinc resistant clones, plasmids of only two clones were sequenced. Sequencing of remaining clones are yet to be performed.

*ΔCot1*PLB3 forward

This clone had shown moderate resistance at 2 mM concentration and shown 60% query cover and 50% homology with Sodium: solute symporter (*Streptomyces lydicus)*.

TTTTGTTTTTTCTTTTTTTTTTTTTTCCCTCACGGTACTGGTTCACTATCGGTCACCAAGTAGTATTTAGCCTTGGGCGATGGTCCGCCC
GGATTCCAACAGGGTTTCTCGTGTCCCGTTGTACTCAGGAACTACCCACGGTCCATTTCGATTTCGACTACGGGGCTATCACCCTCT
TTGGCTGACCTTCCCAGGTCATTCGTCTACCGTCACAGTGTCCGTCGATGCATCTGCCGACGCATCTGGGTAGCCCTACAACACGAT
ATCTACAACGCCGGCAGGCTTGCATAGATACCGTTTGGGCTGCTCCGCGTTCGCTCGCCACTACTAGCGGAATCTCTGATTTGATTT
CTTTTCCTGAGGGTACTGAGATGTTTCAGTTCCCCTCGTTCGCTCTCCCTAGCCTATGAATTCAGCCAGGGAGTGAATCGACATGAC
TCGATTCCGGTTTCCCGATTCGGAGATCGCCGGGTCAACGCTTGTTTACAGCTAACCGGCGCTTATCGCAGTTTTACCACGTCCTTCT
TCGCCTCTTGGTGCCCCGC

Translation of the nucleotide sequence to protein was carried out by using ExPSAY. After the translation following 56 amino acids were obtained.

MVRPDSNRVSRVPLYSGTTHGPFRFRLRGYHPLWLTFPGHSSTVTVSVDASADAS

Description	Max score	Total score	Query cover	E value	Ident	Accession
hypothetical protein [uncultured Chloroflexi bacterium HF0200_09I09]	44.3	44.3	80%	2e-04	53%	ADI17967.1
hypothetical protein BVRB_018590 [Beta vulgaris subsp. vulgaris]	44.7	44.7	82%	3e-04	48%	KMS64603.1
putative uncharacterized protein [Clostridium sp. CAG:508]	38.5	38.5	82%	0.14	39%	CDC31307.1
hypothetical protein EW35_3258 [Staphylococcus aureus]	35.8	35.8	58%	0.21	52%	KFA45280.1
sodium:solute symporter [Streptomyces lydicus]	36.6	36.6	60%	0.75	50%	WP_069567540.1
sodium:solute symporter [Streptomyces lydicus]	36.2	36.2	60%	0.97	50%	WP_077192543.1
sodium:solute symporter [Streptomyces lydicus]	36.2	36.2	60%	0.99	50%	WP_033268429.1
sodium:solute symporter [Streptomyces sp. MOE7]	36.2	36.2	60%	1.1	50%	WP_084771408.1
sodium:solute symporter [Streptomyces sp. NRRL F-4489]	36.2	36.2	55%	1.1	52%	WP_066976063.1
hypothetical protein XD41_1680 [Desulfonauticus sp. 38_4375]	35.8	35.8	41%	1.1	61%	KUJ95332.1

Fig. 5.1 BLASTx analysis for *ΔCot1*PLB3

***Δzrc1*PLB1 forward**

*Δzrc1*PLB1clone was high tolerant at 10 mM Zn concentration and partial protein was obtained which showed 35 % query cover and 37% homologous to unnamed protein product of *Chrondrus crispus*. The analysis was done by BLASTX (eukaryote taxid 2759; Blossom45)

