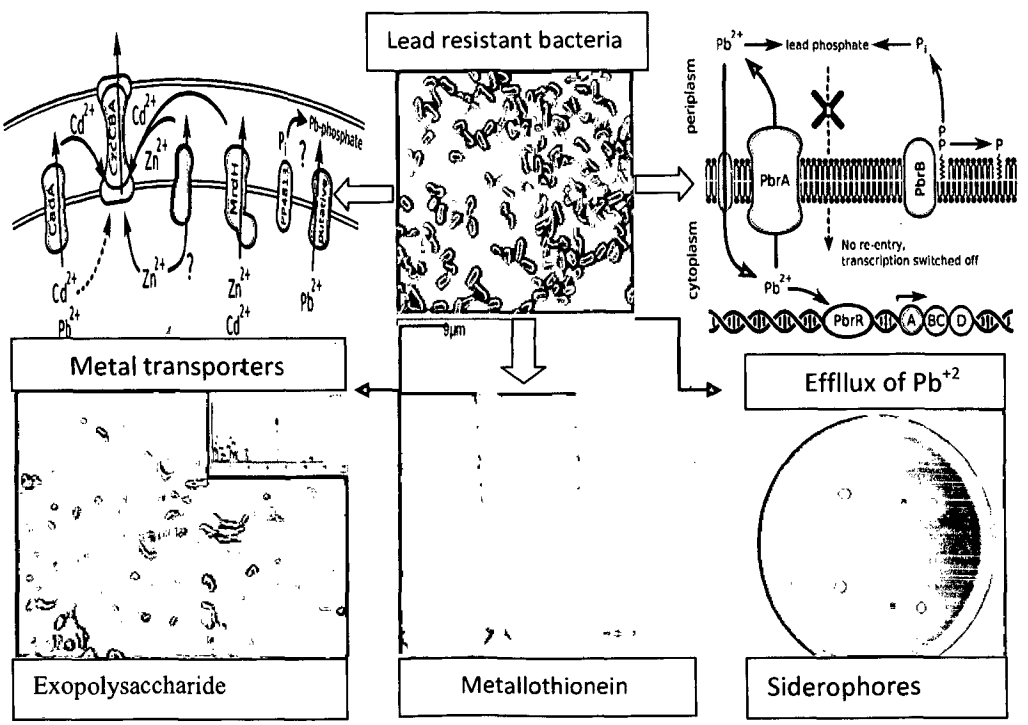
GOA UNIVERSITY
TALEIGAO PLATEAU
GOA-403206

BIOLOGICAL CHARACTERIZATION OF LEAD RESISTANT BACTERIA FROM TERRESTRIAL AND ESTUARINE ECONICHES OF GOA



Ph.D. Thesis
by
Milind Mohan Naik

DEPARTMENT OF MICROBIOLOGY
2012

BIOLOGICAL CHARACTERIZATION OF LEAD RESISTANT BACTERIA FROM TERRESTRIAL AND ESTUARINE ECONICHES OF GOA

THESIS SUBMITTED TO THE

GOA UNIVERSITY

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

MICROBIOLOGY

BY

Milind Mohan Naik
M.Sc. Microbiology

Research Guide

Professor Santosh Kumar Dubey

Department of Microbiology
Goa University, Goa, India

2012

Certificate

This is to certify that Mr. Milind Mohan Naik has worked on the thesis entitled **"Biological characterization of lead resistant bacteria from terrestrial and estuarine econiches of Goa"** under my supervision and guidance.

This thesis, being submitted to the Goa university, Goa, India, for the award of the degree of Doctor of Philosophy in Microbiology is an original record of the work carried out by the candidate himself and has not been submitted for the award of any other degree or diploma of this or any other university in India or abroad.

Prof. Santosh Kumar Dubey, JSPS Fellow
Head
Department of Microbiology
Goa University

Head
Department of Microbiology
GOA UNIVERSITY

Professor Santosh Kumar Dubey
Research Guide
Goa University

Corrections have been incorporated as indicated by the expert (examiners).

Research Guide
21/06/2012

21/6/2012
Dr. D.V. Gokhale
(External Examiner)

STATEMENT

I hereby state that this thesis for Ph.D. degree on "**Biological characterization of lead resistant bacteria from terrestrial and estuarine econiches of Goa"** is my original contribution and that the thesis and any part of it has not been previously submitted for the award of any degree /diploma of any University or Institute. To the best of my knowledge, the present study is the first comprehensive work of its kind from this area.

Milind Mohan Naik
Ph.D. student
Department of Microbiology
Goa University
Goa

CERTIFICATE FROM THE CANDIDATE

I would like to certify that the corrections/modifications have been incorporated in the thesis as suggested by honourable referee and same has been enclosed to the corrected thesis. Corrections have been incorporated on the following pages: page no. 38, 46, 76, 76a, 119, 126, 127, 128 and 136a.

18/5/2012

(Milind Mohan Naik)

ACKNOWLEDGEMENT

No good work is created without joining hands and it will be definitely deluding and improper to say that, I could have completed this study single handed. This effort was not carried out solely by me but was aided by a quite few people whom I wish to thank now because this is the best time and place. This is the only part of the Ph.D. where I could whole heartedly appreciate all those kind and helpful souls.

With deep gratitude, I acknowledge the great debt I owe to my Guide, Professor Santosh Kumar Dubey, for his admirable endurance, guidance, patience and encouragement given to me during the entire period of research. His scientific experience and vast knowledge of the subject, innovative ideas and constructive criticisms have contributed immensely to my research work.

I am thankful to Prof. G.N Nayak, Dean, Faculty of Life Sciences and Dr. Sanjeev Ghadi, V.C's nominee, Faculty research committee, for extending all the facilities during my research work and their valuable criticism.

My sincere thanks go to Prof. Saroj bhosle for extending laboratory facilities and to Prof. Irene Furtado, Dr. Sarita Nazareth, Dr. Sandeep Garg and Dr. Aureen Goudinho for their valuable suggestions during the study.

I express my sincere gratitude to entire non-teaching staff including Mr. Shashikant Parab, Budhaji, Dominic, Ladu, Saraswati, Deepa and Narayan for their constant help and support in various ways.

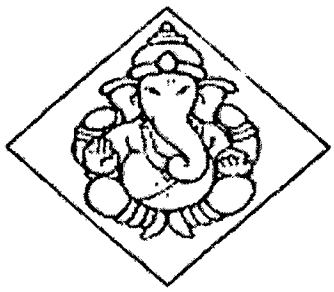
I acknowledge the financial support provided by U.G.C., Government of India as JRF. I am also thankful to Dr. M. S. Prasad and Mr. Vijay Khedekar from National Institute of Oceanography, Goa, India for scanning electron microscopy.

I am highly obliged for zeal, enthusiasm and encouragement provided by Drs Anju Pandey, Vidya Ramachandran, Jeevan Parab, Celisa, Nimali and Ph.D. students Teja, Rahul, Brenda, Swapnil Doijad, Lakshangi, Akshaya, Sanika, Valerie, Pramoda, Trelita, Dnyanada, Cristabell, Subhojit, Sheryanne, Marileou, Shweta, Mufeeda, Ajit, Vassant, Kashif Shamim, Amrita and Sushma throughout my study.

I owe my deepest sense of gratitude to my friends Uday Naik, Amar Degvekar, Shivraj, Rohan, Swapnil, Kuldeep, Renosh, Bhaskar, Prakash, Gautam, Divyaprakash, Bhanudas, Vishwas, Ramprasad, Samit, Srijay, James, Satyajeet, Meghnath, Krishna, Rajat, Pradhan, Sweety, Mahabaleshvar, Santosh, Suryakant, Deepti, Hari, Chinmay, Sandesh, Shrikant, Kashi, Shambhu, Anant, Leena, Vinay and Madhavi, who are not a part of the microbiology department but have provided genuine help in the course of my Ph.D.

But my research endeavor would have remained incomplete without the constant help and untiring, unconditional support of my beloved mother Mrs. Manisha Naik and father Mr. Mohan Naik along with other family members.

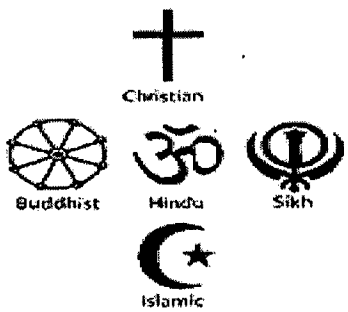
I have successfully compiled my creative and thoughtful research work due to genuine concern and painstaking effort of many more well wishers whose names are not mentioned, but they are still in my heart. So, reward is surely worth for their efforts. Before I end, I offer my prayer of gratitude to the almighty God for his blessings and grace to bestow his benevolence on all.



DEDICATED

TO MY

BELOVED PARENTS



ABBREVIATIONS

Abs	Absorbance	**Kbps**	Kilo base pairs
APS	Ammonium per sulphate	**L**	Litre
b.p.	Boiling point	**LB**	Luria Bertani
CFU	Colony forming unit	**µM**	micromolar
°C	Degree Celsius	**M**	molar
Ca^{+2}	Calcium ion	**µl**	Microlitre
Cd^{+2}	Cadmium ion	**mA**	milli ampere
d/w	Distilled water	**mg**	milli gram(s)
EDTA	Ethylene diamine tetra	**Mg^{+2}**	Magnesium ions
	acetic acid	**min**	minute(s)
EPS	Exopolysaccharide	**mM**	milli molar
Fig.	Figure	**ml**	millilitre
FTIR	Fourier transform infrared	**µg**	microgram
	spectroscopy	**µ**	Micron
gm	Gram	**NH_4NO_3**	Ammonium nitrate
GC	Gas chromatography	**NH_4Cl**	Ammonium chloride
Hrs	Hour(s)	**NA**	Nutrient agar
HCl	Hydrochloric acid	**NaOH**	Sodium hydroxide
H_2SO_4	Sulphuric acid	**nm**	Nanometer
Hg^{+2}	Mercuric ions	**NaCl**	Sodium chloride
KNO_3	Potassium nitrate	**O.D.**	Optical density
K^+	Potassium ions	**PAGE**	Polyacrylamide gel
kDa	Kilo Dalton		electrophoresis

Pb^{+2}	Lead ions	**TEMED**	Tetra methyl
PCR	Polymerase chain reaction		ethylene diamine
rpm	Revolution per minute	**TMM**	Tris-minimal medium
RT	Room temperature	**UV**	Ultra violet
SDS	Sodium dodecyl sulphate	**V**	Volts
sec	Seconds	**v/v**	Volume/Volume
sp.	Species	**w/v**	Weight/Volume
SEM	Scanning Electron	**Zn^{+2}**	Zinc ions
	Microscopy	**ZMB**	Zobell Marine Broth
TBT	Tributyltin	**%**	Percentage
TBTC	Tributyltin chloride		

INDEX

LIST OF TABLES

CHAPTER I

CHAPTER II

LIST OF FIGURES

CHAPTER I

CHAPTER II

CHAPTER III

CHAPTER IV

CHAPTER I: INTRODUCTION

CHAPTER II: **Screening, identification and physiological characterization of lead resistant bacteria from terrestrial and estuarine econiches of Goa**

CHAPTER II

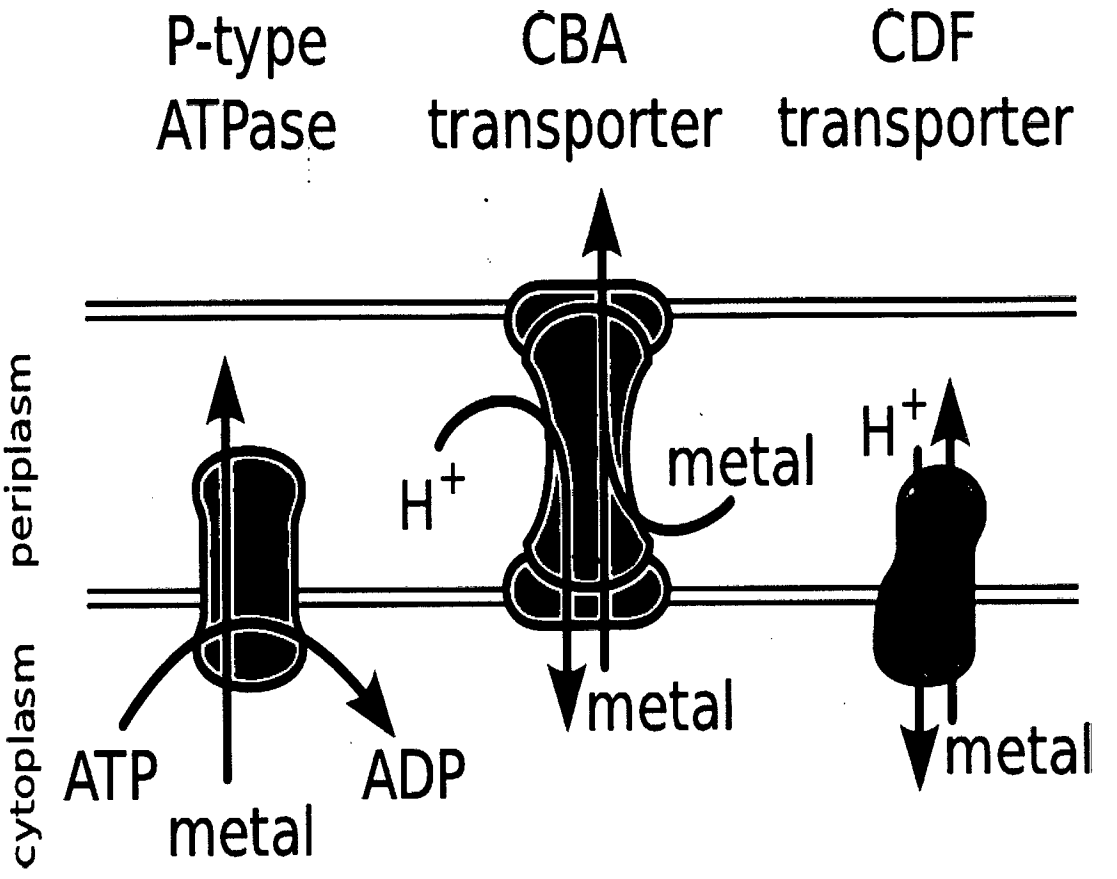
CHAPTER III : **Biochemical characterization of lead resistant bacterial strains**

CHAPTER III

CHAPTER IV: **Molecular and genetic characterization of lead resistant bacteria to explore lead and multiple drug (antibiotic) resistance**

CHAPTER IV

RESULTS AND DISCUSSION **Page No.**



Various heavy metal transporters in resistant bacteria

(Hynninen et al., 2009)

CHAPTER-I

CHAPTER I

INTRODUCTION

1.1 Lead in the environment and applications

Lead (Pb) obtained mainly from galena (PbS) is known to humans for about 7,000 years, and its poisoning has been recognized for at least 2,500 years (Nriagu, 1978; Eisler, 1988) (Fig.1.1). Lead has used in wide range of applications in various industries viz. petroleum, electronics, acid storage batteries, paints, ceramics, stained glass, biocides, ammunition, alloys, toys, antifouling agents (Eisler, 1988; Gummersheimer and Giblin, 2003). Lead arsenate is used extensively as a biocide to reduce bird hazards near airport runways by controlling earthworm population and also to control pests in fruit orchards that ultimately results in lead contamination of terrestrial environment.Toxic metals viz. cadmium, lead and mercury without any known biological functions are one of the most serious environmental pollutants prevalent in industrial wastes and their release into natural water bodies and terrestrial ecosystems poses serious threat to the health and bioproductivity of aquatic and terrestrial biota (Skei, 1978; Nies, 1999; Watt et al., 2000; Tong et al., 2000; Coombs and Barkay, 2004; De et al., 2007, 2008; Velea et al., 2009; Jayaraju et al., 2011). Environmental levels of lead have increased more than 1000-fold over the past three centuries as a result of anthropogenic activities. Heavy metals including lead exert toxic effects on living organisms in a variety of ways which include DNA damage, inactivation of proteins, essential metabolic enzymes and lipids (Roane, 1999; Nies, 1999; Asmub et al., 2000; Hartwig et al., 2002). Therefore U.S. Environmental protection agency (EPA) has included lead, mercury and cadmium in their list of hazardous inorganic wastes (Cameron, 1992). As per WHO guidelines permissible level of lead in drinking water is <10 µg/L (Watt et al., 2000). Lead is persistent environmental pollutant, slowly accumulates and results in biomagnification in food chain and referred as cumulative poison (Dauvin, 2008; Flora et al.,

2008; Lombardi et al., 2010). In case of humans, lead (Pb^{+2}) inactivates many enzymes, causes renal failure, neurodegenerative diseases, reproductive impairment, anemia and weakening of bones but when blood level exceeds 70 µg/dl results in coma and death (Fowler, 1998; Tong et al., 2000; Gummersheimer and Giblin, 2003; Turkdogan et al., 2003; Lam et al., 2007; Flora et al., 2008).Wastes from industries, sewage sludge, power plants and incineration plants contain substantial amounts of toxic heavy metals viz. lead, cadmium, arsenic, chromium and mercury which are of serious environmental concern and need to be removed from the source of pollution itself (Fig.1.2).

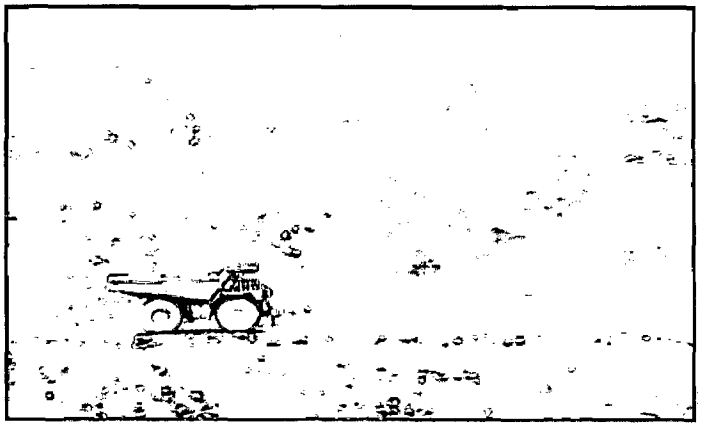


Fig.1.1 Red Dog mine in Alaska: largest lead mine in the world

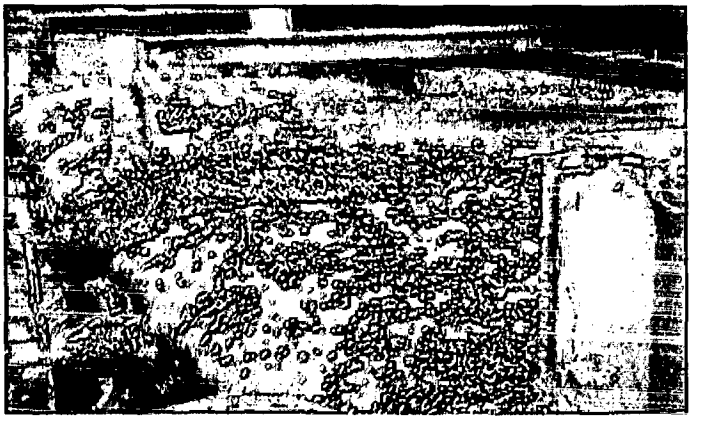


Fig.1.2 Effluent of lead smelter in Alaska

1.2 Chemical characteristics of lead

Elemental lead is a bluish-gray, soft metal with atomic weight 207.19 and atomic number 82 which melts at 327.5°C, boils at 1,749°C and has density of 11.34 g/cm^3 at 25°C. Metallic lead is sparingly soluble in hard, basic water upto 30 µg/l, and up to 500 µg/l in soft acidic water. Lead has got four stable isotopes: Pb^{204} (1.5%), Pb^{206} (23.6%), Pb^{207} (22.6%) and Pb^{208} (52.3%). Of its 24 radioactive isotopes Pb^{210} with half life 22 years and Pb^{212} with half life 10 hours have been used in tracer experiments exclusively. Lead occurs in four different valency states: Pb^{0}, Pb^{+}, Pb^{2+} and Pb^{4+} which are environmentally important. In nature, lead occurs mainly as Pb^{2+} which is oxidized to Pb^{4+} only under strong oxidizing conditions and only few compounds of Pb^{4+} are stable. Some lead salts are comparatively more soluble in water (e.g. lead acetate, 443 g/l; lead nitrate, 565 g/l; lead chloride, 9.9 g/1), whereas others are only sparingly soluble (e.g. lead sulfate, 42.5 mg/l; lead oxide, 17 mg/l; lead sulfide, 0.86 mg/1). Solubility of lead salts is greatest at elevated temperatures in the range 0 to 40° C. Of the organoleads, tetraethyl lead (TEL) and tetramethyl lead (TML) are the most stable and most important because of their widespread use as an antiknocking fuel additives. Both are clear, colorless, volatile liquids, highly soluble in many organic solvents; however, solubility in water is only 0.18 mg/l for TEL and 18.0 mg/l for TML (Harrison and Laxen, 1981; Eisler, 1988).

Chemistry of lead is complex, as in aqueous environment Pb^{+2} is more soluble and bioavailable under conditions of low pH, low organic content, low levels of particulate matter and low concentrations of the salts of calcium, iron, manganese, zinc, and cadmium. (Harrison and Laxen, 1981; Scoullos, 1986).

1.3 Lead in the terrestrial environment

Soil pollution is an undesirable change in the physical, chemical and/or biological characteristics of the soil which reduces the area of cultivable land and habitation. Human health is closely related to the quality of soil and especially to its degree of pollution (Romic and Romic, 2003; Velea et al., 2009). Soil acts as a sink as well as source of pollution with the capacity to transfer pollutants to groundwater and food chain ultimately affecting human health (Facchinelli et al., 2001). Heavy metal contamination of terrestrial environment has attracted great deal of attention worldwide due to non-biodegradable nature and long term persistence of toxic heavy metals in the environment (Raghunath et al., 1999; Li et al., 2004). Average concentration of lead in earth crust is 13μg/g. Soil contaminated with lead is unsuitable for agriculture due to accumulation and adverse effects of lead on crop plants and may also result in biomagnifications at higher tropic levels (Khan et al., 2010).

Amount and bioavailability of lead, cadmium and mercury in soil to natural biota is related to the degree of risk to them. Total lead concentration does not necessarily reflect the amount of lead that is biologically toxic or bio-available. Soluble lead is toxic as it is in free ionic form and may penetrate more readily the cellular membranes (Roane, 1999; Pike et al., 2002). The bioavailability of lead depends upon various soil constituents and pH. Lead may precipitate in soil if soluble concentration exceeds 4 mg/l at pH 4 and 0.2 mg/l at pH 8. In the presence of phosphate and chloride, these solubility limits may be as low as 0.3 mg/l at pH 4 and 0.001 mg/l at pH 8. Therefore, experiments with lead level exceeding these values may reflect precipitation reactions rather than adsorption reactions. Anionic constituents of soil viz. phosphate, chloride and carbonate are known to influence bioavailability of lead either by precipitation of minerals of limited solubility or by reducing adsorption through complex formation (Rickard and Nriagu, 1978). Several adsorption studies have indicated that lead adsorption in soil increased with increasing pH ranging from 4 to 11. Adsorption of lead also

increases with increasing organic matter content of soil (Hildebrand and Blum, 1974; Scrudato and Estes, 1975; Griffin and Shimp, 1976; Zimdahl and Hassett, 1977).

Lead adsorption pattern in soil vis-a-vis soil constituents viz. clay minerals, oxides, hydroxides, oxyhydroxides and organic matter has been studied extensively. Lead adsorption studies using 12 different soils from Italy have clearly revealed that soil organic matter and clay content are two major factors influencing lead adsorption in soil (Soldatini et al., 1976). Similarly lead adsorption characteristics of 7 different alkaline soils from India have also been determined (Singh and Sekhon, 1977) which indicated that clay, organic matter, and calcium carbonate influenced lead adsorption. Recent studies have also shown that chloride ions cause precipitation of lead as solid PbOHCl (Bargar et al., 1998). Solid organic matter such as humic materials in soil and sediments also adsorb lead significantly (Rickard and Nriagu, 1978; Zimdahl and Hassett, 1977). In soil lead is found as different compounds viz. litharg (PbO), cerussite ($PbCO_3$), hydrocerussite ($PbCO_3$-$PbOH_2$), angelsite ($PbSO_4$) and lead phosphate ($Pb_5(PO_4)_3Cl$) (Dermatas et al., 2004; Nadagouda et al., 2009). Despite the apparent immobility of lead, organic soils or sediments retain approximately 70% of the total lead present in ecosystem (Johnson et al., 1995). Consequently, soil serves as a major source of lead with serious impact on survival and bioproductivity of terrestrial macro and microrganisms.

