ABSTRACT

Heavy metals are stable and persistent environmental contaminants since they cannot be degraded or destroyed unlike organic pollutants and subsequently bioaccumulate in the food chain (Verma et al., 2001). Natural sources contribute to heavy metal pollution to a less extent; whereas artificial sources resulted from different human activities, contribute decisively to the pollution of the environment. Control of environmental pollution has become the major and one of the most difficult tasks for the developing countries like India. In spite of Government regulations, lot of such waste is dumped around the cities and metropolis and deposited in natural water resources. In Pune, Mula river is contaminated with such waste materials (Imandoust et al., 2007). Khatat et al. (2003) reported that elevated levels of toxic component in river water, indicated by fish population in the river are reduced drastically. Therefore, we isolated heavy metal tolerant bacteria from this river and further mechanism of heavy metal tolerance was determined.

Proteomic approach has become a powerful tool which is used extensively to investigate the protein expression pattern under several abiotic stresses. Although the first proteomic analyses were conducted 30 years ago, renewed interest in this field has been fuelled by several recent advances, including the availability of public genome and protein databases, the development of database search engines capable of exploiting these databases, and the introduction of high-sensitivity, easy to-use mass spectroscopy (MS) techniques. An important aspect of proteomics is to study the expression levels and characterization of proteins in cells when exposed to different environmental stress conditions. Rather than targeting a particular family of proteins a comparison of protein profiles from control and stressed conditions allow the identification of adaptive proteins and provides an insight of metabolic pathways and unveils the molecular mechanism of stress response in various microorganisms. Proteomics approach has been used to study the effect of heavy metals on bacteria (Weiss et al., 2009; Belfiore et al., 2013; Zhang et al., 2007). Most investigations dealing with the interactions between heavy metal and bacteria have focused exclusively on the mechanisms of resistance that include the transformation of metal by methylation, reduction or oxidation. In contrast, the other cellular functions involved in the adaptation of these microorganisms to toxic
concentrations of heavy metal remain largely unknown. The species *Klebsiella pneumoniae* has been known for its resistance and survival in the presence of several toxic compounds such as cyanide, tetracyanonickelate and heavy metals like cobalt and lead. However, little is known about tolerance and adaptation of *K. pneumoniae* to arsenic. In view of this, altered proteins identified through proteomic approach are preferred to recognize the mechanism of tolerance in *K. pneumoniae* in response to arsenic.

**Aims and objectives**

The goal of this work was to isolate and identify the heavy metal resistant microorganisms and identify heavy metal resistant proteins in control and test so as to evaluate the metal tolerance mechanism in the isolate using the proteomic approach.

The specific goals of the undertaken study were:

1) To isolate and characterize bacteria with potential heavy metal tolerance.
2) To understand the heavy metal tolerance mechanism.
3) To find out differentially expressed proteins under heavy metal stress and their identification by proteomic approach.
4) To evaluate the role of differentially expressed proteins under heavy metal stress.
5) To establish the genetic basis of heavy metal resistance in isolated bacterial strain.

The thesis is divided into four chapters followed by list of references

**Chapter 1: Introduction and review of literature**

This chapter covers an overview of heavy metals, the literature on metal tolerance, toxicity and resistance in microorganisms. It includes literature on arsenic, its toxicity and resistance mechanisms in bacteria. Also describes heavy metal resistant bacteria and their response to heavy metals by proteomic approach. The objectives of the study are included in this chapter.

**Chapter 2: Isolation and characterization of heavy metal tolerant bacteria**

This chapter involves the screening of organisms showing promising results against
heavy metal treatments by microtitre plate. Further, the selected isolate (MR4) was characterized and identified by 16S rDNA nucleotide sequencing. From the findings obtained in the present study, it is evident that, *K. pneumoniae* (MR4) isolated from river Mula in Pune, Maharashtra, India tolerant to multiple heavy metals such as As(V), As(III), Cu(II), Co(II), Ni(II), Hg(II) and sensitive to almost all antibiotics tested except ampicillin and vancomycin. The *K. pneumoniae* showed higher resistance to As(V), whereas As(III) is the form of arsenic so both metals were selected for further study. The morphological changes in the presence of arsenic were studied by scanning electron microscopy. The arsenic resistant gene in *K. pneumoniae* was determined and later, mechanism of arsenic tolerance was studied.

**Chapter 3: Effects of arsenate stress on proteome of *K. pneumoniae***

This chapter incorporates the response of *K. pneumoniae* to LD$_{50}$ concentration of arsenate (200mM). Any change in the environmental conditions would affect the physiological response given by the cell. This would in turn be reflected in the protein expression levels of the cell. Therefore, it was relevant to ascertain the expression levels of proteins which could be responsible for arsenic tolerance in *K. pneumoniae*. The changes were visualized via SDS-PAGE followed by 2-Dimensional electrophoresis. Since, it can separate the proteins through its isoelectric points and molecular weight and it is the most sorted application used for studying metal stress responses. The protein identifications were done through MALDI TOF/TOF and differentially expressed proteins were categorized into their functional classes viz., Outer membrane proteins, cell membrane biosynthesis proteins and transport and binding proteins: Amino acid metabolism; Antioxidant proteins; Stress proteins; Transcription Translation and Purine and pyrimidine metabolism; Carbohydrate metabolism and have discussed their role in protecting cell from stress. Also changes in antioxidant enzymes activity in presence of As(V) were determined by spectrophotometric assay as well as activity staining.

**Chapter 4: Effects of arsenite stress on proteomic expression of *K. pneumoniae***

This chapter describes the effect of LD$_{50}$ concentration of arsenite (2.5mM) on proteome of *K. pneumoniae*. The changes were visualized via 2-Dimensional electrophoresis and the protein identifications were done through MALDI TOF/TOF. The differentially expressed
proteins were categorized into different functional classes and discussed the role of each protein. These identified proteins were classified into the following functional categories viz., outer membrane proteins, membrane transport and binding proteins; antioxidant proteins; stress response proteins; transcription and translation proteins; carbohydrate metabolism; amino acid metabolism and have discussed their role in protecting cell from stress. Also the alteration in antioxidant enzymes activity in presence of As(III) were determined by spectrophotometric assay as well as activity staining.

Proteomic approach to understand the arsenic tolerance mechanism has been studied for the first time in \textit{K. pneumoniae}.

**SUMMARY AND CONCLUSION**

This includes overall summary, main findings and conclusions of research work.