GCGGGGGGGCAAGCAGACATAAAAGGATATTTTTGGTAATTCTAAAACAAATACATGAGGAAAGGCGGAGTACTTGAATGGTGC
GTCGCTTCATATATTAGTTTGTTGGAAGAGAATCGAGAAACGAACCAGCGGTAAAATGATGTGTTCTTTTCATTGATGATTCATAA
TATATATCAGATAATCAAAGCAATTTGATGACTCCTATTTGATTCTGAACTATCAAATCTAGAAGATAGTGTATTGGACTATCTTG
TTTATAACGGTTACGAGAAATTAGAGTTTGATTTCGGAGAAAACGCATTTAGAGACGGCGATTATTTCCAAGGAAAGCAGCAGGC
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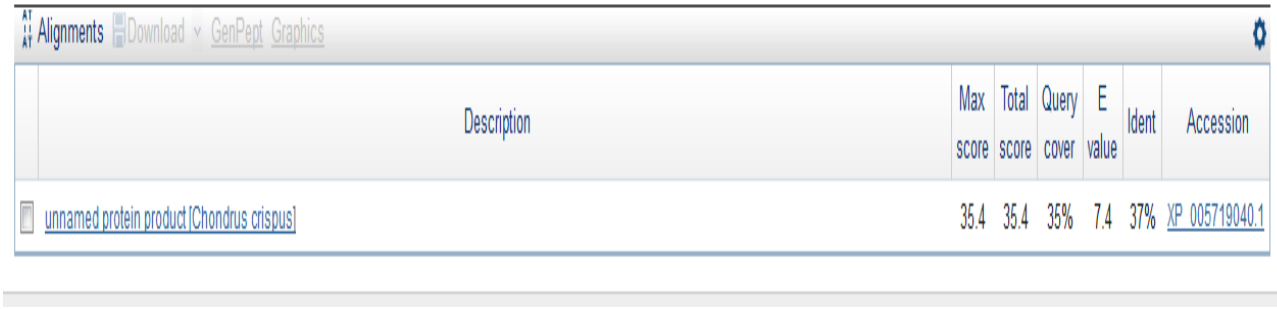

Fig. 5.2 Homologous sequences found in BLASTx analysis for *ΔZrc1*PLB1

CONCLUSION

Present study included the screening of metatranscriptomic libraries A (0.1-0.5Kb) and B (0.5 – 1Kb) constructed from contaminated soil by using a eukaryotic host i.e. *S. cerevisae* through yeast transformation. This study concludes that total 65 transformants were resistant to cobalt metal at 2 mM concentration and 10 transformants 3 mM concentration whereas two clones provided resistant upto 4 mM conc. in case of zinc, only three clones were resistant at 10 mM $ZnSO_4$ These transformants were further characterized by liquid medium growth assay where similar results were obtained as in drop out assay. Furthermore, plasmids responsible for the resistant were isolated and sent for sequencing.

This study demonstrates the significance of functional metatranscriptomics in the field of environmental ecology, biotechnology, bioremediation and biomarker development. By using this approach various underneath phenomena would be unveiled to play important role in modern era of industrialization. These genes could also be helpful in bioremediation of polluted soil or land or in development of biomarkers to detect the contamination level of various heavy metals in a particular environment. Development of various genetic modified microorganisms or genetically modified crops by using the gene screened through metatranscriptomics could be an important measure to increase the fertility of metal polluted agricultural land. Hence metatranscriptomics not only has potential to indentify significant functional metatranscripts from unknown host in an environmental sample but also for heterologous production of biotechnology relevant biomolecules

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APPENDIX I

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
Beef extract	5
Trypton	10

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

LB plates with ampicillin/IPTG/X-Gal

Make the LB plates with ampicillin as above; 100 µl of 100 mM IPTG and 20 µl of 50 mg/ml X Gal may be spread over the surface of an LB ampicillin plate and allowed to absorb for 30 minutes at 37°C prior to use.

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 minutes.

SD medium

Ingredient	Quantity
Yeast nitrogen base (without amino acid)	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-Uracil	200 mg/l
L-Valine	1500 mg/l

L-Tyrosine 300 mg/l

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

IPTG stock solution (0.1M)

1.2 g IPTG add into water to made 50 ml final volume. Filter sterilizes and store at 4°C.

X-Gal (2ml)

100 mg 5-bromo-4-chloro-3-indolyl-Dgalactoside dissolve in 2ml N, N'-dimethylformamide. Cover with aluminum foil and store at 20°C.

APPENDIX II

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