Table 1.1 Marked lead contaminated terrestrial sites in the world

S. N.	Location/site	Type of sample	Lead concentrations	References
1	Raipur and Korba region, Chhattisgarh State,India	Soil from metal smelting and coal burning sites	12.8-545 µg/g	Patel et al., 2006
2	Dandora municipal waste dumping site, Nairobi Kenya	i. Soil sample ii. waste dump	i. 50-590 ppm ii. 13,500 ppm	Kimani, 2010

3	Hawaii, USA	Roadside soil	4 – 1,750 µg/g	Sutherland, 2003
4	Marmorilik, W. Greenland	Soil	8,922±622 µg/g	Larsen et al., 2001
5	Belize	Soil	1,572 µg/g	Walker et al., 2003
6	Battery recycling plant of Haina, Dominican Republic	i. Soil ii. Children's blood	3,115 mg/g Mean blood lead levels: 71 µg/dl	Grant et al., 2006 Blacksmith Institute, New York, USA, 2006
7	Lead mining, Rudnaya pristan, Russia	1.Residential garden soil 2. Road side soil	476-4,310 mg/kg 2020-22900 mg/Kg	Grant et al., 2006 Blacksmith Institute,New York, USA, 2006
8	Mining and smelting, Kabwe, Zambia	i. Soil ii. Children's blood	2,400 mg/g Blood lead level >200 µg/dl	Blacksmith Institute, New York, USA, 2006
9	Lead mining, La Oraya, Peru	i. Soil ii. Children's blood	1,620 mg/g 33.6 µg/dl	Blacksmith Institute, New York, USA, 2006

1.4 Lead in the aquatic environment

Several industries inadvertently release effluents with high concentrations of lead which enters into aquatic environment posing serious threat to the natural biota. In surface waters lead exists in three forms: i. dissolved labile (e.g. Pb^{2+}, $PbOH^{+}$ and $PbCO_3$); ii. dissolved bound (e.g. colloidal or complexed lead) and iii. particulate lead (Benes et al., 1985). Lead based antifouling paints are also used to paint the hulls of boats, ships and many static structures that are submerged, including pontoons, piers, aquaculture nets, buoys, pipelines and drilling platforms to control fouling organisms. With phasing out and ultimate ban on tri-organotin based antifouling paints, Cu(I) and Pb(II) based antifouling paints with cuprous oxide/ cuprous thiocyanate, zinc oxide and lead oxides became potential alternatives

(Sanchez-Bayo and Goka, 2005; Turner, 2010). Slow and un-controlled leaching of these antifouling biocides from the painted surface of ships, boats and other submerged structures results in elevated levels of lead in the aquatic ecosystem viz. harbours, marinas and estuaries (Turner, 2010).

Lead is transported in the estuarine environment either in the form of suspended particles or dissolved ions which are subsequently removed from water and adsorbed finally into the sediments (Fig.1.3).Thus estuarine sediments are major reservoirs of heavy metals including lead. The contamination level and distribution characteristics of heavy metals in coastal waters and sediments from Tianjin Bohai Bay, China revealed that Pb and Zn were the main heavy metal pollutants in the coastal waters of the bay. High levels of Pb and Zn appeared especially near the estuary indicating that river discharge was the main source of lead pollution and Pb pollution by atmospheric deposition had also increased due to the use of leaded petrol in motor cars (Meng et al., 2008).

Sediment sample of Mandovi estuary of Goa revealed appreciable levels of lead ranging from 4.5-46.5 $\mu g\ g^{-1}$ respectively at different source points (Alagarsamy, 2006).The Pollution Load Index (PLI) for Pb, Fe, Mn, Zn, Cu, Co, and Cr for Divar sediments (Mandovi estuary) was far greater (i.e. 1.65 - 2.19) than that of Tuvem (Chapora estuary) (0.91-1.3) reflecting the intensity of anthropogenic inputs into the ecosystem due to transport of ferromanganese ore along the Mandovi river (Atri and Kerkar, 2011). Antarctic waters from the Indian side were examined for the incidence of metal and antibiotic-resistant bacteria during the austral summer (13^{th} Indian Antarctic expedition) along the cruise track extending from 50°S and 18°E to 65°S and 30°E. The bacterial isolates from these waters showed resistance to multiple heavy metals including lead prove that even Antarctic waters which are considered relatively more pristine than the other oceanic waters are not free from heavy

metal contamination (De Souza et al., 2006). Mercury and TBT resistant bacteria have already been isolated from west coast of India which are also resistant to cadmium and lead along with common antibiotics (Dubey and Roy, 2003; Roy et al., 2004; Bramhachari, 2006; Dubey et al., 2006; Krishnamurthy et al., 2008; De et al., 2008; Ramachandran, 2009).

Table.1.2 Marked lead polluted rivers and estuaries in the world

S. N.	Location/site	Type of sample	Lead concentration	Referrence
1	Ganga river, Varanasi, U. P., India	Sub surface water	3.6 - 107.34 g/ ha/ y	Pandey et al., 2010
2	Kabini river, Karnataka, India	Sediment	4.6 mg/g	Hejabi et al., 2011
3	Cauvery river, Karnataka India	i. Water (Down stream) ii. Sediment (Down stream)	9.95 ppm 450.52 ppm	Begum et al., 2009
4	Yamuna river in Delhi and Agra, India	Sediment	22-856 mg/Kg	Singh, 2001
5	North sea, UK	Estuarine sediment	52–207 µg/g	Smith and Orford, 1989
6	Jurujuba Sound, South east Brazil	River sediment	64–174 µg/g	Neto et al., 2000
7	Newark bay, New Jersey, USA.	Estuarine Sediment	64 mg/kg - 2.5 g / kg	Bonnevie et al., 1992
8	Western coast of Mauritius	Estuarine sediment	27 mg/ kg	Ramessur, 2004

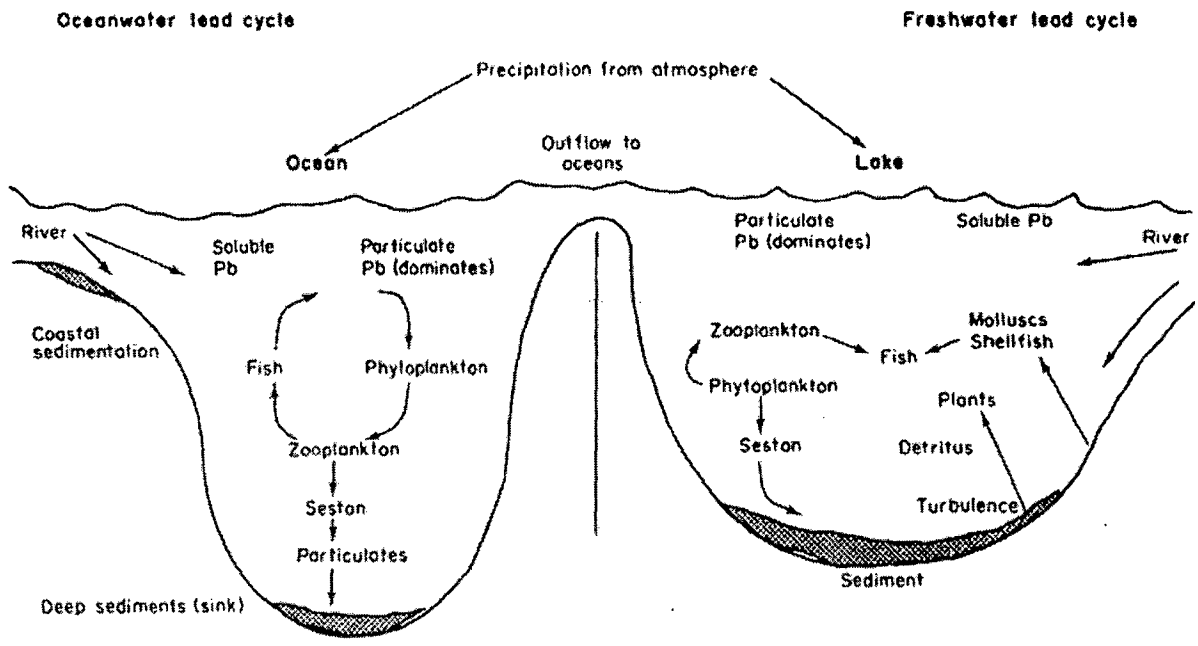


Fig.1.3 Biogeochemical cycle of lead in fresh water and in salt water ecosystems (Jaworski et al., 1987)

1.5 Lead resistant bacteria

Metals are natural constituents of earth and some of them such as Zn^{+2}, Ni^{+2}, Cu^{+2} are even essential for many living organisms, but become toxic to microorganisms and macro organisms at higher concentrations (Nies, 1999). Lead, cadmium and mercury being non-essential to bacterial cells are toxic even at low concentrations (Trajanovska et al., 1997; Nies, 1999). Generally, both natural and anthropogenic sources are responsible for terrestrial contamination with toxic heavy metals and interestingly spatial variation in the level of contamination has been noticed (Sutherland, 2003; Kashem et al., 2006; Patel et al., 2006; Khan et al., 2008; Shah et al., 2010). Furthermore, the ingestion of heavy metals viz. Pb, Cd, Cu, Hg, Ni, and Zn can seriously cause depletion of some essential nutrients in the human

body which in turn adversely affects immune system, intra-uterine growth, causes psychosocial dysfunctions, disabilities associated with malnutrition and a high prevalence of upper gastro-intestinal cancer (Trichopoulos, 1997; Iyengar and Nair, 2000; Turkdogan et al., 2003). But, interestingly some natural microbial strains employing a variety of protective mechanisms can survive at very high concentrations of these toxic heavy metals including lead without any impact on their growth and metabolism.Various strategies through which they resist high concentrations of heavy metals include efflux, reduction, oxidation, extracellular sequestration, biosoption, precipitation and intracellular bioaccumulation (Trajanovska et al., 1997; Levinson and Mahler, 1998; Nies, 1999; Roane, 1999; Borremans et al., 2001; Blindauer et al. 2002; Zucconi et al., 2003; De et al., 2007, 2008; Desai et al., 2008; Taghavi et al., 2009; Wang et al., 2009) (Fig. 1.4). It is interesting to note that they possess genetic determinants (genes) conferring metal resistance either on chromosomal genome, plasmid or transposons (Silver, 1981; Bopp et al., 1983; Lebrun et al., 1994; Silver and Phung, 1996; Trajanovska et al., 1997; Crupper, 1999; Nies 1999; Borremans et al., 2001; Bruins et al., 2003; Coomb and Barkay, 2005; Taghavi et al., 2009). The genome of *Pseudomonas putida* KT1440 contains 61 open reading frames involved in resistance to several metals (Canovas et al., 2003). Resistance to multiple metals viz. Pd, Cd, Zn, Sn, Cu, and Hg was found in the tributyltin resistant 250 bacterial sp. (Pain and Cooney, 1998). This unique characteristic of heavy metal resistant microbes including bacteria makes them an ideal tool for bioremediation of metal contaminated environmental sites.

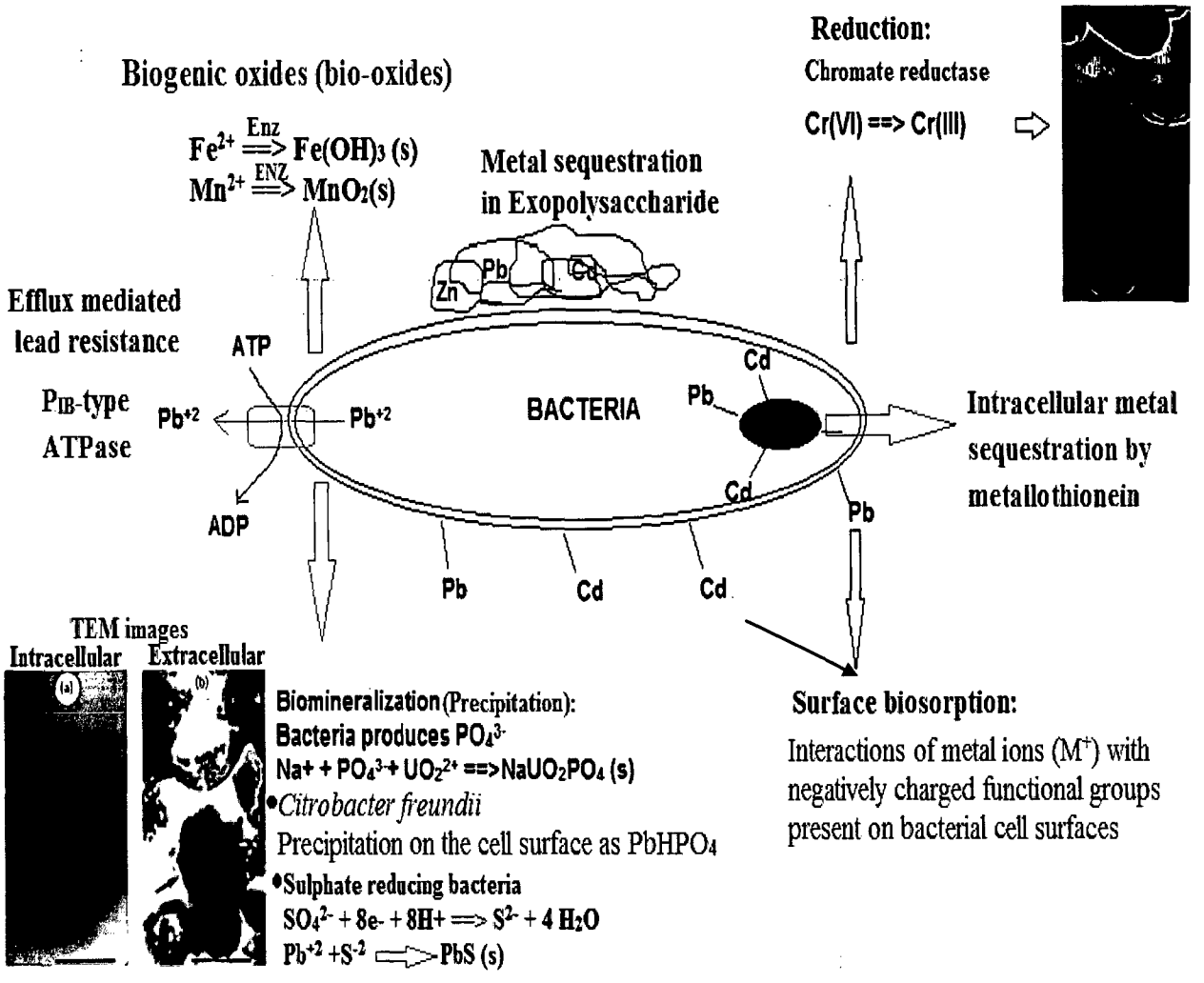


Fig.1.4 Various heavy metal (including lead) resistance mechanisms operational in bacteria

1.6 Biochemical and molecular mechanisms of heavy metal (including lead) resistance in bacteria

1.6.1 Efflux mechanism

In order to maintain heavy metal homeostasis, intracellular level of toxic heavy metal ions has to be tightly controlled (Nies, 1999). Soft metal transporting P_{IB}-type ATPases are group of proteins involved in transport of heavy metals outside the cell membrane and governing bacterial heavy metal resistance (Nies and Silver, 1995; Rensing et al., 1999) (Fig.

1.5). These transporter proteins prevent over-accumulation of highly toxic and reactive metal ions viz. Pb (II), Cu (I), Ag (I), Zn (II) and Cd (II). P_{IB}-type ATPases can be divided into two subgroups: i) Cu (I)/Ag (I)-translocating ATPases encoded by gene *copA* in *Enterococcus hirae*, *Helicobacter pylori* and *E. coli* ; ii) Zn (II) /Cd (II) / Pb(II)-translocating ATPases encoded by gene *zntA* in *E. coli* and gene *cad A* in *Staphylococcus aureus* plasmid, pI258 (Nies and Silver, 1995; Rensing et al., 1999).

In *Ralstonia metallidurans* CH34 complete operon *pbrUTRABCD* conferring efflux mediated lead resistance has already been sequenced (Borremans et al., 2001; Taghavi et al., 2009). Several P_{IB}-type ATPases are associated with mobile genetic elements and plasmid mediated lead resistance has been reported for *Staphylococcus aureus* and *Ralstonia metallidurans* (Rensing et al., 1999; Borremans et al., 2001). Genes encoding P_{IB}-type ATPases are found in majority of sequenced bacterial and archaeal genomes (Coomb and Barkay, 2004, 2005). P-type ATPases belong to the family of transmembrane transporters responsible for movements of ions and small organic molecules in and out of the cell membranes. The subfamily of transmembrane transporters which includes P_{IB}-type ATPases regulates efflux of toxic heavy metals outside the cell membranes and prevents the over-accumulation of highly reactive and toxic soft-metals thus play an important role in heavy metal resistance (Coomb and Barker, 2004, 2005). The genes *cadA, zntA* and *pbrA* encoding ATPases are members of the superfamily of P-type cation-translocating ATPases, but belong to a group of soft metal transporters. P-type ATPases and cation diffusion facilitator (CDF) transporters export metal ions from the cytoplasm to the periplasm, whereas CBA, a three-component trans-envelope efflux pump acts as chemiosmotic ion-proton exchanger to extrude periplasmic metal ions (Nies, 2003; Hynninen et al., 2009). CBA efflux pumps driven by proteins of the resistance nodulation cell division superfamily. The novel lead resistance mechanism of *Cupriavidus metallidurans* CH34 involves P-type ATPase for removal of Pb^{2+}

ions from the cytoplasm and phosphatase which produces inorganic phosphate for lead sequestration in the periplasm (Hynninen et al., 2009).

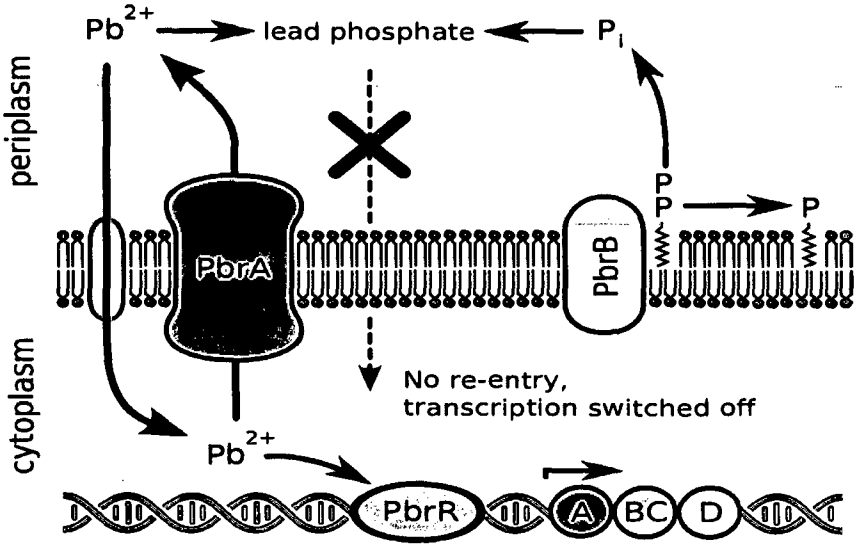


Fig.1.5 Efflux and Precipitation mediated lead resistance in *Cupriavidus metallidurans* CH34 (Hynninen et al., 2009).

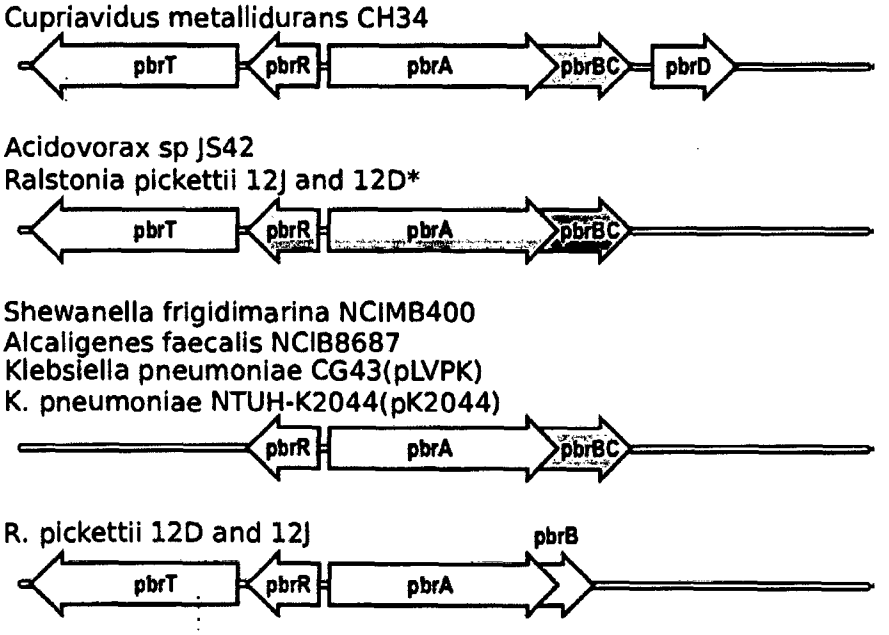


Fig.1.6 *pbr* operons showing various genes in different lead resistant bacteria (Hynninen et al., 2009)

1.6.2 Intracellular bioaccumulation

Microorganisms have evolved several resistance mechanisms to withstand the toxic effects of heavy metals and organometals. One of the common mechanisms is induction of specific metal binding proteins facilitating the sequestration/bioaccumulation of toxic metals inside the cell. These well studied metal binding proteins are referred as metallothioneins (MTs).Intracellular metal bioaccumulation and homeostasis in cell cytosol involves these low molecular weight, cystein-rich metallothioneins which range from 3.5 to 14 kDa (Hamer, 1986). These unique proteins also demonstrate induction in response to specific heavy metals such as Cd, Pb, Zn, and Cu (Gadd, 1990; Turner et al., 1996; Blindauer et al., 2002; Liu et al., 2003).

Metallothioneins play an important role in immobilization of toxic heavy metals thereby protecting bacterial metabolic processes catalysed by enzymes (Blindauer et al., 2002; Liu et al.,2003).Several cyanobacterial and bacterial strains have been reported to encode metallothioneins for maintaining cytosolic metal homeostasis viz. *Synechococcus* PCC 7942 (SmtA), *Anabaena* PCC 7120 (SmtA), *Oscillatoria brevis* (BmtA), *Pseudomonas aeruginosa* (BmtA) and *Pseudomonas putida* (BmtA) (Turner et al., 1996; Blindauer et al., 2002; Liu et al., 2003). Two copper-inducible supernatant proteins viz. CuBPl and CuBP2 with molecular mass 21 kDa and 19 kDa were identified in marine bacterium, *Vibrio alginolyticus* which were 25-46 times amplified in the supernatant of copper-challenged culture as compared with control. Thus these proteins facilitated copper accumulation and homeostasis (Harwood-Sears and Gordon, 1990). *Pseudomonas fluorescens* exposed to lead although showed 18 differentially expressed proteins, but only one protein could match significantly to spoVG protein which expressed Pb-induced upregulation (Sharma et al., 2006). It has already been reported that spoVG is involved in sporulation process when cells are under stress. Similarly, *Bacillus megaterium* resists 0.6 mM lead by sequestering lead

intracellularly possibly by metallothionein like proteins (Roane, 1999). Aickin and Dean, (1977) investigated uptake of lead by microorganisms which are capable of removing toxic metals from sewage sludge and effluents. This characteristic of heavy metal resistant bacteria makes them ideal tool for bioremediation of heavy metal contaminated sites.

1.6.3 Extracellular sequestration

Bioavailability of toxic metals is an important factor regulating metal toxicity as soluble metals can more readily penetrate cellular membranes (Roane, 1999). Therefore metal immobilisation strategy is applied by microbes to counteract toxic effects of heavy metals. Extracellular high molecular weight biopolymers secreted by bacterial cells referred as exopolysaccharides (EPS) consist of macromolecules such as polysaccharides, proteins, nucleic acids, humic substances, lipids and other non polymeric constituents of low molecular weight(Bramhachari and Dubey, 2006; Bramhachari et al.,2007). These exopolysaccharides are chemically diverse and are mostly acidic heteropolysaccharides with functional groups viz. hydroxyl, carboxyl, amides and phosphoryl which exhibit high affinity towards heavy metals (Bhaskar and Bhosle, 2006; Bramhachari et al., 2007; Braissant et al., 2007) (Fig.1.5). Bacterial EPS play a key role in initial attachment of cells to different substrata, cell-to-cell aggregation, protection against desiccation and resistance to harmful exogenous materials (Decho, 1990; Iyer et al., 2004; Pal and Paul, 2008). Various microbial biopolymers have been shown to possess potential to bind heavy metals with different degree of specificity and affinity (Bhaskar and Bhosle, 2006; De et al. 2008; Pal and Paul, 2008). Bacterial EPS and its possible role in bioaccumulation of Cu and Pb in marine food chain was investigated using a partially purified and chemically characterized EPS isolated from *Marinobacter* sp. (Bhaskar and Bhosle, 2006).Exopolymer binding process is important in the downward transport of metals in the ocean environment (Decho,1990). In marine *Pseudomonas aeruginosa* CH07 lead was entrapped in EPS indicating it as a possible resistance mechanism (De et al., 2007,

2008). EPS are high molecular weight polyanionic polymers which bind metals by electrostatic interaction between metal cation and negatively charged components of EPS resulting in metal immobilisation within the exopolymeric matrix (Roane, 1999; van Hullebusch et al., 2003). *Pseudomonas marginalis* was able to resist 2.5 mM lead by sequestering lead in an exopolymer (Roane, 1999). Exopolysaccharide produced by *Paenibacillus jamilae* can biosorb 303.03 mg lead/g EPS from lead solution (Morillo et al., 2008). *Paenibacillus jamilae* is able to use toxic olive-mill wastes as the fermentation substrate for the production of the exopolysaccharide which showed preferential binding to lead in multi-metal sorption system (Morillo, et al., 2006). *Pseudomonas* sp. S8A isolated from mine tailing contaminated soil was resistant to cadmium up to 200 mg/ l and lead up to 300 mg/ l and produced both exopolymer and biosurfactant (Kassab and Roane, 2006). Enzymatic activities in bacterial EPS also assist degradation of organic recalcitrants and transformation of heavy metals followed by their precipitation and entrapment in the biopolymer (van Hullebusch et al., 2003; Pal and Paul, 2008).

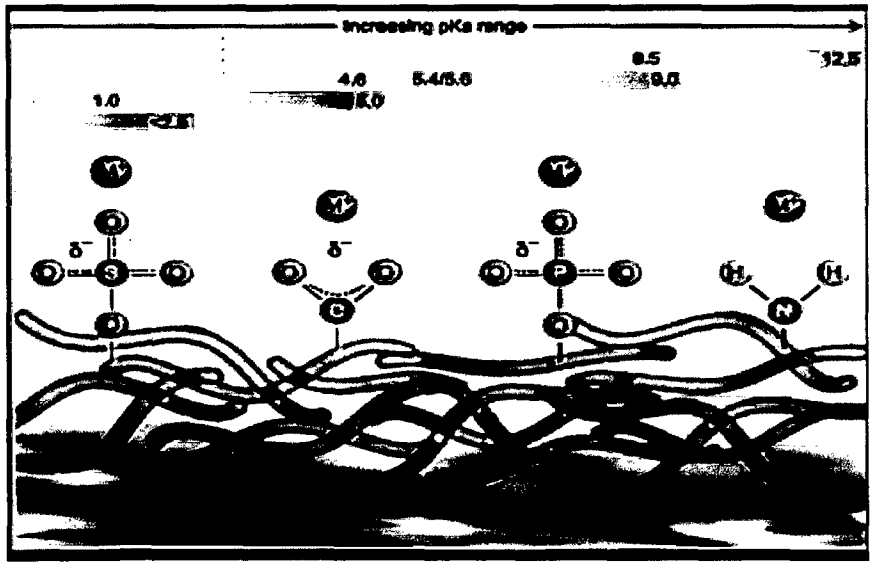


Fig.1.7 Functional groups commonly associated with exopolymeric substances (EPS) and their possible interactions with metal ions (Braissant et al., 2007)

Surface biosorption is also a mechanism of extracellular sequestration of heavy metals to prevent its entry inside bacterial cells and maintain metal homeostasis. Biosorption of metals is mediated by several mechanisms viz. ion exchange, chelation, adsorption and diffusion through cell walls and membranes (Voleski, 1994; Chang et al., 1997). *Pseudomonas aeruginosa* PU21 biomass has potential to biosorb lead, copper and cadmium from metal solution (Chang et al., 1997). This surface biosorption is due to various negatively charged chemical groups present on the bacterial cell surface (Fig. 1.8). The carboxyl group of the peptidoglycan serves as main metal binding site at the cell wall of gram positive bacteria, whereas phosphate groups contribute significantly in case of gram negative bacteria (Gadd and White, 1993).

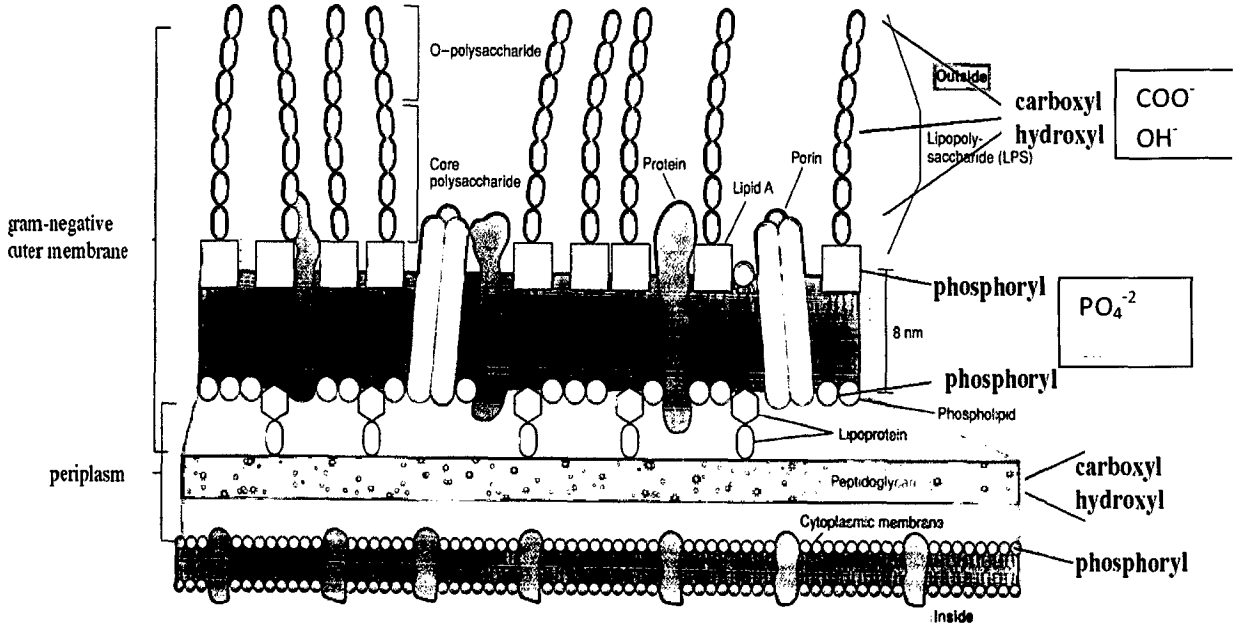


Fig.1.8 Surface biosorption of heavy metals by various negatively charged chemical groups present on the bacterial cell surface

1.6.4 Bioprecipitation

The precipitation of toxic metals to an insoluble complex reduces their bioavailability and toxicity. There are very few reports on microbial precipitation of lead. Aickin et al., (1979) reported precipitation of lead on the cell surface of *Citrobacter* sp. as $PbHPO_4$ which was revealed by electron microscopy and X ray microanalysis, whereas Levinson et al., (1996) suggested intracellular accumulation and precipitation of $Pb_3(PO_4)_2$ by *S. aureus* grown in the presence of high concentrations of soluble lead nitrate. Similarly, *Vibrio harveyi* is also capable of precipitating lead as an unusual phosphate i.e. $Pb_9(PO_4)_6$ (Mire et al., 2004). *Klebsiella* sp. cultured in phosphate-limited medium has also been reported to precipitate lead as PbS (Aiking et al., 1985). In *Staphylococcus aureus* lead precipitation occurred in both lead sensitive and lead resistant strains; however the resistant strains were more effective in precipitation (Levinson and Mahler, 1998). Insoluble compound generated by *Pseudomonas* sp. contained both lead and phosphorus indicating that the product was lead phosphate (Al-Aoukaty et al., 1991). Alkaline phosphatase encoding gene, *phoK* from *Sphingomonas* sp. BSAR-1 was cloned in *E. coli* and over expressed alkaline phosphatise bioprecipitated uranium as insoluble, nontoxic $H_2(UO_2)_2(PO4)_2 .8H_2O$ from alkaline solution (Nilgiriwala et al., 2008). Lead resistant *Bacillus iodinium* GP13 and *Bacillus pumilus* S3 precipitates lead as lead sulphide (PbS) (De et al., 2008). Phosphate solubilising *Enterobacter cloacae* resists lead by immobilizing lead as insoluble lead phosphate mineral i.e. pyromorphite (Park et al., 2011). Reclamation of heavy metal polluted environment using microbial precipitation method has been effective, affordable and ecofriendly technological solution.

1.6.5 Redox reactions

The *mer* operon that confers mercury resistance to bacteria is widely distributed in mercury resistant bacterial population (Osborn et al., 1997; Barkey et al., 2003). The *merA* encodes mercury reductase enzymes which detoxify mercury by reducing Hg^{+2} to volatile Hg^{0} (De et al., 2008). A deep sea sedimentary manganese-oxidizing bacterium, *Brachybacterium* sp. strain Mn 32, showed high Mn(II) resistance (MIC 55 mM) and Mn(II)-oxidizing/removing abilities. This bacterial strain removed Mn (II) employing a simple pathway involving oxidation of soluble Mn (II) to insoluble biogenic Mn oxides (Wang et al., 2009). *Pseudomonas* sp. G1DM21 isolated from Cr (VI) contaminated industrial landfill reduce Cr (VI) to Cr (III) through chromate reductase activity (Desai et al., 2008). Till date there are no reports of lead oxidising or reducing enzymes involve in lead resistance in microorganisms including bacteria.

1.6.6 Alteration in cell morphology

In order to counteract frequent exposure to toxic heavy metals and organic compounds bacteria also exhibit significant alterations in cell morphology (Neumann et al., 2005; Chakravarty et al., 2007; Chakravarty and Banerjee, 2008). Change in morphology is one of the strategies that bacteria adopt to cope up with environmental stresses. It was observed that the maximum alterations in size occurred when the bacterium *Acidiphilium symbioticum* H8 was exposed to sub-inhibitory concentrations of Cu and Cd. Loosely packed coccobacillus-type normal cells formed characteristic chains of coccoidal, lenticular shape with constrictions at the junctions between them in the presence of Cd, whereas Cu induced their transformation into round cells. Ni caused cell aggregation, but Zn showed no effect (Chakravarty and Banerjee, 2008). Cadmium-exposed *Pseudomonas putida* showed extensive blebbing of the outer membrane along with polyphosphate granules containing Cd^{2+} which

was revealed by electron microscopy. Cells from exponential phase cultures of cadmium adapted *P. putida* were found in clusters and were much smaller than control cells grown without cadmium and contained electron dense aggregates also (Higham et al., 1986). Scanning probe atomic force microscopy (AFM) analysis indicated that exposure of *Pseudomonas* sp. G1DM21 to 1 mM Cr (VI) for 24 h, leads to an increase in cell length and height (Desai et al., 2008).

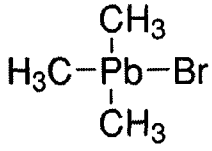
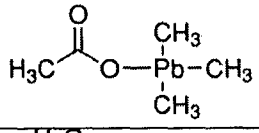
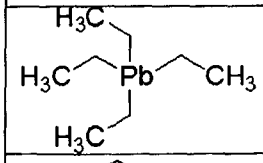
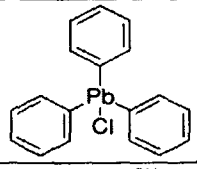
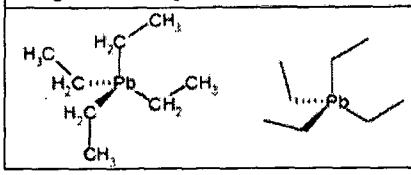
1.6.7 Role of pigments in metal detoxification

Pigmented bacteria are predominant in areas subjected to stress conditions such as high concentration of organic pollutants, heavy metals, drugs and high salt concentrations (Hermansson et al., 1987; Nair et al., 1992; Sun et al., 2006).The yellowish-green pyoverdine isolated from the bacteria *Pseudomonas chlororaphis* has significant role in triphenyltin (TPT) degradation (Yamaoka et al., 2002). *Pseudomonas aeruginosa* is known to secrete two chemically distinct iron chelators (siderophore pigment) viz. pyoverdine and pyochelin to solubilise Fe^{+3} and transport into the bacterial cells via specific receptors (Cox and Adams, 1985; Namiranian et al., 1997). Besides sequestering Fe^{+3} these microbial siderophores also form stable complexes with metals viz. Cd^{+2}, Pb^{+2}, and Zn^{+2} (Gilis et al., 1998; Hepinstall et al., 2005; Namiranian et al., 1997). Induction of bacterial siderophore synthesis in response to Cd^{+2}, Zn^{+2} and Cu^{+2} stress has also been reported which is responsible for detoxification of these heavy metals as a consequence of chelation (Clarke et al., 1987; Rossbach et al., 2000; Sinha and Mukherjee, 2008). Since microbial siderophores form stable metal–ligand complexes and influence the metal mobility in the environment thus prove to be an important strategy to sequester toxic heavy metals. Lead resistant *Pseudomonas vesicularis* and *Streptomyces* sp. showed red and red-brown pigmentations respectively in the presence of lead nitrate (Zanardini et al., 1998).

1.6.8 Biotransformation of organo-lead

Among the organo-lead compounds tetraethyl lead (TEL) and tetramethyl lead (TML) are the most stable and important because of their widespread use as an anti-knocking petrol additive. Due to use of leaded gasoline, lead particles are emitted in the atmosphere from automobile exhaust as lead halides. Organo-leads are very toxic in nature due to their mutagenic and teratogenic characteristics (Jarvie, 1988). In nature, tetra-alkyl lead compounds, such as tet-ethyl lead and tetra-methyl lead are subject to photolysis and volatilization. Degradation proceeds from tri-alkyl to di-alkyl species and eventually to inorganic lead oxides. Interestingly, some natural microorganisms are capable of degrading organo-leads using their biotransformation mechanism.Microbial consortia has also been reported to degrade tetra-ethyl lead in the soil (Teeling and Cypionka, 1997).

Table.1.3 Organo-lead compounds

Structure	Chemical Name	Chemical Formula
	Bromo-tri-methyl lead	C_3H_9BrPb
	Acetoxytri-methyl lead	$C_4H_{12}O_2Pb$
	Tetra-methyl lead	$C_4H_{12}Pb$
	Tri-phenyl lead cloride	$C_{18}H_{20}PbCl$
	Tetra-ethyl lead	$C_8H_{20}Pb$

1.7 Bioremediation of heavy metals

With rapid industrialization and urbanization enormous amount of industrial waste containing toxic heavy metals have been generated which need special treatment before they are released into the natural environment viz. terrestrial, aquatic and atmospheric environment. Bioremediation processes are cost effective, ecofriendly and highly efficient as compared to physicochemical methods for heavy metal removal. Therefore for last several decades metal resistant microorganisms including bacteria have been considered a potential alternative for clean up and bioremediation of heavy metal contaminated environmental sites. Many bioremediation technologies have been developed for detoxification and removal of toxic heavy metals from metal contaminated aquatic sites and industrial wastes employing various indigenous metal resistant bacteria from heavy metal contaminated sites (Francis and Tebo, 1999; Chen et al., 1999; Gutnick and Bach, 2000; Rathgeber et al., 2002; van Hullebusch et al,. 2003; Iyer et al., 2005; Morillo et al., 2006; De et al., 2007, 2008; Pal and Paul, 2008; Jayabarath et al., 2009; Wang et al., 2009).

1.8 Microbial sensors for monitoring heavy metals

Several whole cell bacterial bioreporters have been developed which serve as a convenient biological device for monitoring and quantifying bioavailable heavy metal contaminants in environmental samples with great accuracy, specificity and sensitivity (Table.4). Hynninen et al., 2010, have reported an improvement in the limit of detection of bacterial bioreporters by tinkering the natural metal transport systems of the host bacterium. The limit of detection of a *Pseudomonas putida* KT2440 based Zn/Cd/Pb biosensor was improved up to 45-fold by disrupting four main efflux transporters for Zn/Cd/Pb, thereby causing the metals to accumulate inside the cell. The specificity of the bioreporter may also be modified by changing the sensor element. A Zn-specific bioreporter was designed using

the promoter of gene *cadA1* from *P. putida* as a sensor element. The constructed transporter deficient *P. putida* reporter strain detected Zn^{2+} ions approximately 50 times lower than other available Zn bioreporters. The detection limit of this biosensor was significantly below the permitted limit for Zn and Pb in water and soil. Recently, it has been shown that a lead(II)-regulatory protein, PbrR691 from *Ralstonia* (or *Cupriavidus*) *metallidurans* CH34, binds lead(II) almost 1000-fold more selectively over other metal ions such as mercury(II), cadmium(II), zinc(II), cobalt(II), nickel(II), copper(I), and silver(I) (Chen et al., 2007). This regulatory protein can be used in development of lead specific biosensor. Despite the fact that several bioreporters have been constructed for measuring heavy metals, their applications to environmental samples have remained minimal.

Table. 1.4 Zn, Cd and Pb-sensing bioluminescent bacterial bioreporters and their detection limits

Bacterial Bioreporters	Limit of detection (μM)			References
	Pb^{+2}	Cd^{+2}	Zn^{+2}	
P. fluorescens (OS8::Kn cadRPcadA lux)	0.3	0.03	4	Ivask et al., 2009
E. coli MC1061 (pSLzntR/pDNPzntAlux)	0.7	0.01	5	Ivask et al., 2009
S. aureus RN4220 (pTO 024)	0.03	0.01	1	Tauriainen et al., 1998
P. putida KT2440 (pDNPczc1 lux)	0.41	0.49	0.08	Hynninen et al., 2010
P. putida KT2440.2431 (pDNPczc1 lux)	0.02	0.05	0.05	Hynninen et al., 2010
Pbr R691-based fluorescent probe	2	Not tested	Not tested	Chen et al., 2005

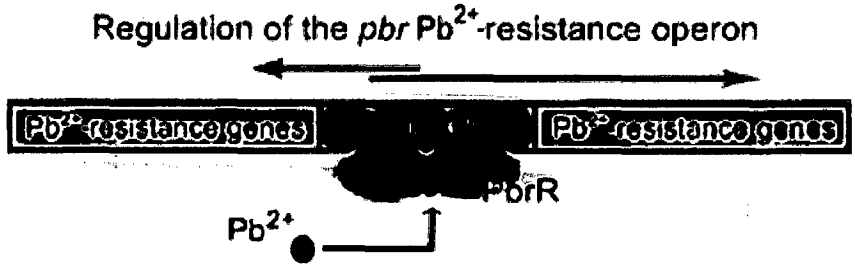


Fig.1.9 *PbrR* as lead sensing element in the development of lead biosensor

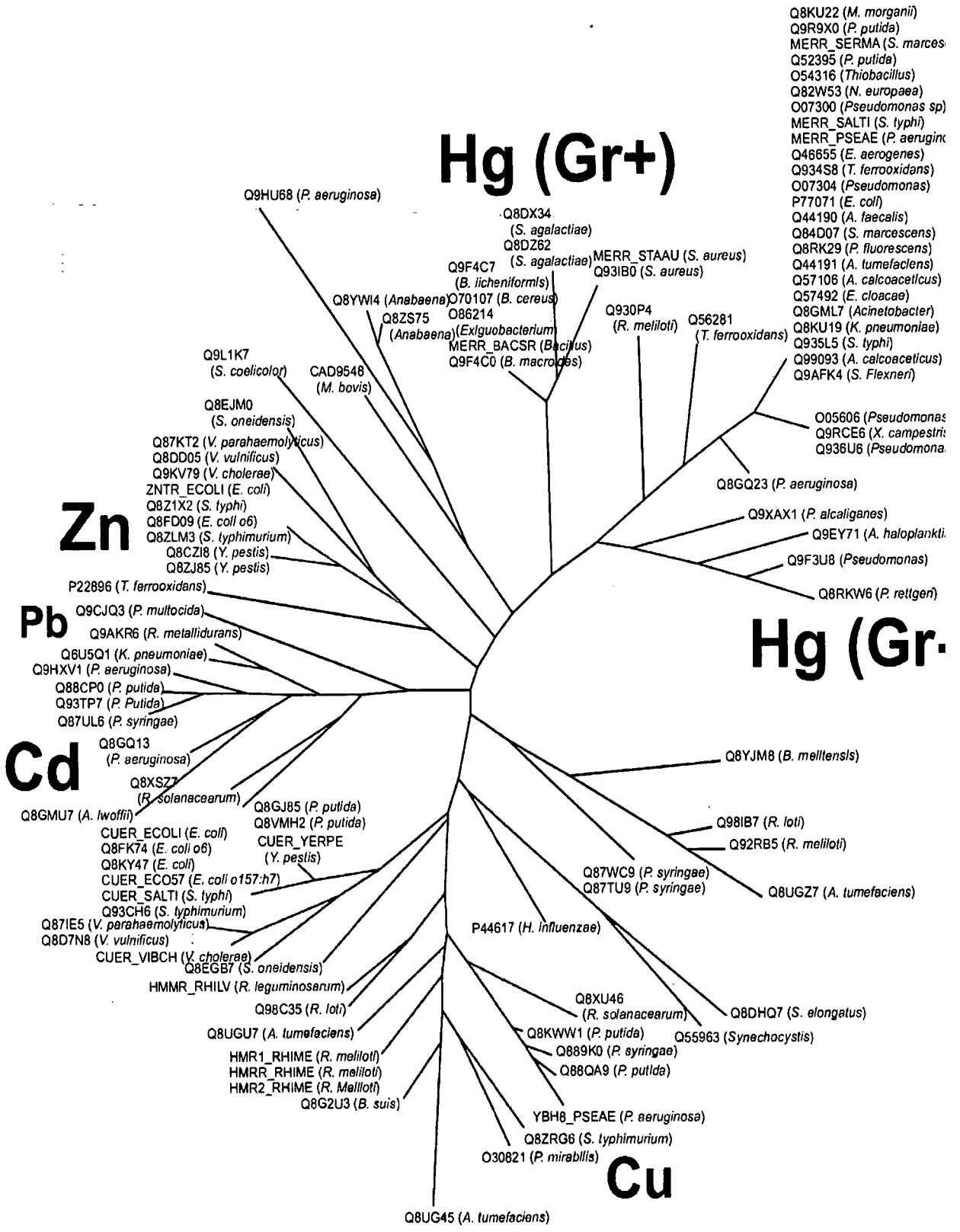


Fig.1.10 Phylogenetic tree of various metal sensing specific gene regulators (with gene accession no.) which may be used for biosensor development and metal specificity has been colour coded (Permin et al., 2006).

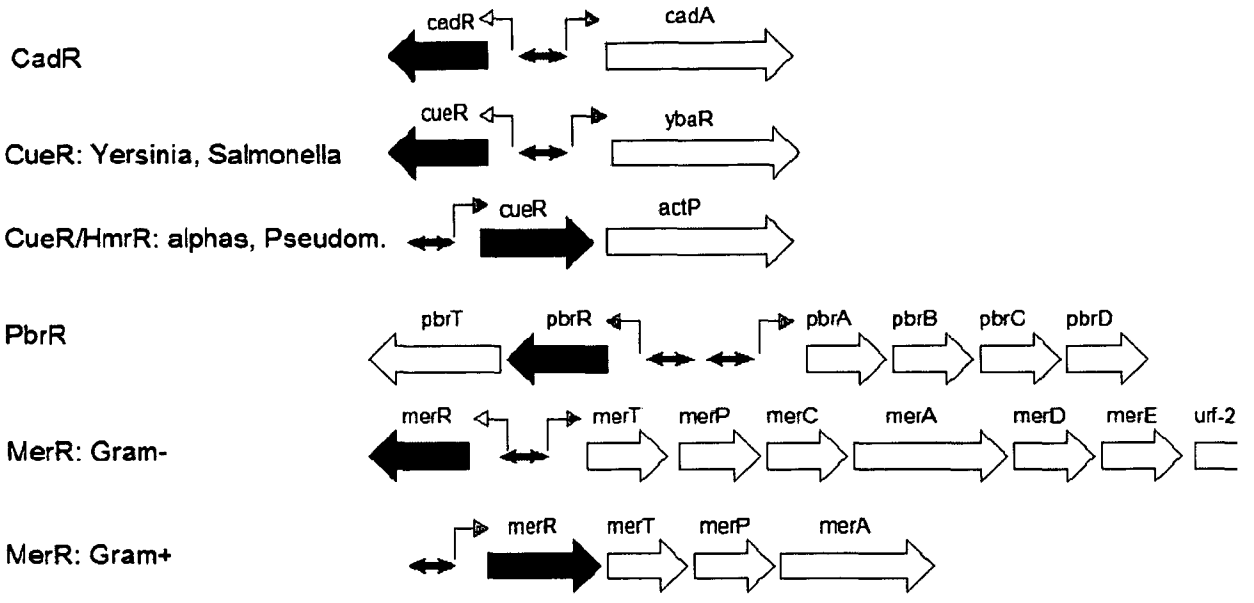


Fig.1.11 Operon organization of loci regulated by MerR, CadR/PbrR, CueR and ZntR. Red arrow indicates regulator gene whereas green double arrow indicates direction of transcription by RNA polymerase (Permin et al., 2006)

1.9 Association of metal resistance with multiple drug resistance

Heavy metal resistance in bacteria is closely associated with multiple antibiotic (drug) resistance as genes encoding antibiotic resistance and heavy metal resistance are often present on the same plasmids or transposons (Clark et al., 1977; Calomiris et al., 1984; Roane and Kellogg, 1996). Improper and extensive use of antibiotics and antimicrobial drugs has become a major environmental concern due to their persistence in the natural environment (Khetan and Collins, 2007) and a long term exposure of microbes to antibiotics may lead to antibiotic resistance conferred by genetic determinants (Martinez, 2008). Bacteria can resist multiple antibiotics along with various heavy metals viz. Zn, Cd, Pb and As employing multi-drug resistance (MDR)/metal resistance efflux pumps and also by horizontal gene transfer between different bacterial species (Martinez, 2006; Martinez, 2008). A good correlation

exists between the occurrence of antibiotic resistance and occurrence of metal tolerance (McArthur and Tuckfield, 2000).

1.10 Cross resistance in heavy metal resistant bacteria

Xanthomonas campestris showed cadmium-induced cross-resistance to lethal concentrations of Zn (Banjerdkij et al., 2003). Low level pre-exposure of *Xanthomonas campestris* to Cd induced high level of protection against Zn. Pretreatment of cadmium resistant *Ralstonia* sp.TAK1 with an inducing level of Cd conferred cross resistance against subsequent exposure to the lethal levels of Zn (Prapagdee and Watcharamusik, 2009). It is interesting to note that gene cluster, *czr is* involved in both cadmium and zinc resistance in *Pseudomonas aeruginosa* CMG103 (Hassan et al., 1999). Similarly Zn (II) /Cd (II) / Pb(II)-translocating P_{IB}-type ATPase encoded by gene *znt A* in *E. coli* and gene *cad A* in *Staphylococcus aureus* plasmid, pI258 confers resistance to Zn, Cd and Pb (Nies and Silver, 1995; Rensing et al., 1999). Pretreatment of *Pseudomonas aeruginosa* BC15 with sub-lethal concentrations of Cd induced adaptive resistance to lethal doses of Cd and cross resistance to lethal concentrations of zinc (Raja et al., 2008).

1.11 Organometal resistance in heavy metal resistant bacteria

Majority of TBT resistant bacteria are also resistant to Hg, Cd, Zn, Sn, Cu and Pb which suggests that resistance to these heavy metals may be associated with oraganotin resistance (Pain and Cooney, 1998). Bacteria isolated from fresh water and estuarine environment are resistant to Zinc as well as TBT (Wuertz et al., 1991). Organomercurial such as methyl-mercury is detoxified by microbial organo-mercurial lyase encoded by the gene *merB*, resulting in Hg^{+2} which is subsequently reduced to elemental mercury (Hg^0) by mercuric reductase enzyme encoded by gene *merA* (Osborn et al., 1997; Barkay et al., 2003).

Environmental pollution by heavy metals is caused by various industrial and agricultural activities. Heavy metals viz. lead, mercury and cadmium are used by several industries and are discharged as industrial effluents and wastes into natural water bodies and terrestrial environment resulting in contamination of these sites with these toxic heavy metals. These non-essential heavy metals are toxic to microorganisms along with macroorganisms inhibiting enzymatic activities, disrupting membrane functions and damaging nucleic acids (Trajanovska et al., 1997; Roane, 1999). At the community level, heavy metals may repress overall metabolic activities, biodiversity and population density of organisms (Sobolev and Begonia, 2008; Khan et al., 2009). Natural bacterial population plays a pivotal role in major elemental cycles. Since these heavy metal pollutants pose a serious threat to natural bacterial populations, the adaptive response of bacteria in the contaminated environment has been studied extensively. Investigations have revealed that bacterial strains survive and indeed flourish in such environments possess inherent genetic mechanisms to withstand high levels of heavy metals. Although there are several reports on heavy metal resistant bacteria, in general very little is known about lead resistant bacteria, their mechanism of resistance and their potential use as lead bioremediator in lead contaminated environmental sites. Lead resistant *Vibrio harveyi* is capable of precipitating lead as an unusual phosphate i.e. $Pb_9(PO_4)_6$ (Mire et al., 2004) where as *Ralstonia metallidurans* CH34 resists lead through its efflux outside the cell (Borremans et al., 2001; Taghavi et al., 2009; Hynninen et al., 2009) and a bacterial consortia degraded Tetra-ethyl lead in soil (Teeling, 1997). Similarly lead resistant *Arthrobacter* sp. and other firmicutes were isolated from the waste site of a lead battery manufacturing factory (Trajanovska et al. 1997), *Bacillus megaterium* showed intracellular lead accumulation and *Pseudomonas marginalis* showing extracellular lead exclusion were also isolated from lead contaminated soils (Roane, 1999). Pb(II) resistant strains of *Staphylococcus aureus* and *Citrobacter freundii* have also been isolated which accumulated

lead as intracellular lead-phosphate (Levinson and Mahler, 1998). Putative entrapment of lead and cadmium in extracellular polymeric substances secreted by *Pseudomonas aeruginosa* CHO7 as revealed by SEM-EDX and precipitation of lead as PbS by *Bacillus iodinium* GP13 (De et al., 2008) and *Klebsiella* sp. mediate precipitation of lead as PbS has also been reported (Aiking et al., 1985).

As lead, cadmium and mercury are present in different environments, remediation of these toxic metals is of great ecological concern. Bioremediation technology involving microbes is simple, cost effective, highly efficient and environmentally sustainable as compared to physico-chemical methods. For last several decades metal resistant microorganisms including bacteria have been considered a potential alternative for clean up and bioremediation of heavy metal contaminated environmental sites.

Prompted by these facts and information, I have made attempts to screen, identify and biologically characterize lead resistant bacterial strains from various lead contaminated econiches of Goa to explore their diverse biochemical or molecular resistance mechanisms. The broader context of this work is the application of genetic systems in biomonitoring of lead and potential use of the bacteria in bioremediation of lead from lead polluted environmental sites.

1.12 Main objectives of research

1) Screening and identification of lead (Pb^{+2}) resistant bacteria from terrestrial and estuarine econiches of Goa.

2) Physiological characterization of lead (Pb^{+2}) resistant bacterial strains with reference to:

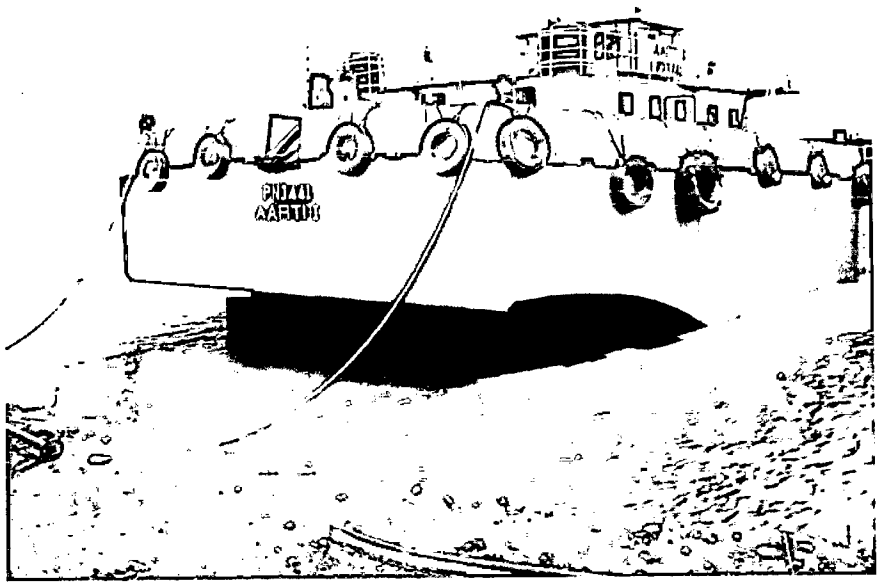
- Growth behavior in different medias and optimas (Temperature, pH, NaCl %, carbon sources).
- Lead (Pb^{+2}) tolerance limits and cross tolerance to other toxic metals (Hg^{+2}, Cd^{+2}, Zn^{+2}).
- EPS (Exopolysaccharide) production.
- Pigment production.
- Antibiotic resistance

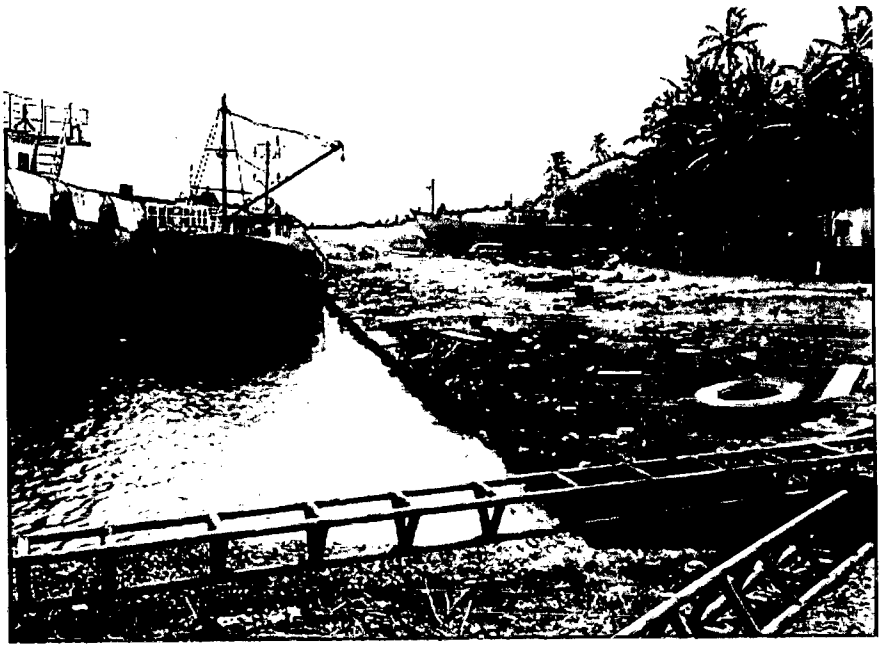
3) Biological characterization of lead (Pb^{+2}) resistant bacterial strains with reference to:

- SDS –PAGE profile of total proteins of lead resistant bacteria to explore lead induce proteins.

4) Genetic and molecular biological characterization of lead resistant bacterial isolates with reference to:

- Plasmid profile
- Localization of lead resistance conferring gene on plasmid or chromosomal genome.
- Screening of lead resistance encoding gene from plasmid /chromosomal genome of lead resistant bacterial isolates using PCR technology.





Sampling sites from Mandovi and Zuari estuary

CHAPTER-II

CHAPTER II

Screening, identification and physiological characterization of lead resistant bacteria from terrestrial and estuarine econiches of Goa

MATERIALS AND METHODS

2.1 Collection of environmental samples:

2.1.1 Sampling sites

(A) Terrestrial sites

Soil and liquid waste samples contaminated with lead were collected from different lead battery manufacturing companies and electronic industries from Goa, India (Table 2.1). Soil samples were collected from several solid waste dumping sites and liquid samples from various points of waste discharge. Samples were collected in sterile polycarbonate bottles and used within 24 hrs of collection for physicochemical and bacteriological analysis.

(B) Estuarine sites

Surface water samples were collected from different sites of Mandovi and Zuari estuaries such as ship building areas, vicinity of ship painting yards and ships along with other estuarine sites. All samples were collected in sterile polycarbonate bottles and used within 24 hours of collection for physicochemical and bacteriological analysis. The bottles containing water samples were mechanically shaken prior to use and kept for 10 minutes to allow the heavy particles to settle down. Physicochemical analysis of water samples was done to determine pH (using digital pH meter), temperature (using mercury thermometer) and salinity (using salinometer) as per the standard procedures. The appropriate volume (100 µl-10 ml) of water sample was taken for physiochemical and bacteriological analysis.

2.1.2 Detail of sampling sites

In the present study 10 sampling sites were selected for collecting water samples from Mandovi and Zuari estuaries of Goa. Zuari estuary includes sampling sites viz. WISL (Western India shipyard ltd.), GSL (Goa shipyard ltd.), Manmad shipyard, Dona Paula and Kakra beach. Whereas sampling sites of Mandovi estuary include Kala academy jetty, Ferry point at Panjim, below Mandovi Bridge, Ribandar ferry point and Dempo shipyard (Fig.2.1).

Table 2.1 Terrestrial sampling sites

S.N.	Sampling sites
	Car battery manufacturing industries
1.	Germania batteries Pvt. Ltd. Corlim, Goa
2.	Permalite batteries Pvt. Ltd. Corlim, Goa
3.	Shrine industries Pvt. Ltd. Kakoda, Quepem, Goa
4.	United Lead oxide Pvt. Ltd. Kundaim, Goa
	Electronics industries
1.	Bharti electronics Pvt. Ltd. Mapusa, Goa
2.	D-Link, Verna, Goa

2.2 XRD analysis of soil samples from car battery waste

X ray diffraction analysis of soil samples contaminated with lead from waste dump of lead battery industries was done to analyze various forms of lead present in these contaminated soil samples. Soil was dried, grounded and smeared on glass slide pre-coated with ultra-thin layer of petroleum jelly. Scintag X3 diffractometer coupled with Cu K radiation and Li-drifted germanium detector were used in this study and data was collected at a speed of 2 degrees/min. XRD data was analysed using library supplied by Joint Commission on Powder Diffraction Software (JCPDS) and earlier reports (Dermatas et al., 2004; Vantelon et al., 2005; Nadagouda et al., 2009).

2.3 Atomic absorption spectroscopic (AAS) analysis of soil samples

Lead content of soil samples was analysed using AAS (Varian AA240 FS, Australia) (Khan et al., 2010). One gram of dried and powdered soil sample was taken into Teflon beaker and 15 ml of aqua regia was added. The sample was kept overnight followed by heating on the hot plate until no brown fumes were produced. Concentrated $HClO_4$ (5 ml) was added to the sample and heated again until the solution was evaporated near to dryness. Samples were diluted using appropriate volume of deionised ultrapure water and analysed by AAS.

2.4 Isolation of lead resistant bacteria

2.4.1 Soil and liquid waste samples from lead battery manufacturing company

Isolation of lead-resistant bacterial strain was done from soil and liquid waste samples contaminated with car battery waste from battery manufacturing and electronics industries of Goa, India. These samples were plated on PYT80 agar (appendix A.4) plates amended with 100 µM lead nitrate by dilution plating technique (Konopka et al., 1999). Filter sterilized lead nitrate (1M stock) was amended to the PYT80 agar medium after sterilization. Discrete bacterial colonies were further spot inoculated on fresh PYT80 agar plates with different concentrations of lead nitrate and the bacterial colony which appeared at highest concentration of lead nitrate was selected for further characterization. Viable count of lead resistant bacteria was determined as colony forming units (cfu/ml).

2.4.2 Water samples from estuaries

Isolation of lead-resistant bacterial strain was also done using surface water samples from various sampling sites of Mandovi and Zuari estuaries Goa, India. Water samples were plated on Zobell marine agar (Appendix A.3) plates amended with 500 µM lead nitrate by dilution plating technique (Zobell, 1941). Filter sterilized lead nitrate (1 M stock) was amended to the Zobell marine agar after sterilization. The isolated bacterial colonies which appeared

were further spot inoculated on fresh Zobell marine agar plates with different concentrations of lead nitrate and the bacterial colony which appeared at highest concentration of lead nitrate was selected for further characterization. Viable count of lead resistant bacteria was also determined as colony forming units (cfu/ml).

2.5 Determination of environmental optimas (pH, temperature, salinity and carbon sources) for growth of lead resistant bacteria

2.5.1 Carbon source

Several carbon sources viz. glucose, gluconate, fructose, succinate, galactose, xylose, malonate, ribose, maltose, mannose, rhamnose and sucrose were tested in Tris-buffered minimal medium amended with 100 µM lead nitrate in order to select best carbon source required for lead resistance. Overnight grown culture was inoculated in fresh growth media (pH 7) to a starting optical density of 0.02 at 600 nm. Bacterial isolates were grown in the presence of different carbon sources (0.4 %) at 30°C , 150 rpm and growth was monitored every 2 hrs by measuring absorbance at 600 nm.

2.5.2 pH

Bacterial growth at various pH values from 5.5 to 8.5 were tested to investigate best pH required by the lead resistant bacterial isolate for growth and lead resistance in Tris-minimal media amended with 100 µM lead nitrate. Bacterial isolates were grown at different pH values at 30°C and 150 rpm. Here 0.4% glucose was used as carbon source and growth was monitored every 2 hrs by measuring absorbance at 600 nm (O.D).

2.5.3 Temperature

Bacterial growth at various temperatures ranging from 20-43°C were tested to investigate best temperature required by the lead resistant bacterial isolates for growth and lead resistance in Tris- minimal media amended with 100 µM lead nitrate. Here 0.4% glucose was

used as carbon source and growth was monitored every 2 hrs with constant shaking at 150 rpm by measuring absorbance at 600 nm (O.D).

2.5.4 Salinity (as % NaCl)

Bacterial growth at various NaCl% ranging from 0.5-3.5% were tested to investigate best NaCl% required by the lead resistant bacteria for growth and lead resistance in Tris-minimal media amended with 100 µM lead nitrate. Bacterial isolates were grown at different NaCl% at pH 7.0 and 30°C with constant shaking at150 rpm. Here 0.4% glucose was used as carbon source and growth was monitored every 2 hrs by measuring absorbance at 600 nm.

2.6 Lead tolerance in Tris-minimal media

Total metal concentration does not necessarily reflect the amount of metal biologically toxic or bioavailable. Thought to be primarily toxic in their free ionic form, soluble metal can more readily penetrate cellular membranes (Roane, 1999; Pike et al., 2002). Complex media precipitates lead and bioavailability of lead decreases therefore minimal media without any organic ingredient or precipitating agent is mostly used. Complex media viz. Nutrient broth, Luria bertani broth and Zobell marine broth (Appendix A2, A3 and A7) gave white precipitation when lead nitrate is added, probably due to complexation of Pb^{+2} to organic components and phosphate ions in the medium therefore growth behaviour and lead resistance limit of the selected lead-resistant bacterial isolates was studied in Tris-minimal medium (TMM) (Mergeay et al., 1985) (Appendix A.1.), supplemented with different concentrations of lead nitrate at optimum temperature, pH and NaCl% required for growth and lead resistance with constant shaking at 150 rpm. Absorbance of the culture suspension was recorded at definite time intervals as O.D at 600 nm using UV–Vis spectrophotometer (Shimadzu, UV-2450, Japan) and graph was plotted between absorbance and time interval. Slight modification in Tris-minimal medium was done by using 0.05% β-glycerol phosphate instead of inorganic

phosphate in order to avoid lead precipitation. **I have used $Pb(NO_3)_2$, $CdCl_2$, $ZnCl_2$ and $HgCl_2$ in heavy metal resistance study since these are the most soluble salts of lead, cadmium, zinc and mercury respectively and are most toxic. Most soluble metal salts (bioavailable) give the real picture of metal resistance concentration, since soluble metals are easily bio-available.** MIC values of lead, mercury, cadmium and zinc were also recorded. The lead resistant isolates were then maintained on TMM supplemented with 100 µM lead nitrate to avoid bacterial contamination and preserved at 4°C in cold room and sub cultured every month.

2.7 Identification of lead resistant bacterial isolates

Identification of the selected lead-resistant bacterial isolates was done based on morphological and biochemical characteristics following Bergey's manual of systematic bacteriology (Krieg and Holt, 1984) (Appendix B1 and C).Further confirmation was done by FAME (Fatty acid methyl ester) analysis (Sherlock version 6.0B) and 16S rDNA sequencing followed by BLAST search (Altschul et al., 1997).

2.7.1 Identification based on morphological and biochemical characteristics

Morphological characterization of bacterial isolates is based on colony morphology, pigmentation and motility. Further identification of isolates is based on biochemical tests such as gram staining which reveals cell wall composition and oxidative-fermentative test determines whether organism is oxidative, fermentative or facultative anaerobe. Carbohydrate fermentation test confirms fermentative utilization of sugars with production of acid and gas. Methyl Red test detects formation of large quantities of acid in the medium resulting from fermentation of glucose and Voges Proskauer's test detects the production of non-acidic or neutral end products i.e. butanediol and acetoin. Citrate utilization test determines that a bacterium can use citrate as the sole carbon source and indole production test determines ability of bacteria to convert tryptophan into indole. Production of several microbial enzymes viz. urease, amylase, catalase, gelatinase, oxidase and nitrate reductase by the test bacterial isolates was also determined (Krieg and Holt, 1984).

2.7.2 Identification of the isolates based on 16S rDNA Sequence

16S rDNA of the bacterial isolates was PCR amplified as per the standard molecular biological procedure using genomic DNA of isolates as template and following eubacterial primers: 8F (5'-AGAGTTTGATCCTGGCTCAG-3')

1492R (5'-ACGGCTACCTTGTTACGACTT-3')

PCR amplification reaction was performed using PCR amplification kit (Bangalore Genei, India) and sequencing was done at Xcelris laboratories, Ahmedabad, Gujarat, India. 16S rDNA sequence of bacterial isolates was compared with 16 S rDNA data base of GenBank using NCBI- BLAST search (Altschul et al. 1997).

2.7.3 Identification of isolates based on Fatty acid methyl ester (FAME) analysis

Bacterial isolates were grown on trypticase soy agar (TSA) at their optimum growth conditions. Whole cell fatty acids were extracted from cell material according to the MIDI protocol (Sasser, 1990). Overnight grown bacterial culture was taken (approx. 40 mg pellet) in a clean screw capped glass tube and 1 ml of Reagent I (45 gm NaOH + 150 ml CH_3OH+ 150 ml d/w) was added to it. The tube was sealed with teflon lined screw caps, vortexed briefly and heated in a boiling water bath for 5 minutes. The tube was vigorously vortexed for 5-10 seconds and returned to the water bath (100°C) to complete the 30 minute heating (Saponification step). The tube was cooled uncapped and 2 ml of Reagent II was added (325 ml of 6N HCl + 275 ml CH_3OH). The tube was capped again and briefly vortexed. After vortexing, the tube was heated for 10 minutes at 80° C. (This methylation step is critical with time and temperature). Addition of 1.25 ml of Reagent III (200 ml hexane + 200 ml methyl tertbutyl ether) to the cooled tube was followed by recapping and gentle tumbling on a clinical rotator for about 10 minutes. The tube was uncapped again and the aqueous (lower) phase was pipetted out and discarded (extraction step). About 3 ml of Reagent IV (10.8 gm NaOH + 900 ml d/w) was added to the organic phase remaining in the tube, the tube was recapped, and

tumbled for 5 minutes. Following uncapping, about 2/3 of the organic phase was pipetted into a GC vial which was capped and ready for analysis. Gas chromatographic analysis was performed on a GC Sherlock microbial identification system (New York, USA) fitted with cross-linked methyl silicon fused capillary column (25 m, 0.2 mm i.d.), flame ionization detector (FID) and a sampler. Helium was used as carrier gas. The sample was injected at oven temperature of 50°C. After 1 min, the oven temperature was raised to 170°C at the rate of 30°C/min and then to 270°C at the rate of 2°C/min and finally to 300°C at 5°C/min.

2.8 Antibiotic susceptibility test

Antibiotic susceptibility test of lead resistant bacterial isolates was performed following Kirby–Bauer disc diffusion method (Bauer et al., 1966) using Muller–Hinton agar (Appendix A6) and antibiotic discs (Himedia, India).Various antibiotics tested are as follows: chloramphenicol (C), gentamycin (G), clindamycin (Cd), erythromycin (E), ampicillin (A), vancomycin (Va), oxacillin (Ox), cephalothin (Ch), co-trimoxazole (Co), bacitracin (B), penicillin G (P), tetracycline (T), neomycin (N), polymyxin B (Pb), nitrofurantoin (Nf) , tobramycin (Tb), kanamycin (K), sulphatriad (SI), colistin methane sulphonate (Cl), methicillin (M), lincomycin (L), oleondamycin (Ol), amikacin (AK), streptomycin (S), nalidixic acid (Na), cephalexin (Cp), ciprofloxacin (Cf), norfloxacin (Nx), cephaloridine (Cr) and mecillinam (Mc). Diameter of zone of inhibition of test isolates caused by individual antibiotics was compared with standard chart (Himedia) and based on these results resistance or sensitivity of bacterial isolates to the tested antibiotics was determined.

RESULTS AND DISCUSSION

The coastal region of Goa is seriously affected due to extensive mining operations, ship building and shipping activities, disposal of industrial effluents and solid wastes and various other natural biogeochemical and anthropogenic activities. Ten major mines are located in the Zuari basin of Goa generating 1,000–4,000 tons of mine rejects/day/mine (Dessai and Nayak, 2009).Goa shipyard ltd. (GSL) and Western India shipyard ltd. (WISL) situated at Vasco-da-gama, Goa are among the biggest shipyards in the west coast of India, which substantially contribute several heavy metal and organometal pollutants in the surrounding estuarine and marine environment of Goa. The environmental pollutants generated by Goan mines and shipyards include heavy metals viz. Fe, Mn, Hg, Cr, As, Pb, Zn, Sn, and organometals such as Tributyltin (TBT) and Dibutyltin (DBT) (Meena et al., 2003; Alagarsamy, 2006; Bhosle, 2007; Dessai and Nayak , 2009; Turner, 2010; Atri and Kerkar, 2011). These environmental pollutants often show critical properties such as toxicity, high level persistence in the environment and non-biodegradability (Tong et al., 2000; De et al., 2007, 2008; Dauvin, 2008; Lombardi et al., 2010; Jayaraju et al., 2011).

Toxic heavy metals viz. Pb, Hg and Cd released in the solid waste and industrial effluents find their way ultimately to the aquatic and terrestrial environment and pose a serious threat to survival and metabolic activities of natural biota including microbial flora (Nies, 1999; Watt et al., 2000; Tong et al., 2000; Hartwig et al., 2002; Coombs and Barkay, 2004; Lam et al., 2007; De et al., 2007, 2008; Velea et al., 2009; Khan et al., 2010; Jayaraju et al., 2011). Lead polluted environmental sites need special attention since lead is a potential cumulative poison (pollutant) showing high toxicity, persistence and bioaccumulation in the environment (Trajanovska et al., 1997; Lam et al., 2007; Dauvin, 2008; Flora et al., 2008; Lombardi et al., 2010).

2.9 Physicochemical characteristics of estuarine water samples

Physicochemical analysis of estuarine water samples clearly revealed variation in pH ranging from 7.7 to 8.2, temperature from 28^0 C to 31^0 C and salinity from 13.16 PSU to 32.23 PSU respectively at different sampling points (Table 2.2). It is interesting to note that pH of estuarine water samples was alkaline and maximum alkalinity i.e. 8.2 was observed for water samples collected from GSL and WISL situated at Vasco since these sites are close to open sea. Similarly salinity was also significantly high for these samples (i.e. 30.33-32.23 PSU).

Table 2.2 Physicochemical characteristics of estuarine water samples

Sr. No.	Sampling stations	pH	Temperature (°C)	Salinity (PSU)
1	Kala academy jetty	7.8	28	24.43
2	Ferry point, Panjim	7.8	30	17.79
3	Below Mandovi bridge	7.8	30	17.88
4	Ribandar ferry point	7.8	30	15.35
5	Dempo shipyard ltd.	7.7	28	13.16
6	Kakra beach	7.7	31	26.12
7	Dona Paula	7.9	29	28.37
8	Western India Shipyard ltd.	8.2	30	32.23
9	Goa shipyard ltd.	8.2	30	30.30
10	Manmad shipyard ltd.	7.8	28	22.41

2.10 Lead content of soil samples

AAS analysis of soil samples revealed that soil samples from waste dump of battery manufacturing companies were highly contaminated with lead. Soil sample from Germania batteries contained 7,767 ppm lead whereas sample from Permalite batteries contained 5,497 ppm lead. Frequent exposure of bacteria to toxic pollutants such as Cd, Zn and lead in environments induce stress response proteins in bacteria (Laplase et al., 2000; Blindauer et al.,

2002; Liu et al., 2003; Sharma et al., 2006; Lacerda et al., 2007; Taghavi et al., 2009) therefore high concentrations of lead in soil impart stress on natural bacterial population which result in evolution of genetic determinants responsible for high level of lead resistance in terrestrial bacterial isolates.

2.11 Lead compounds in terrestrial (soil) samples contaminated with lead

XRD analysis of soil samples (solid waste) from Germania batteries Pvt. ltd. Corlim, Goa and M/S Permalite batteries Pvt ltd. Corlim, Goa clearly revealed presence of several lead compounds viz. litharge (PbO), cerussite ($PbCO_3$), hydrocerussite ($PbCO_3$-$PbOH_2$), angelsite ($PbSO_4$), lead phosphate ($Pb_5(PO_4)_3Cl$) and metallic or free lead (Fig. 2.2 and 2.3). XRD analysis of terrestrial samples conducted earlier goes hand in hand with our report (Dermatas et al., 2004; Vantelon et al., 2005; Nadagouda et al., 2009). Frequent exposure of bacterial isolates to these lead compounds and free lead ions in soil may induce stress responsive genes which encode Pb induced proteins responsible for high level lead resistance (Laplase et al., 2000; Blindauer et al., 2002; Bruins et al., 2003; Sharma et al., 2006, Taghavi et al., 2009).

2.12 Viable count of bacteria

2.12.1 Viable count of bacteria in estuarine water samples

The total viable count of lead resistant bacteria of all the water samples from Zuari estuary ranged from 1.6-3.7 $\times$ 10^3 cfu/ml when water samples were plated on Zobell marine agar amended with 500 µM lead nitrate whereas viable count of Mandovi estuary ranged from 0.43-3.61$\times 10^3$ cfu/ml (Tables 2.3, 2.4).These studies clearly demonstrated that approximately 3.72 % and 2.54 % of natural bacterial population from Zuari estuary and Mandovi estuary is resistant to lead nitrate due to appreciably high levels of lead contributed by mining, industrial and other anthropogenic activities. Exposure of bacterial isolates of estuarine environment to

high levels of lead activates these microbes to evolve resistance mechanisms to withstand the metal stress.

2.12.2 Viable count of bacteria in terrestrial samples

Viable count of lead resistant bacteria from waste sample of various battery manufacturing industries on PYT80 agar plates containing 100 μM lead nitrate ranged from 0.38-2.25 × 10^3 cfu/ml for soil samples whereas 0.86-3.16×10^3 for liquid waste samples. Waste sample from two electronics companies showed viable count ranging from 0.046 to 0.42 × 10^3 cfu/ml for soil samples and 0.01-0.16 × 10^3 cfu/ml for liquid waste (effluent) samples (Table 2.5.). This clearly revealed that several lead resistant bacterial isolates are present in the waste samples of car battery manufacturing and electronics industries as they may have inherent genetic and biochemical mechanisms to cope up high levels of lead. Various strategies through which they resist high levels of lead along with other heavy metals may include efflux, reduction, oxidation, extracellular sequestration, biosoption, precipitation and intracellular bioaccumulation (Trajanovska et al., 1997; Levinson and Mahler, 1998; Nies, 1999; Roane, 1999; Blindauer et al., 2002; De et al., 2007, 2008; Desai et al., 2008; Taghavi et al., 2009; Wang et al., 2009; Maldonado et al., 2010).

2.13 Environmental optimas for growth of lead resistant bacterial strains

Six lead resistant bacterial strains were selected for further study and designated as P2B, 4EA and 2EA (terrestrial isolates) and WI-1, M-9 and M-11 (estuarine isolates).

2.13.1 Optimum temperature

The temperature is an important factor to which bacteria show a wide pattern on growth behaviour (Gikas et al., 2009). Studies have revealed that optimum temperature of bacterial strains P2B and 4EA for growth in presence of lead ranged from 30°C to 34°C, for isolate

2EA 34°C whereas estuarine isolates WI-1, M-9 and M-11 showed best growth at 30°C (Figs. 2.4 a-f).

2.13.2 Optimum pH

Optimum pH for growth in the presence of lead for bacterial strains P2B, 2EA and 4EA is pH 7.0 whereas estuarine strains WI-1, M-9 and M-11 grew well at pH range 7-7.5 (Figs. 2.5 a-f). Estuarine isolates grow best at neutral to alkaline pH since pH of estuarine water samples was alkaline. Terrestrial isolates grew best in the presence of lead nitrate (100 μM) at neutral pH but growth of terrestrial as well as estuarine bacterial isolates was repressed at acidic pH (<7) because at acidic pH lead is more soluble and bioavailable to the bacterial cells (Babich and Stotzky, 1979; Harrison and Laxen, 1981; Scoullos, 1986; Dubey and Rai, 1990). Interestingly not much adverse effect on growth of bacterial strains in presence of lead nitrate was observed at alkaline pH (>7.5) because under alkaline conditions bioavailability of lead ions to the bacterial cells is less as compared to acidic conditions (Cunningham et al., 2010). Thus it is evident from these experiments that lead is cytotoxic at acidic pH and it is interesting to note that reduction in pH of medium results in enhanced lead toxicity possibly due to increased bioavailability of lead cation in the medium.

2.13.3 Optimum salinity (as % NaCl)

Sodium chloride level in the environment is the major contributor to the osmotic effect of ions on the growth of microorganisms. Optimum NaCl levels for growth in presence of lead nitrate (100 μM) for bacterial strains P2B and 4EA was 1%, for 2EA 0.5%, for WI-1 and M-9 1.5 %, whereas for M-11 ranged from 1.5 to 2 % (Figs. 2.6 a - f). These estuarine bacterial strains required more sodium chloride for growth in the presence of lead than terrestrial strains, because estuarine sites from which we have isolated bacteria, salinity of water samples ranged from 13.16-32.23 PSU (1.3-3.2 %) which is quite high. Amelioration of

metal toxicity in the presence of NaCl could possibly be due to formation of anionic co-ordination with Cl^-, which could be comparatively less toxic to microbes than the free metal cations (Babich and Stotzk, 1978; Dubey and Rai, 1990).

2.13.4 Carbon sources

Among various carbon sources tested viz. glucose, gluconate, fructose, succinate, galactose, xylose, malonate, ribose, maltose, mannose, rhamnose and sucrose the best carbon source for growth in presence of lead for all the strains was found to be glucose (Fig.2.7. a-f).This clearly indicated that glucose is metabolized faster as compared to other carbon sources and protects the strains against lead toxicity possibly better than others. Glucose is very simple sugar and most of the bacteria in environment can utilise easily either by oxidative, fermentative or both processes. Enzymes involved in glucose catabolism of all bacterial strains are constitutively expressed (Mandelstam, 1962). Under stress conditions viz. oxidative, salt and heavy metal stress bacterial and cyanobacterial strains prefer glucose more than any other carbon source (Pickett and Dean, 1976; Rai and Dubey, 1988; Brynhildsen et al., 1988). In a similar experiment *chlorella vulgaris* exposed to cadmium showed significant protective effect by glucose (El-Naggar et al., 1998). In the present investigation all the lead resistant bacterial strains have also showed preference for glucose as compared to other carbon sources viz. fructose, succinate, galactose, gluconate, xylose, malonate, ribose, maltose, mannose, rhamnose and sucrose (Fig.2.7. a-f). **Glucose (0.4%) was optimum concentration for the growth of lead resistant isolates whereas glucose above 0.4% caused precipitation of lead in the growth medium (Fig. 2.24).Only bioavailable heavy metal exert toxic effect on bacterial cells and not sequestered metals.**

2.14 Lead tolerance of the bacterial strains in Tris-minimal medium (TMM).

Tris-minimal medium is suitable for lead resistance studies as compared to other complex media viz. NB, ZMB and LB since there is no complex organic component in this medium to influence the lead bioavailability and toxicity (Mergeay et al., 1985; Roane, 1999,

Pike et al., 2002). Complex media such as NB, ZMB and LB precipitate lead as a result of sequestration with organic moieties thus reduce the bioavailability of lead to the bacterial strains. Nutrient broth, Luria bertani broth and Zobell marine broth gave white precipitate when lead nitrate was added, probably due to complexation of Pb^{+2} ions to organic components and phosphate ions in the medium. Therefore TMM with β-glycerol phosphate and glucose (0.4 %) was used for lead resistance studies in order to avoid precipitation of lead and maintain lead availability in this medium at maximum level.

Bacterial strains M-9 and M-11 resist lead nitrate up to 0.8 and 1.2 mM with MIC values of 1mM and 1.4 mM respectively (Figs. 2.8, 2.9). MIC of cadmium and mercury for M-9 was 0.6 mM and 0.03 mM, whereas for M-11, 0.6 mM and 0.05 mM respectively (Fig. 2.10). These strains resist high levels of lead but very low levels of cadmium and mercury since they are more sensitive to these toxic metals as compared to lead (Osborn et al 1997; Nies, 1999; Roane, 1999; Roane and Pepper, 1999; Naz et al., 2005; De et al., 2007, 2008; Taghavi et al., 2009). Lead resistant strain P2B was resistant to 1.6 mM lead nitrate with MIC of 1.8 mM (Fig. 2.11.). It also showed tolerance to $CdCl_2$, and $HgCl_2$ as MIC for cadmium chloride, and mercuric chloride was 0.2 mM and 0.03 mM respectively but in the presence of 100 µM lead nitrate MIC for cadmium and mercury increased to 0.3 mM and 0.05 mM respectively exhibiting enhanced cross tolerance to these toxic metals (Figs. 2.12, 2.13). Similarly strain 4EA showed resistance up to 0.8 mM lead nitrate in TMM with MIC of 1 mM (Fig. 2.14.). In addition to lead resistance this strain also showed enhanced cross tolerance to $ZnCl_2$, $CdCl_2$, and $HgCl_2$ as MIC for zinc chloride, cadmium chloride, and mercuric chloride increased from 0.4, 0.2 and 0.04 mM to 0.7, 0.4, and 0.06 mM respectively in presence of 100 µM lead nitrate (Figs. 2.15, 2.16). MIC of cadmium and mercury for strains P2B and 4EA increased in the presence of 100 µM lead nitrate in the culture medium which may be due to lead induced cross tolerance to other toxic metals. Similar results were showed by *Xanthomonas campestris*

wherein cadmium-induced cross-resistance to lethal concentrations of Zn (Banjerdkij et al., 2003). Low level pre-exposure of *Xanthomonas campestris* to Cd induced high level of protection against Zn. Pretreatment of cadmium resistant *Ralstonia* sp.TAK1 with an inducing level of Cd conferred cross resistance against subsequent exposure to the lethal levels of Zn (Prapagdee and Watcharamusik, 2009). Pretreatment of *Pseudomonas aeruginosa* BC15 with sub-lethal concentrations of Cd induced adaptive resistance to lethal doses of Cd and cross resistance to lethal concentrations of zinc (Raja et al., 2008).

Bacterial strain 2EA showed resistance to lead upto 1.4 mM with MIC of 1.5 mM (Fig. 2.17.). This bacterial strain also showed tolerance to cadmium, mercury and zinc with MIC values 0.2 mM, 30 µM and 0.5 mM respectively (Fig. 2.18). Similarly strain WI-1 resists 0.6 mM lead nitrate with MIC value of 0.8 mM (Fig. 2.19) and tolerates $CdCl_2$ and $HgCl_2$ as their MIC values were 0.5 mM and 0.03 mM respectively (Fig.2.20). These results clearly demonstrated that lead resistant bacterial strains also tolerate cadmium, zinc and mercury possibly due to presence of genetic determinants (genes) on their chromosomal or plasmid genomes which confer metal resistance. Genome of *Pseudomonas putida* has got 61 open reading frames with different types of metal resistance genes which makes this strain resistant to high levels of Cd, Hg, As, Pb, Zn, Ni,Cu etc.(Canovas et al., 2003). Detoxification of toxic heavy metals viz Cd and Pb by marine bacteria highly resistant to mercury was reported by De et al., 2008.

It is well known that majority of antifouling paints possess several organic biocides along with heavy metals (Sanchez-Bayo and Goka, 2005; Turner, 2010) and Mandovi estuary is heavily contaminated with organotin biocide, TBT and several heavy metals viz. Pb, As, Mn, Co, Zn, Cu and Fe (Meena et al., 2003; Bhosle, 2007; Alagarsamy, 2006). Majority of TBT resistant bacteria are also known to resist Hg, Cd, Zn, Sn, Cu and Pb (Pain and Cooney, 1998; Ramachandran, 2009). In the present study lead resistant strain WI-1 also showed

tolerance to TBT in ZMB (MIC-0.5 mM) along with cadmium and mercury which revealed that resistance to these heavy metals may be associated with TBT (organotin) resistance. Some more lead resistant bacterial strain from terrestrial sites contaminated with lead battery waste viz. 4A, GM-10, GM02, GM03 and GM04 also showed tolerance to lead nitrate upto 0.2 mM, 0.4 mM, 0.2 mM, 0.6 mM and 0.3 mM respectively (Fig. 2.21) as their MIC values were 0.3 mM, 0.5 mM, 0.3 mM, 0.7 mM and 0.4 mM respectively.

2.15 Antibiotic resistance in lead resistant bacterial strains

Table 2.6 Multi-drug resistance in lead resistant bacterial strains

Antibiotics / Bacteria strains	A 25 µg	C 25 µg	Nx 10 µg	Co 25 µg	Cp 30 µg	Na 30 µg	E 15 µg	Tb 10 µg	K 30 µg	AK 10 µg	Ol 15 µg	Cr 30 µg	Mc 33 µg	L 10 µg	G 10 µg
P2B	+	+	-	+	+	-	-	+	-	-	+	+	+	+	-
4EA	+	-	-	+	+	+	+	-	-	+	+	+	+	+	-
WI1	+	-	-	+	+	-	+	+	+	+	-	+	+	+	-
M-9	+	+	+	+	+	-	+	-	+	-	-	+	+	+	-
M11	+	+	-	+	+	+	+	-	+	-	+	+	+	+	-
2EA	+	-	-	+	+	-	+	-	-	+	+	+	-	-	-

Antibiotics / Bacterial strains	Cf 30 µg	S 10 µg	M 25 µg	Sl 200 µg	Va 30 µg	Ox 15 µg	Cd 10 µg	P 10 units	Pb 300 units	Nf 30 µg	T 30 µg	Cl 25 µg	Ch 25 µg	N 30 µg	B 30 µg
P2B	-	+	+	-	+	+	+	+	-	-	-	-	+	+	+
4EA	-	-	+	-	+	+	+	+	-	+	+	-	+	-	+
WI1	-	-	+	-	+	+	+	+	-	-	-	-	+	-	+
M-9	-	-	+	+	+	+	+	+	-	-	+	+	+	-	+
M-11	-	-	+	+	+	+	+	+	-	-	-	-	+	-	+
2EA	-	+	+	-	+	+	+	+	+	-	-	-	+	-	+

Key: Resistant (+) Sensitive (-)

Uncontrolled, haphazard and extensive use of antibiotics and anti-microbial drugs in hospitals, aquaculture farms prawn hatcheries and poultry farms has become a major environmental problem due to their ultimate release and persistence in the natural terrestrial

and aquatic environments (Khetan and Collins, 2007). Long term exposure of microbes to these residual antibiotics and anti-microbials leads to evolution of drug resistance which is conferred by genetic determinants on plasmids, transposons or integrons (Martinez, 2008). Heavy metal resistance in bacteria is linked with multiple antibiotic resistance because many metal resistant bacteria also show multiple antibiotic resistance. Genetic determinants (genes) that code for antibiotic resistance and genes which confer heavy metal resistance are often carried on the same plasmid/transposon in addition to their copies in the chromosomal genome too (Clark et al., 1977; Calomiris et al., 1984; Roane and Kellogg, 1996). Majority of antibiotic resistant bacterial strains also exhibit metal tolerance to various toxic metals and organometals (McArthur and Tuckfield, 2000; De Souza et al., 2006; Matyar et al., 2010). Vanadium-induced multidrug resistance in environmental isolate of *Escherichia hermannii* and *Enterobacter cloacae* has been reported (Hernandez et al., 1998). TbtABM, a multidrug efflux pump associated with tributyltin (organometal) resistance in *Pseudomonas stutzeri* (Jude et al., 2004). Similar results were observed in case of all the six selected lead resistant bacterial strains which showed resistance to multiple antibiotics and indicated that genetic determinants for lead and multi-drug resistance may be interlinked (Table 2.6).

2.16 Identification of lead resistant bacterial strains

Lead-resistant bacterial strains were identified as *Pseudomonas aeruginosa* strain 4EA, *Enterobacter cloacae* strain P2B, *Providencia alcalifaciens* strain 2EA, *Pseudomonas stutzeri* strain M-9, *Pseudomonas aeruginosa* WI-1, *Vibrio harveyi* M-11 based on morphological, biochemical characteristics along with 16S rDNA sequence analysis (Table 2.7 and Figs 2.22 a-f). 16S rDNA sequence data of these six lead resistant strains has already been submitted to Genbank and accession numbers have already been allotted (Table 2.8). Based on FAME analysis few more lead resistant bacterial isolates were identified from car battery waste

which includes *Providencia rettgeri* GM04, *Proteus penneri* GM03, *Bacillus subtillis* GM02, *Proteus penneri* GM-10 and *Salmonella choleraesuis* 4A (Figs. 2.23 a-e).

Table 2.8 Lead resistant bacterial strains with their Genbank accession numbers

S.N.	Bacterial strains	Genbank accession no.
1	*Pseudomonas aeruginosa* strain 4EA	JF421616
2	*Enterobacter cloacae* strain P2B	HQ268733
3	*Providencia alcalifaciens* strain 2EA	HQ268734
4	*Pseudomonas stutzeri* strain M-9	HQ268731
5	*Vibrio harveyi* strain M-11	HQ268730
6	*Pseudomonas aeruginosa* strain WI-1	HQ268732

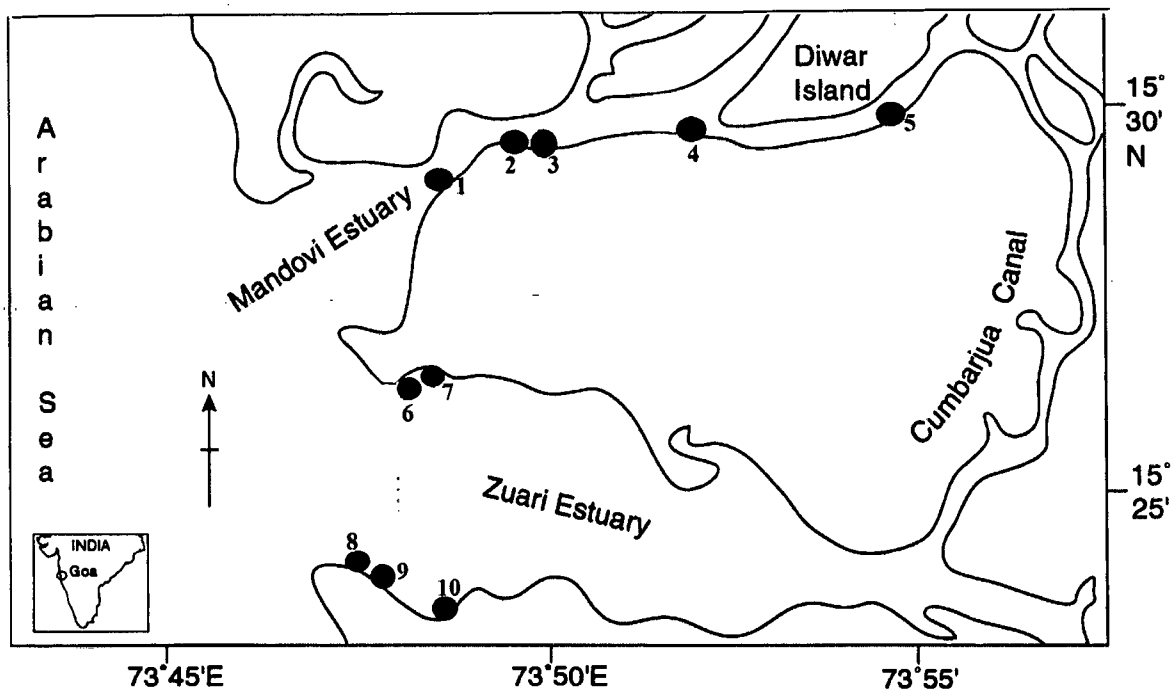


Fig. 2.1 Sampling sites of Mandovi (●) and Zuari (●) estuaries

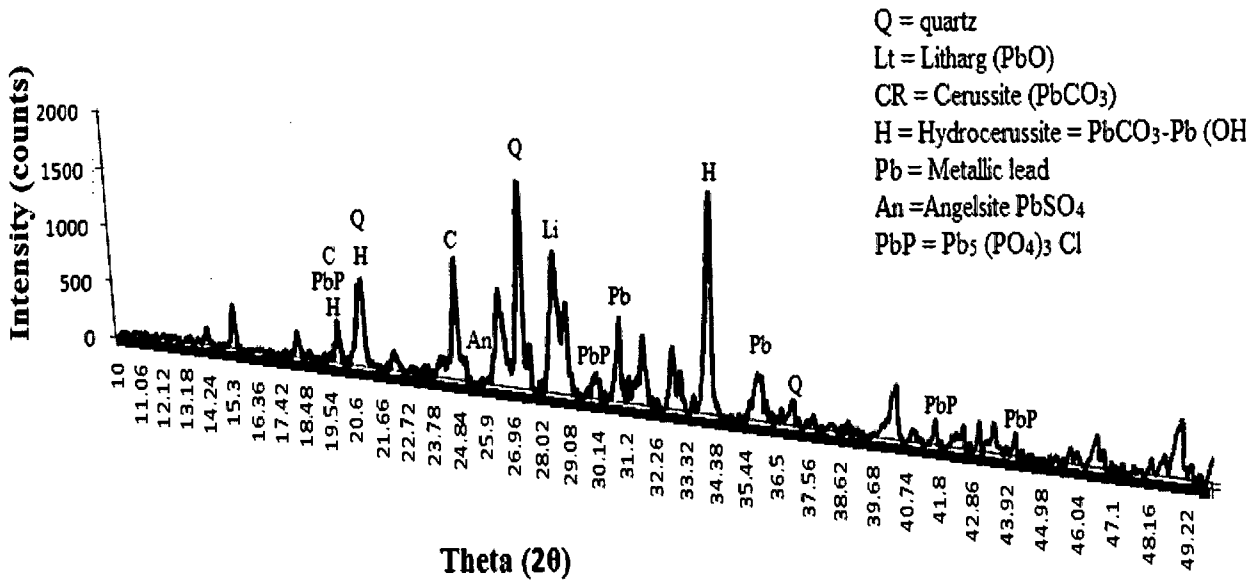


Fig. 2.2 XRD analysis of soil sample from waste dumping site of Germania batteries Pvt. Ltd. Corlim, Goa

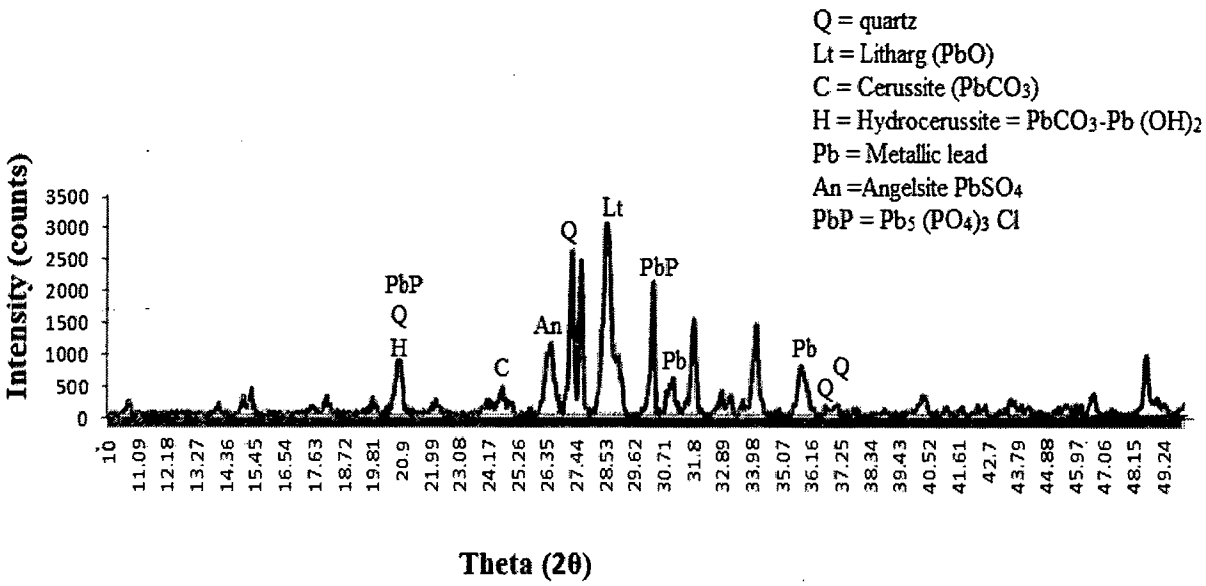


Fig. 2.3 XRD analysis of soil sample from waste dumping site of M/S Permalite batteries Pvt. Ltd. Corlim, Goa

Western India shipyard Ltd.,Vasco, Goa		Goa Shipyard Ltd.,Vasco, Goa		MANMAD Shipyard Ltd.,Vasco, Goa		DONA PAULA beach, Goa		KAKRA beach, Goa	
CONTROL ZMA only	ZMA+ Lead nitrate (0.5 mM)	CONTROL ZMA only	ZMA + Lead nitrate (0.5 mM)	CONTROL ZMA only	ZMA+ Lead nitrate (0.5 mM)	CONTROL ZMA only	ZMA+ Lead nitrate (0.5 mM)	CONTROL ZMA only	ZMA+ Lead nitrate 0.5 mM
67 ± 4.4 $\times 10^3$	3.7± 0.2 $\times 10^3$	93± 6.2 $\times 10^3$	2.4± 0.6 $\times 10^3$	86± 5.3 $\times 10^3$	3.1± 0.8 $\times 10^3$	36± 3.4 $\times 10^3$	1.6± 0.3 $\times 10^3$	79± 2.9 $\times 10^3$	2.6± 0.7 $\times 10^3$

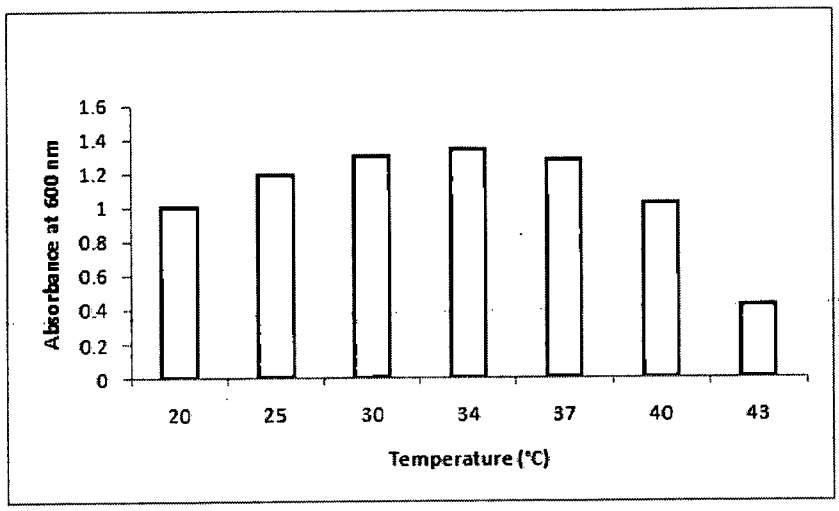
Table. 2.3 Viable count of bacterial population along with lead resistant strains from Zuari estuary

KALA ACADEMY JETTY, Panjim, Goa		FERRY POINT, PANJIM, Goa		MANDOVI BRIDGE, Goa		RIBANDAR FERRY POINT, Ribandar, Goa		DEMPO SHIPYARD Ltd., Old Goa	
CONTROL ZMA only	ZMA + Lead nitrate (0.5 mM)	CONTROL ZMA only	ZMA + Lead nitrate (0.5 mM)	CONTROL ZMA only	ZMA + Lead nitrate (0.5 mM)	CONTROL ZMA only	ZMA + Lead nitrate (0.5 mM)	CONTROL ZMA only	ZMA + Lead nitrate 0.5 mM
84± 6.3 $\times 10^3$	0.43±0.1 $\times 10^3$	56± 4.1 $\times 10^3$	1.65±0.5 2×10^3	72±7.2 $\times 10^3$	1.24± 0.33$\times 10^3$	63± 3.5 $\times 10^3$	2.65±0.9 $\times 10^3$	102±8.9 $\times 10^3$	3.61± 1.7 $\times 10^3$

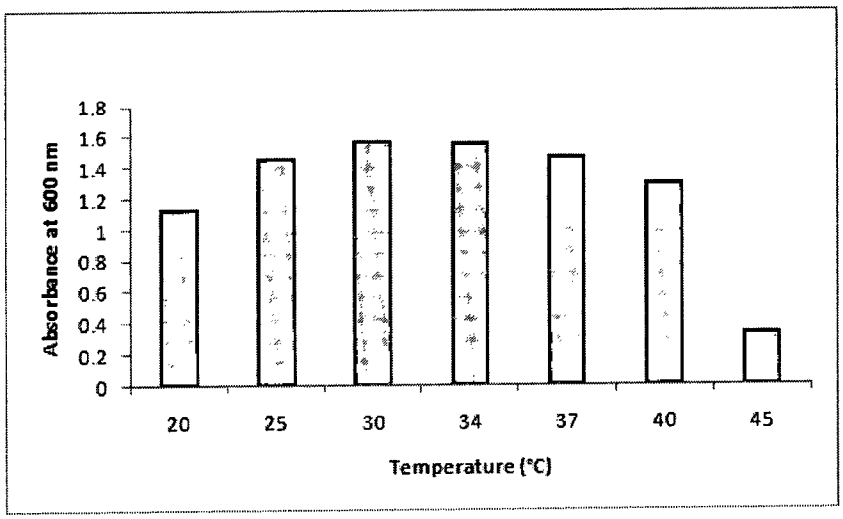
Table.2.4 Viable count of bacterial population along with lead resistant strains from Mandovi estuary

SITES / SAMPLES	Germania	Permalite	Lead ion oxide	Shrine kakoda	Bharati electronics	D-LINK
Soil samples						
Sample 1 control	105 × 10^3	37 ×10^3	43 × 10^3	89 × 10^3	128 × 10^3	79× 10^3
Sample 1 Lead nitrate	2.24 × 10^3	0.46 × 10^3	2.25 ×10^3	0.38 × 10^3	0.046× 10^3	0.42×10^3
Sample 2 Control	80 × 10^3	161 × 10^3	28× 10^3	63 ×10^3	76 ×10^3	86 ×10^3
Sample 2 Lead nitrate	1.79 × 10^3	0.88 × 10^3	0.46×10^3	1.2 ×10^3	0.266×10^3	0.083 ×10^3
Liquid waste samples						
Sample 3 control	23 ×10^3	117×10^3	52×10^3	86×10^3	138×10^3	57×10^3
Sample 3 Lead nitrate	0.93 ×10^3	1.86×10^3	1.48×10^3	1.27×10^3	0.05×10^3	0.013×10^3
Sample 4 control	59×10^3	93×10^3	67×10^3	117×10^3	74×10^3	95×10^3
Sample 4 Lead nitrate	3.16×10^3	0.53×10^3	0.86×10^3	0.83×10^3	0.01×10^3	0.16×10^3

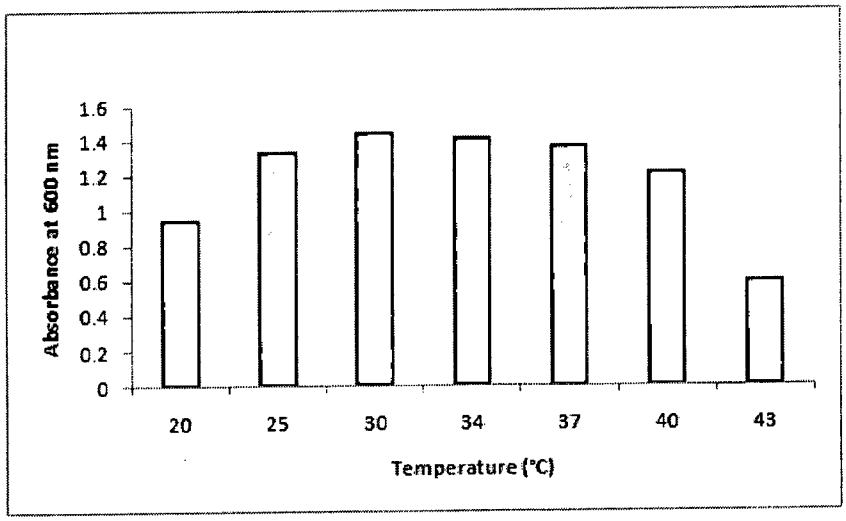
Table.2.5 Viable count of bacterial population along with lead resistant strains from soil and liquid waste samples on PYT80 agar plate amended with 100 µM lead nitrate


Absorbance at 600 nm
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
20
25
30
34
37
40
43
Temperature (°C)

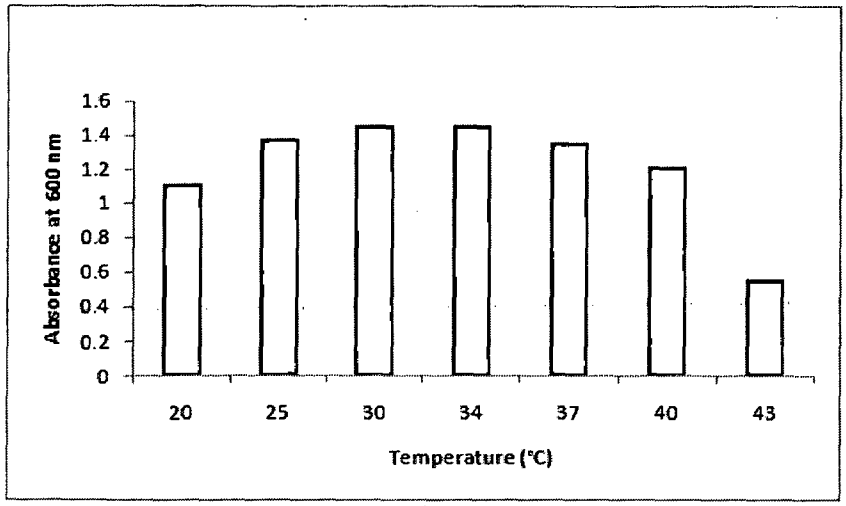
(a)


Absorbance at 600 nm
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
20
25
30
34
37
40
45
Temperature (°C)

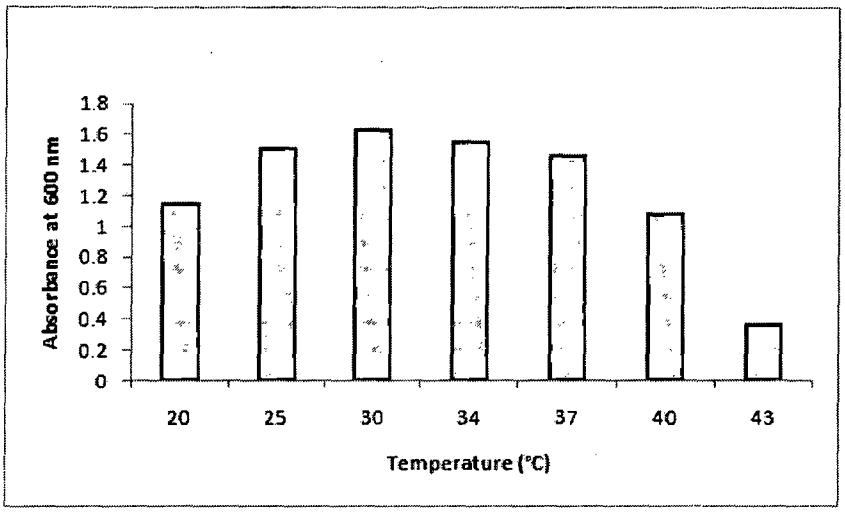
(b)


Absorbance at 600 nm
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
20
25
30
34
37
40
43
Temperature (°C)

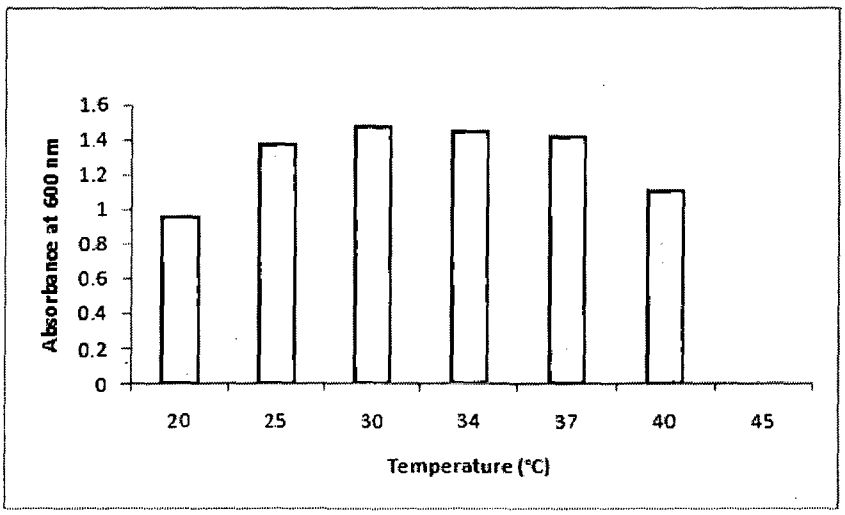
(c)



(d)

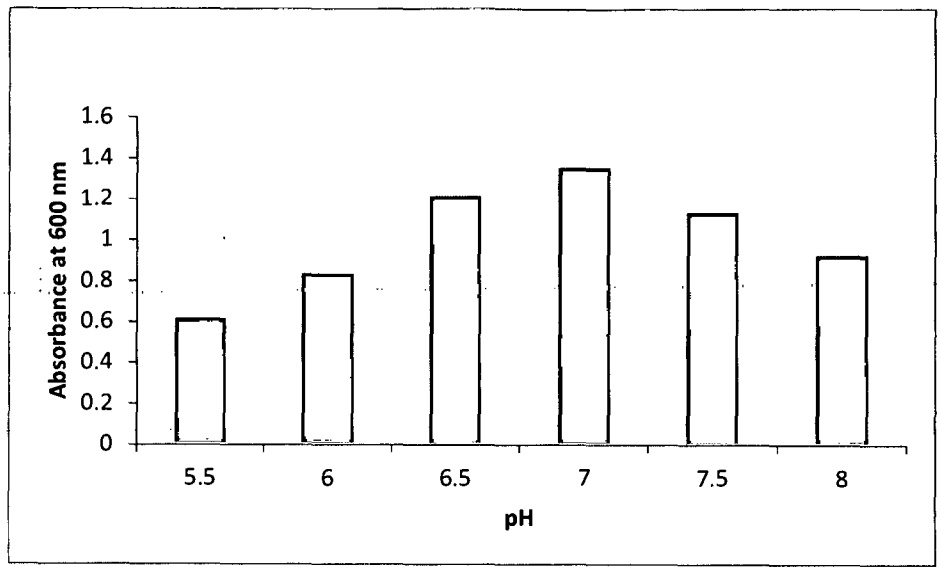


(e)

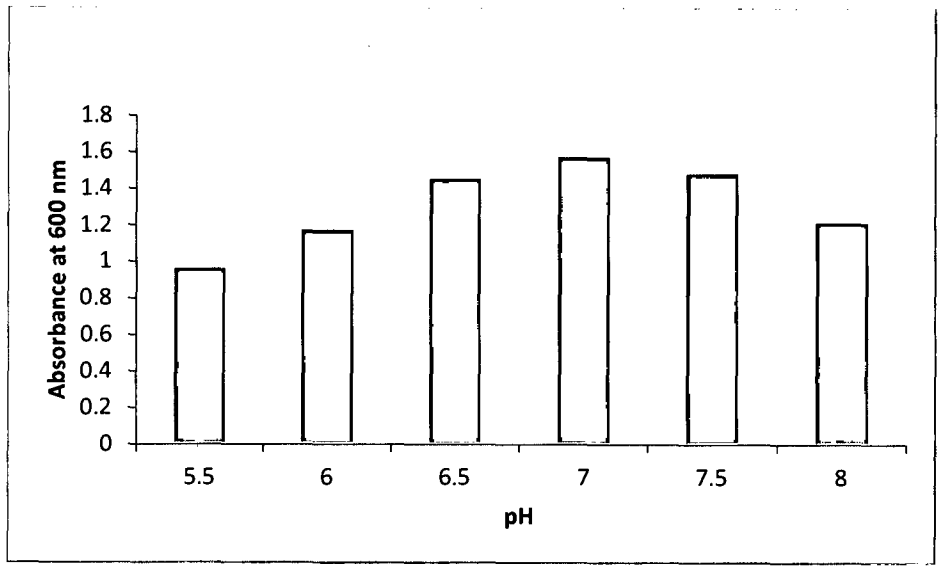


(f)

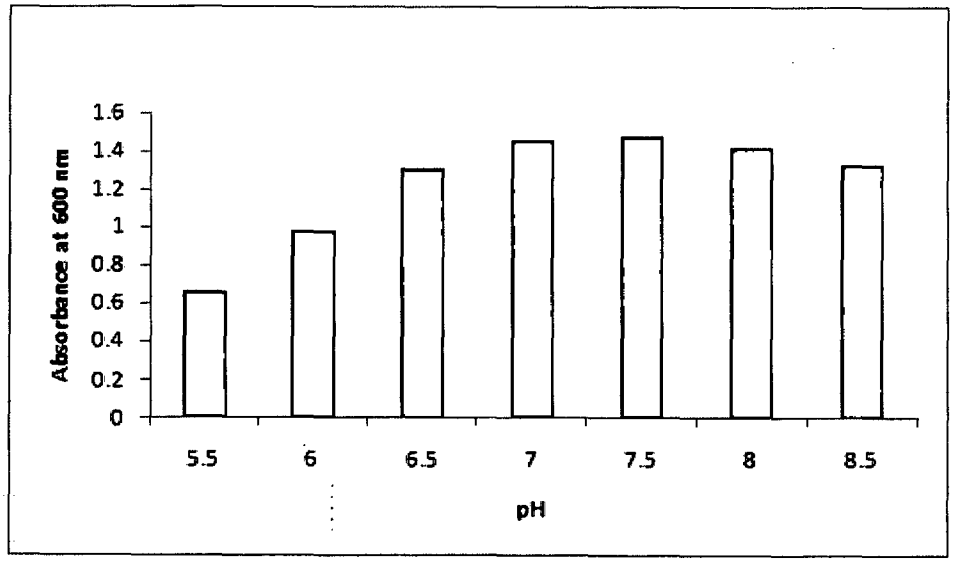
Fig.2.4. (a-f) Growth response of bacterial strains 2EA, P2B, WI-1, 4EA, M-9 and M- 11 at different temperature in TMM amended with lead nitrate (100 µM)


Absorbance at 600 nm
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
5.5
6
6.5
7
7.5
8
pH

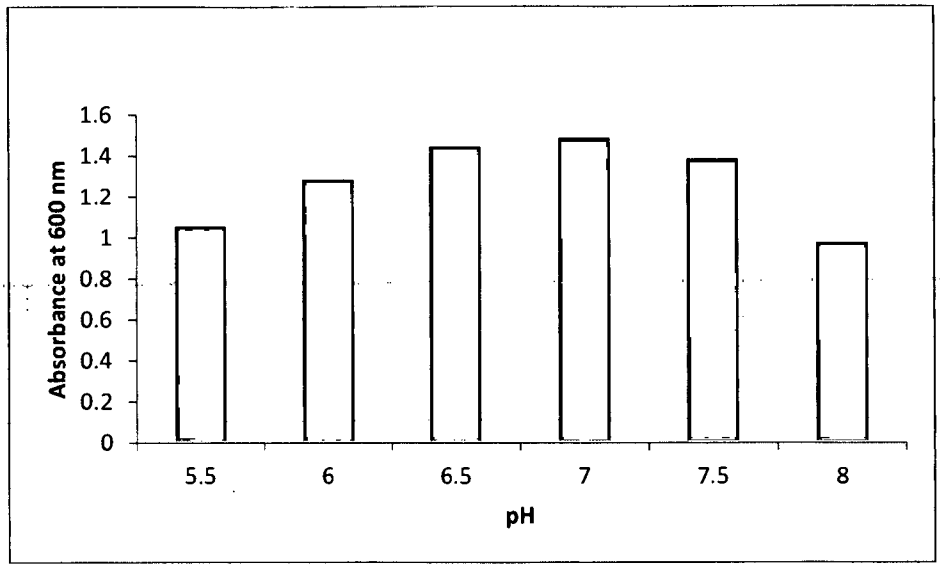
(a)


Absorbance at 600 nm
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
5.5
6
6.5
7
7.5
8
pH

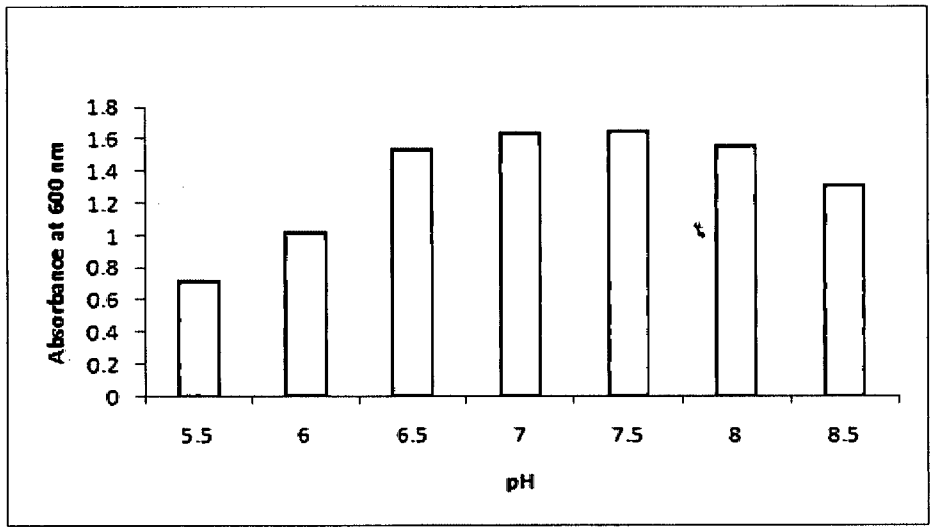
(b)


Absorbance at 600 nm
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
5.5
6
6.5
7
7.5
8
8.5
pH

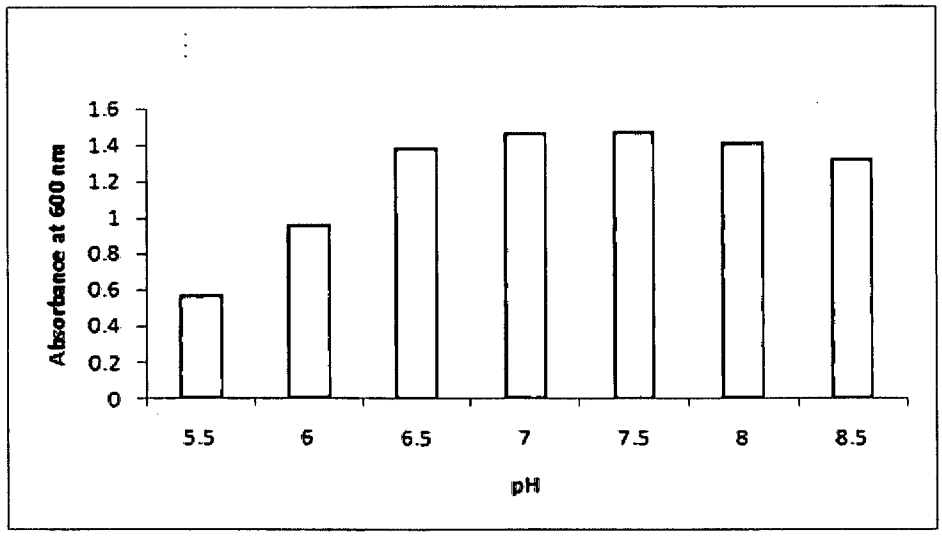
(c)



(d)

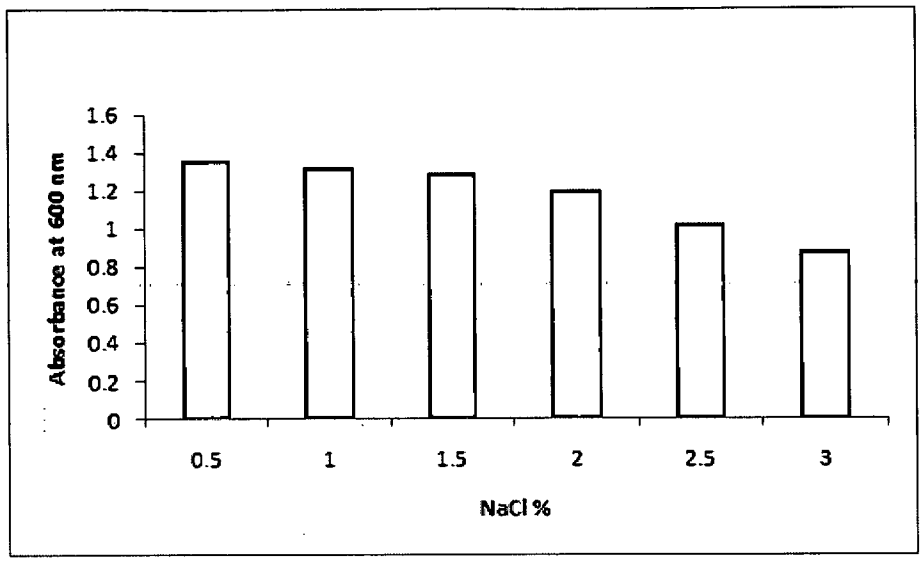


(e)

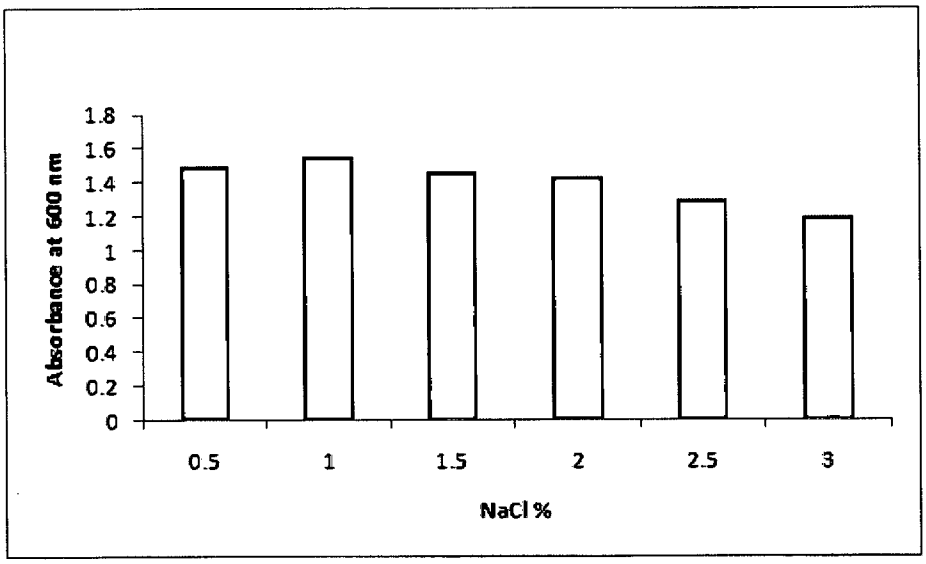


(f)

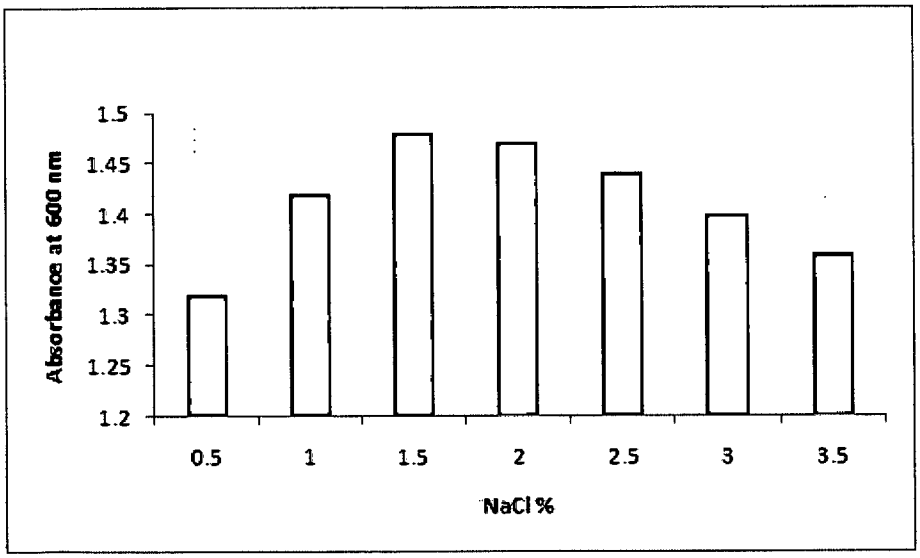
Fig. 2.5.a-f Growth response of bacterial strains 2EA, P2B, WI-1, 4EA, M-9 and M-11 at different pH in TMM supplemented with lead nitrate (100 µM)


Absorbance at 600 nm
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0.5
1
1.5
2
2.5
3
NaCl %

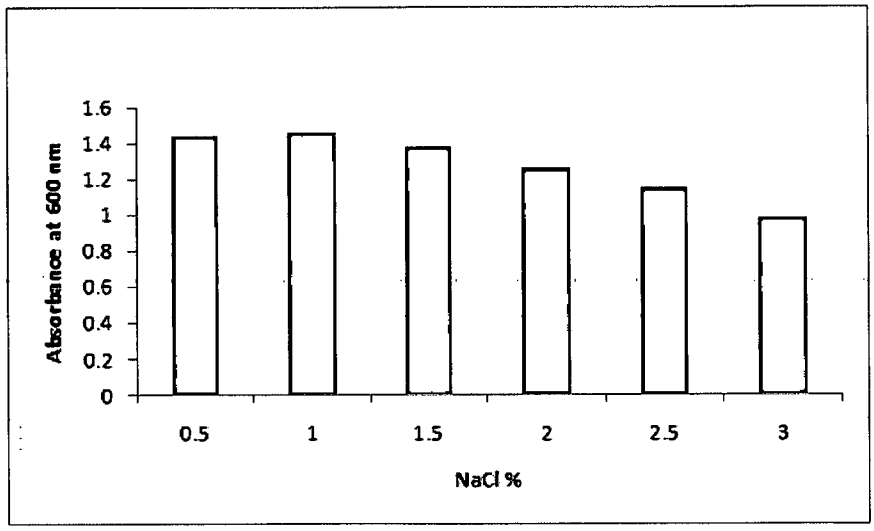
(a)


Absorbance at 600 nm
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0.5
1
1.5
2
2.5
3
NaCl %

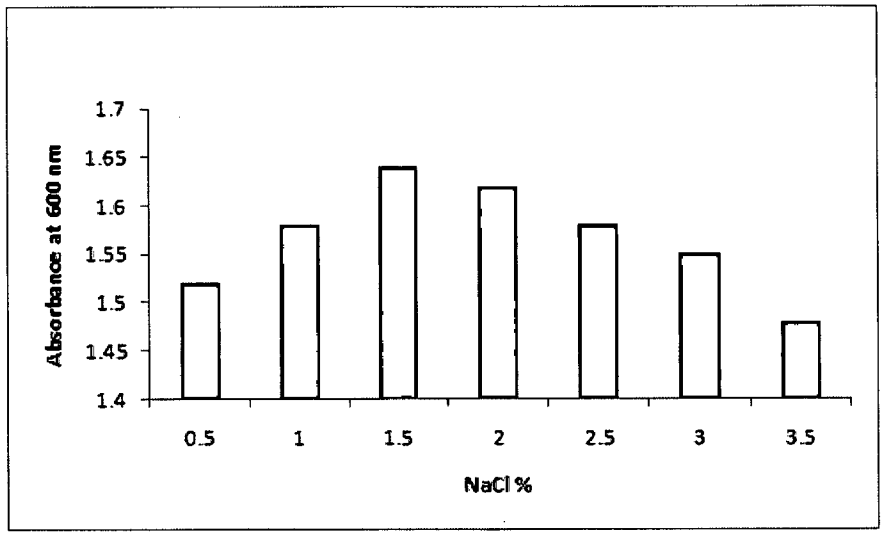
(b)


Absorbance at 600 nm
1.5
1.45
1.4
1.35
1.3
1.25
1.2
0.5
1
1.5
2
2.5
3
3.5
NaCl %

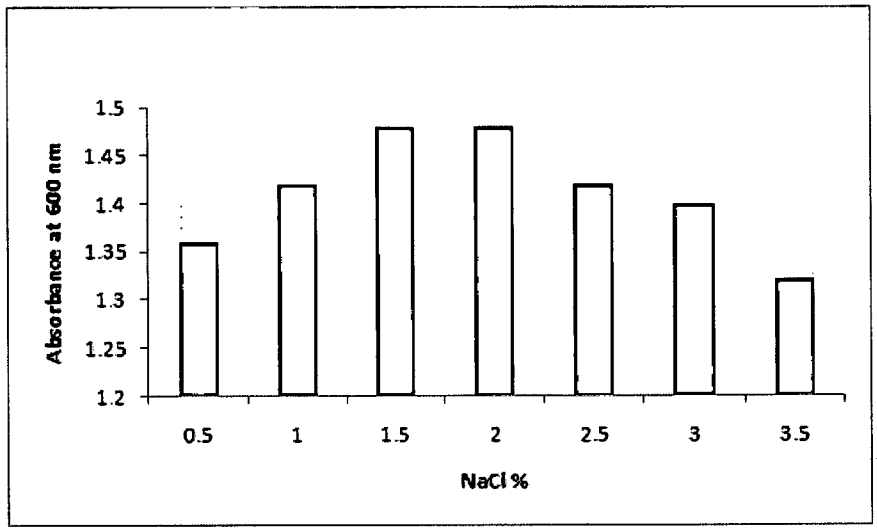
(c)



(d)

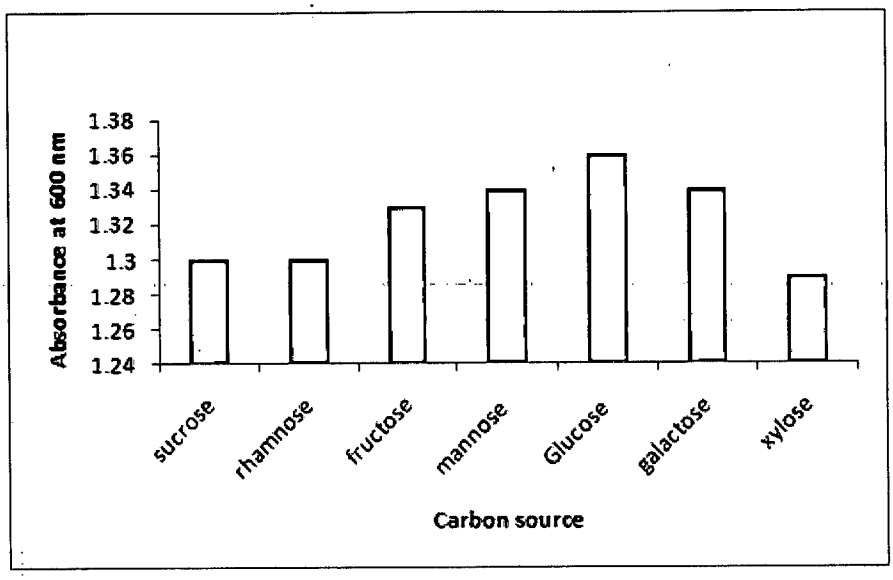


(e)

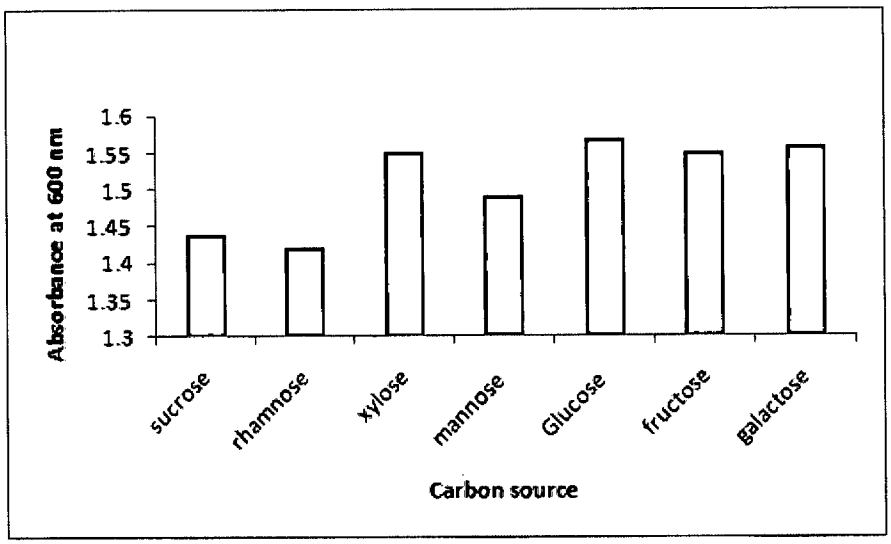


(f)

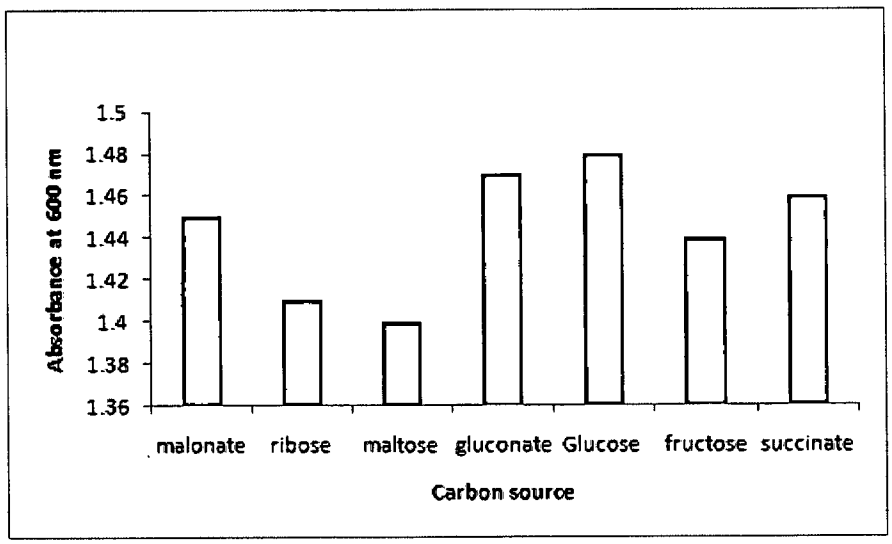
Fig. 2.6.a-f Growth response of bacterial strains 2EA, P2B, WI-1, 4EA, M-9 and M- 11 at different NaCl% in TMM supplemented with lead nitrate (100 µM)


Absorbance at 600 nm
1.38
1.36
1.34
1.32
1.3
1.28
1.26
1.24
sucrose
rhamnose
fructose
mannose
Glucose
galactose
xylose
Carbon source

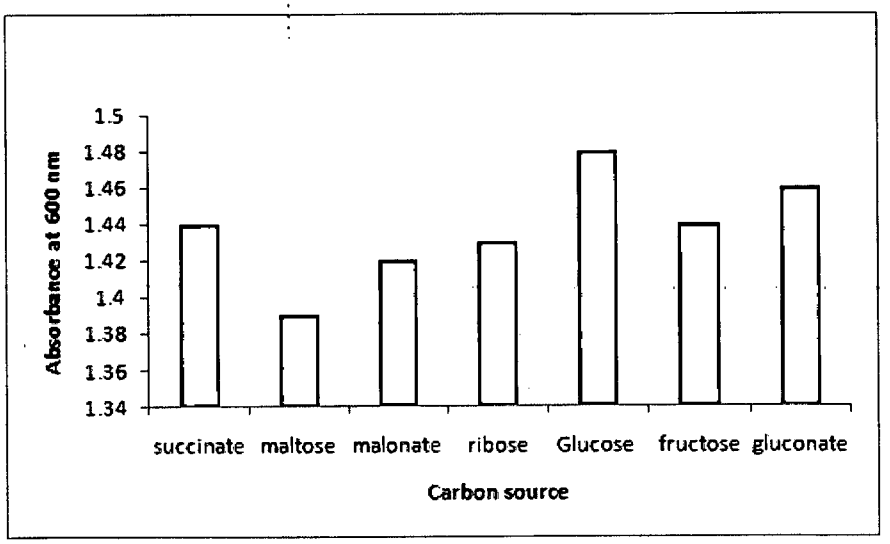
(a)


Absorbance at 600 nm
1.6
1.55
1.5
1.45
1.4
1.35
1.3
sucrose
rhamnose
xylose
mannose
Glucose
fructose
galactose
Carbon source

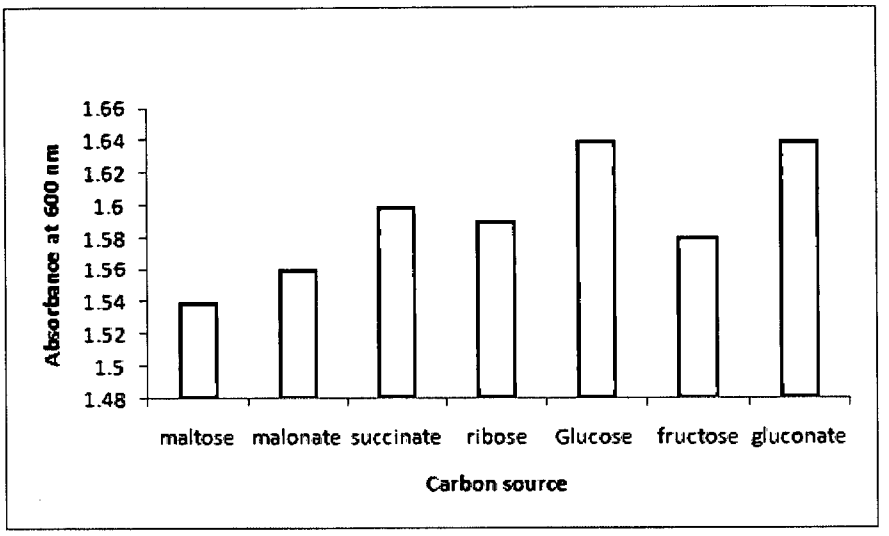
(b)


Absorbance at 600 nm
1.5
1.48
1.46
1.44
1.42
1.4
1.38
1.36
malonate
ribose
maltose
gluconate
Glucose
fructose
succinate
Carbon source

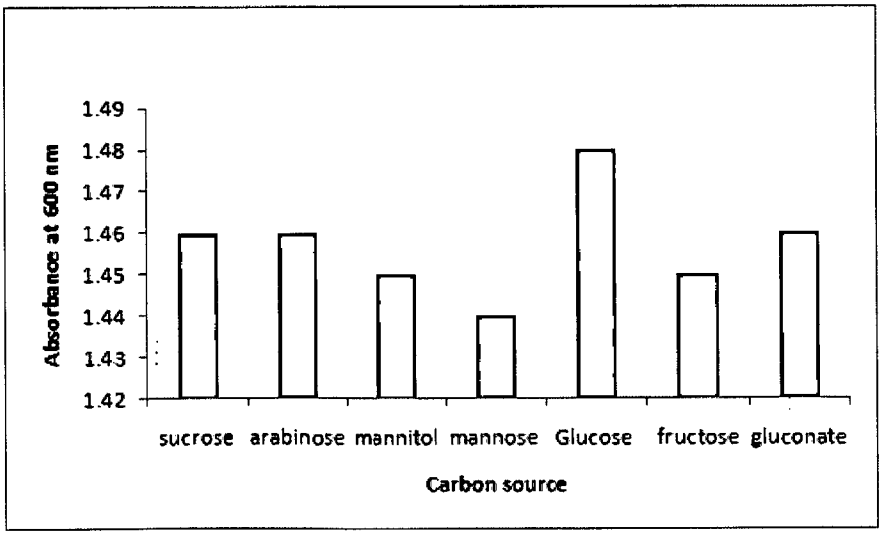
(c)



(d)



(e)



(f)

Fig. 2.7.a-f Growth response of bacterial strains 2EA, P2B, WI-1, 4EA, M-9 and M- 11 on different carbon sources in TMM supplemented with lead nitrate (100 µM)

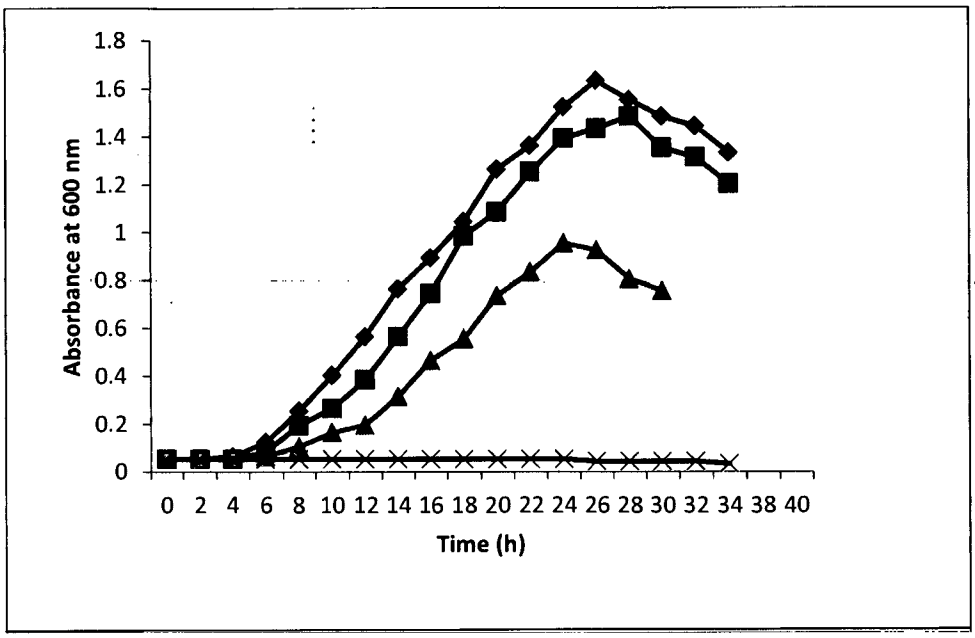


Fig.2.8 Growth behavior of *Pseudomonas stutzeri* strain M-9 in TMM amended with different concentrations of lead nitrate. Filled diamond (◆): 0 mM (control), filled square(■) : 0.5 mM, filled triangle (▲): 0.8 mM, X (✕): 1.0 mM

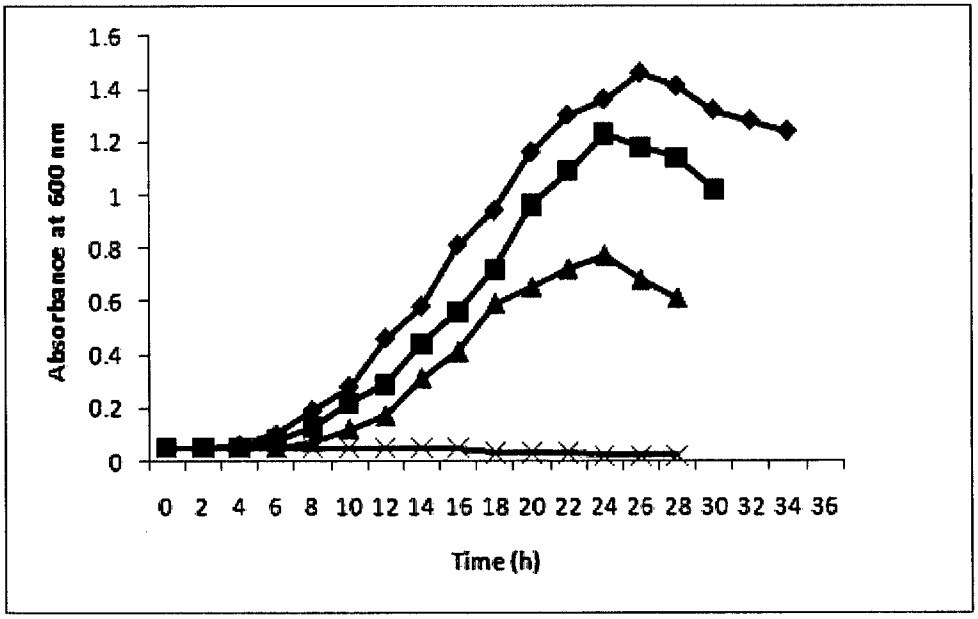


Fig.2.9 Growth behavior of *Vibrio harveyi* M-11 in TMM amended with different concentrations of lead nitrate. Filled diamond (◆): 0 mM (control), filled square (■): 0.5 mM, filled triangle (▲): 1.2 mM, X (✕): 1.4 mM

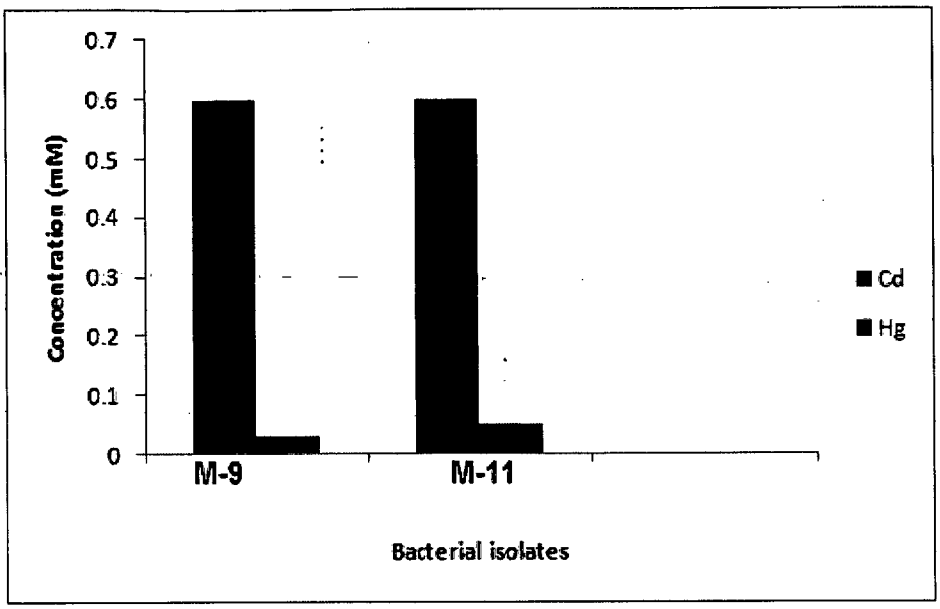


Fig.2.10 MIC of cadmium and mercury for bacterial strains *Pseudomonas stutzeri* M-9 and *Vibrio harveyi* M-11, in TMM

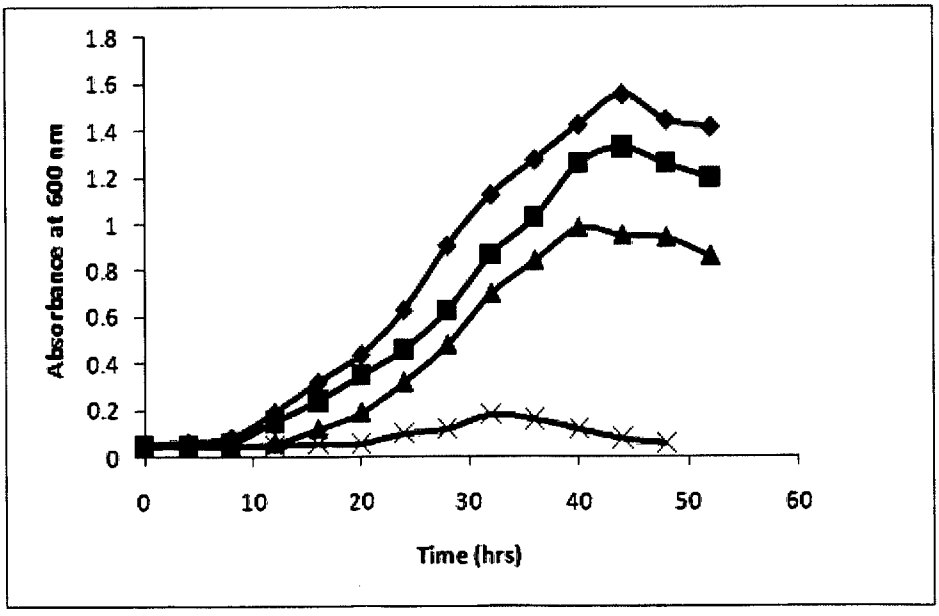


Fig.2.11 Growth behavior of *Enterobacter cloacae* strain P2B in TMM amended with different concentrations of lead nitrate. Filled diamond (◆): 0 mM (control), filled square (■): 0.5 mM, filled triangle (▲): 1.6 mM, X (✕): 1.7 mM

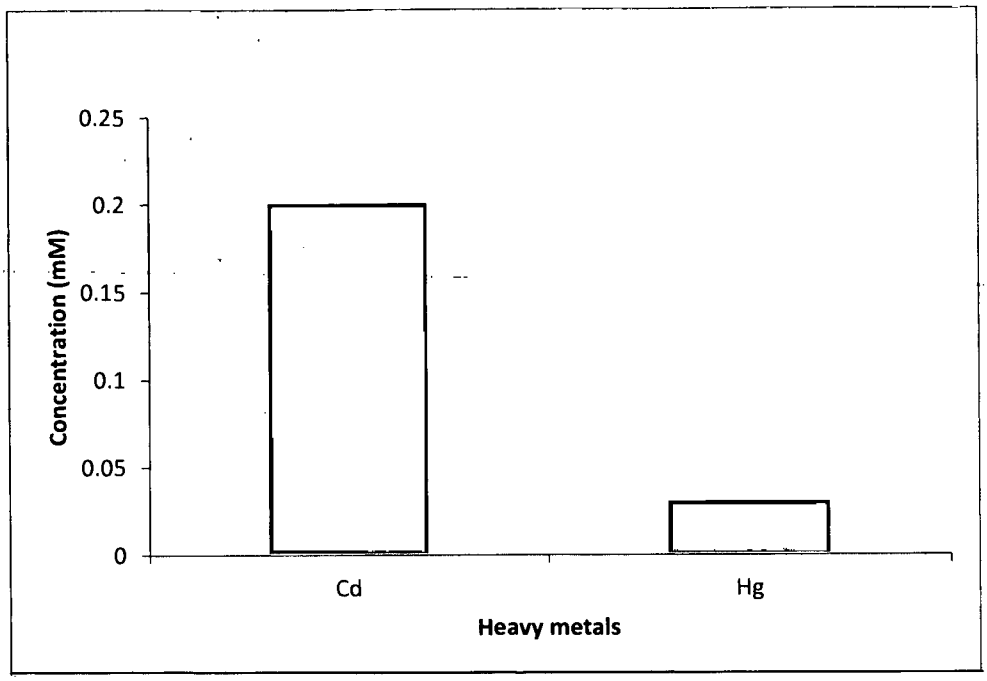


Fig.2.12 MIC of cadmium and mercury for *Enterobacter cloacae* strain P2B in TMM

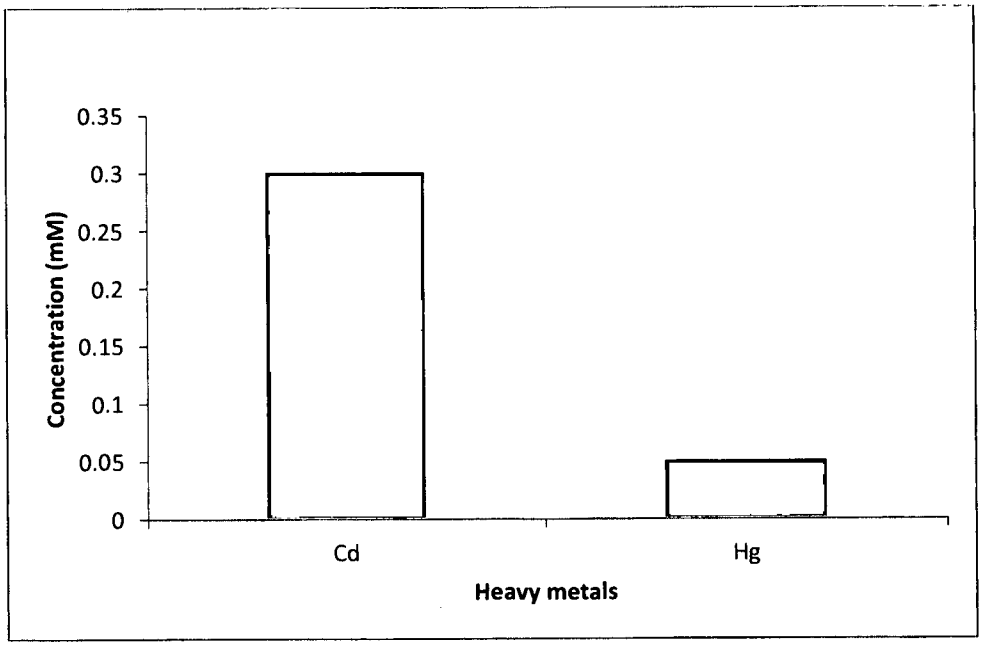


Fig.2.13 MIC of cadmium and mercury for *Enterobacter cloacae* strain P2B in presence of 100 μM lead nitrate

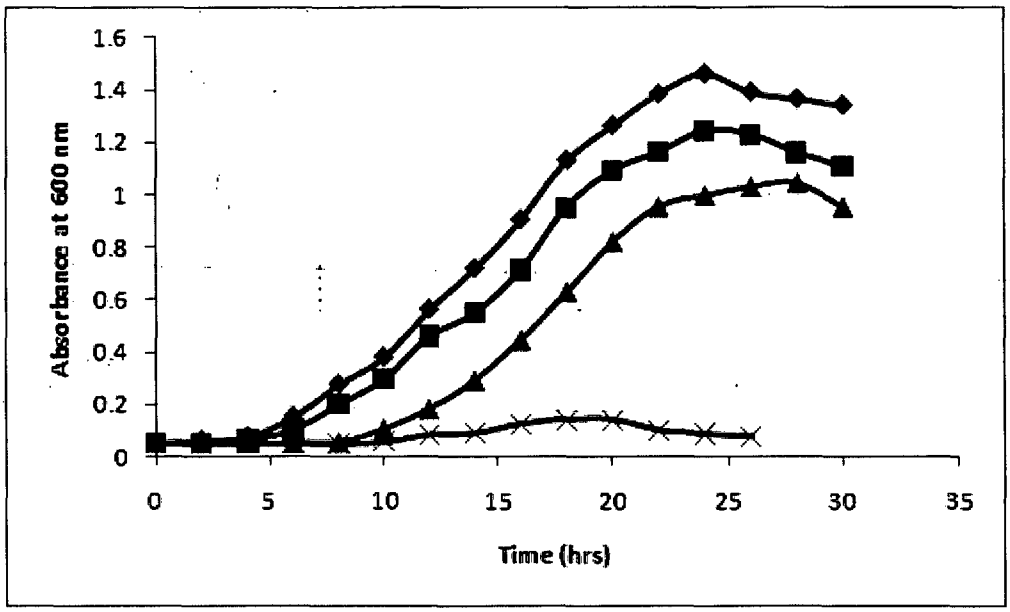


Fig.2.14 Growth behavior of *Pseudomonas aeruginosa* strain 4EA in TMM amended with different concentrations of lead nitrate. Filled diamond (◆): 0 mM (control), filled square (■): 0.5 mM, filled triangle (▲): 0.8 mM, X (✕): 0.9 mM

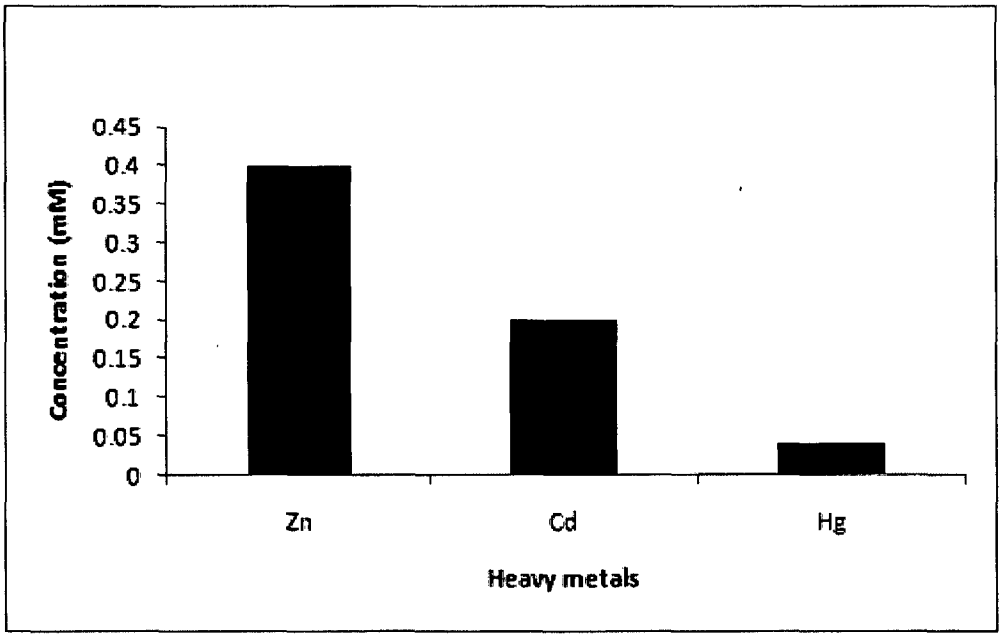


Fig.2.15 MIC of zinc, cadmium and mercury for *Pseudomonas aeruginosa* strain 4EA in TMM

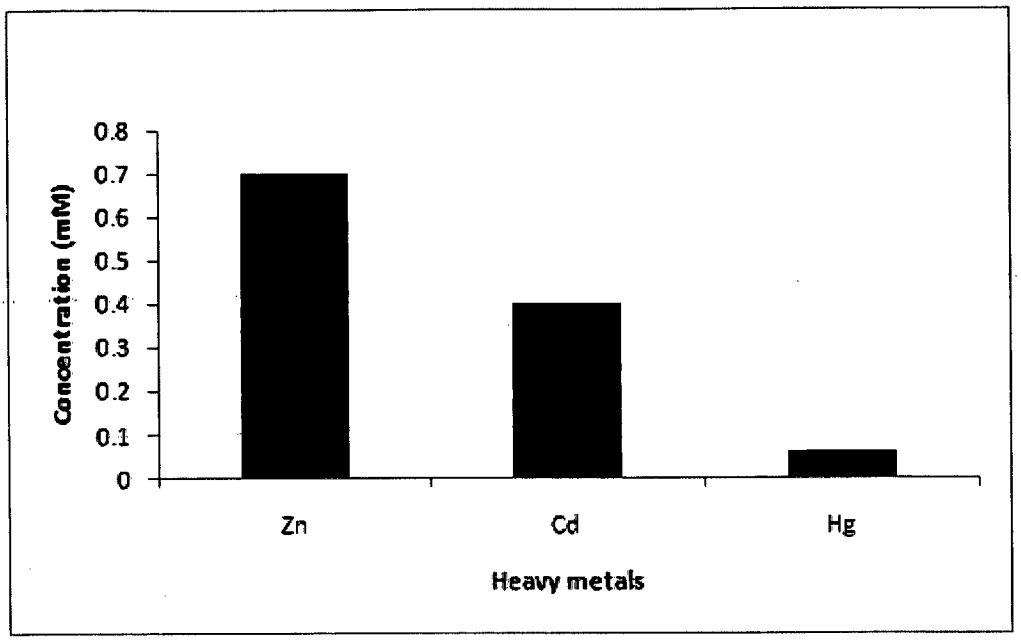


Fig.2.16 MIC of zinc, cadmium and mercury for *Pseudomonas aeruginosa* 4EA in presence of 100 µM lead nitrate

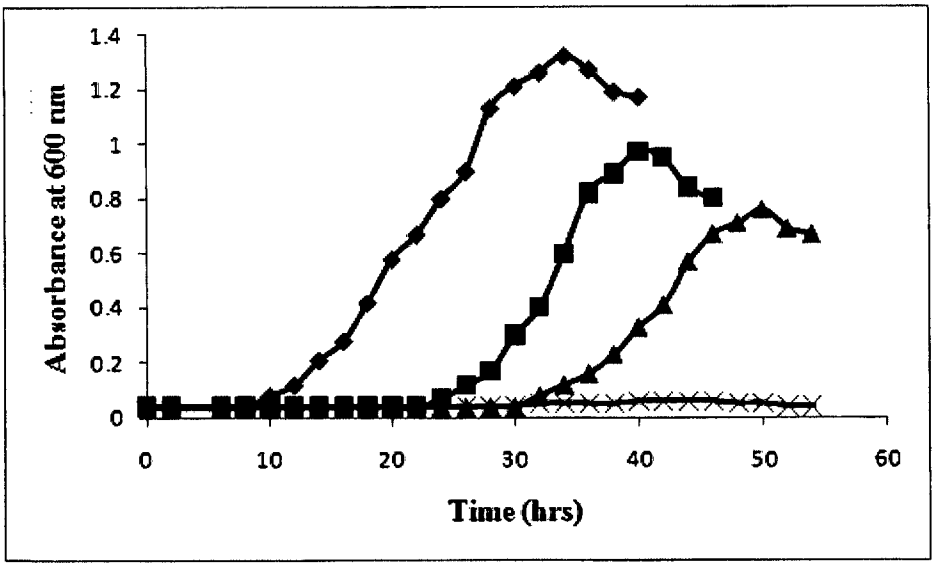


Fig.2.17 Growth behavior of *Providencia alcalifaciens* strain 2EA in TMM amended with different concentrations of lead nitrate. Filled diamond (◆): 0 mM(control), filled square (■): 1.0 mM, filled triangle (▲): 1.4 mM, X (✕): 1.5 mM

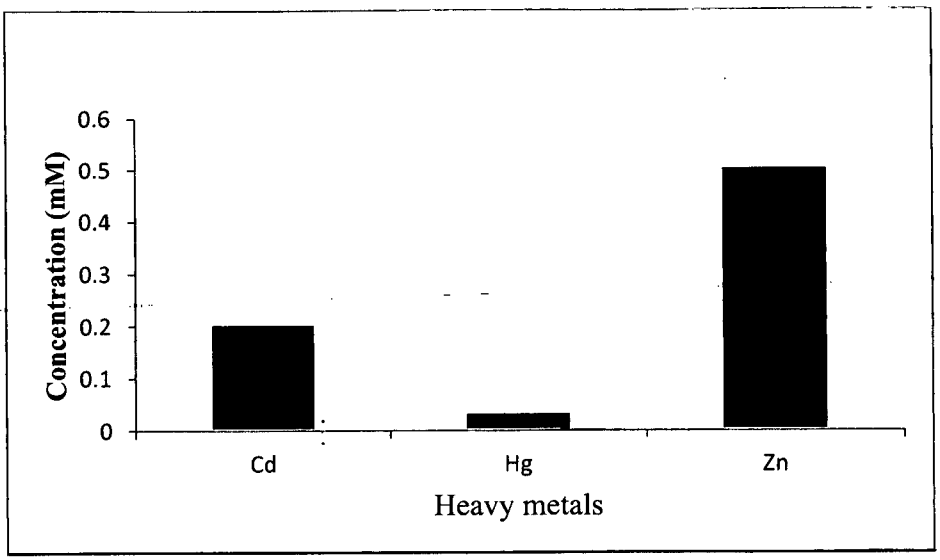


Fig.2.18 MIC of cadmium, mercury and zinc for *Providencia alcalifaciens* strain 2EA in TMM

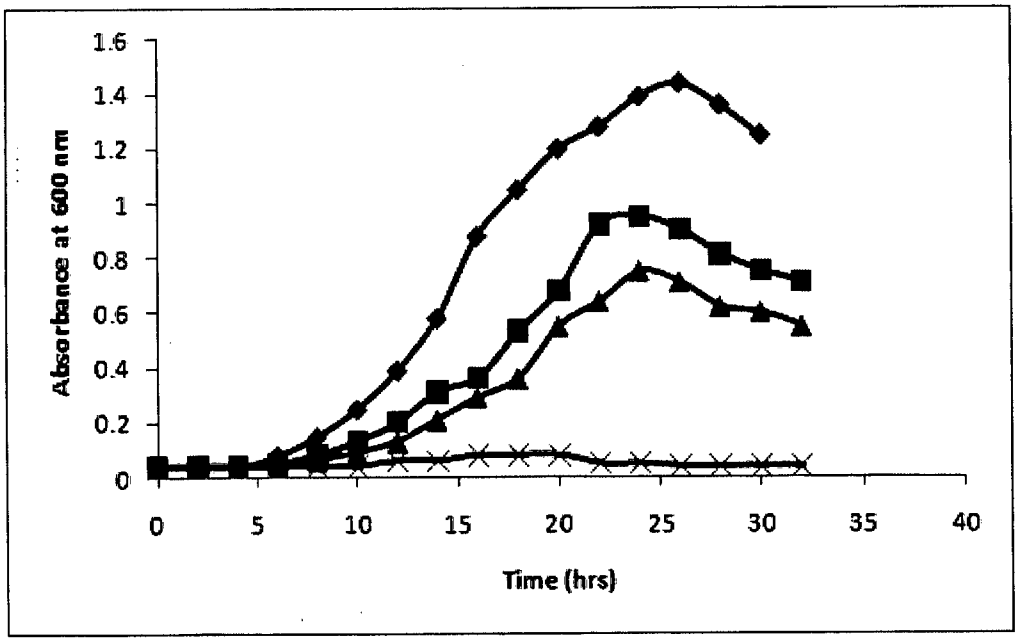


Fig.2.19 Growth behavior of *Pseudomonas aeruginosa* strain WI-1 in TMM amended with different concentrations of lead nitrate. Filled diamond (◆): 0 mM (control), filled square (■): 0.3 mM, filled triangle (▲): 0.6 mM, X (✕): 0.8 mM

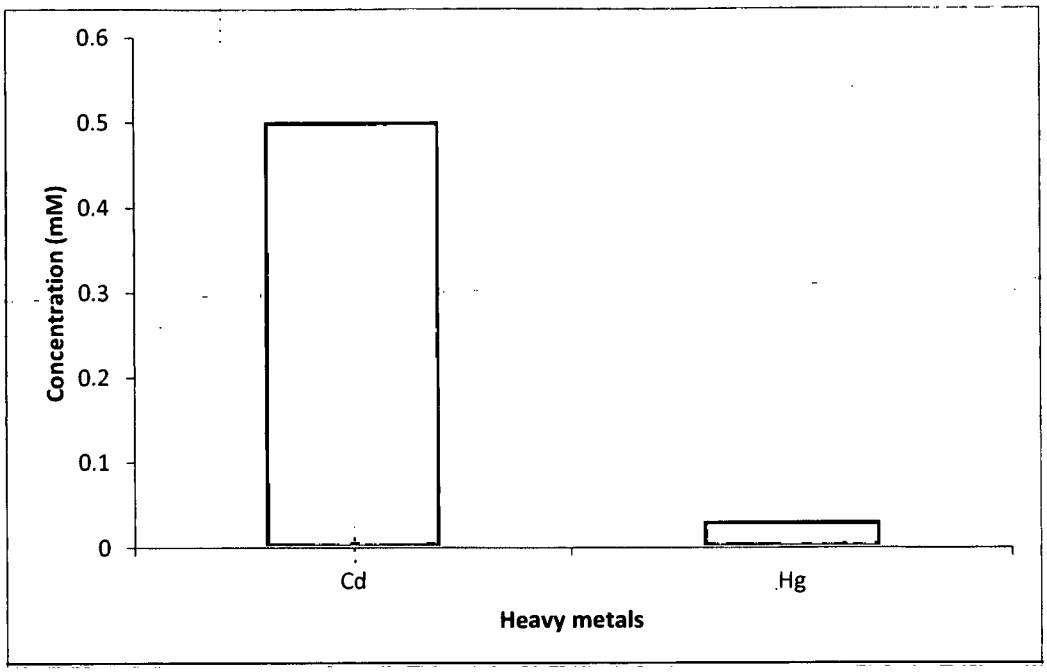


Fig.2.20 MIC of cadmium and mercury for *Pseudomonas aeruginosa* strain WI-1 in TMM

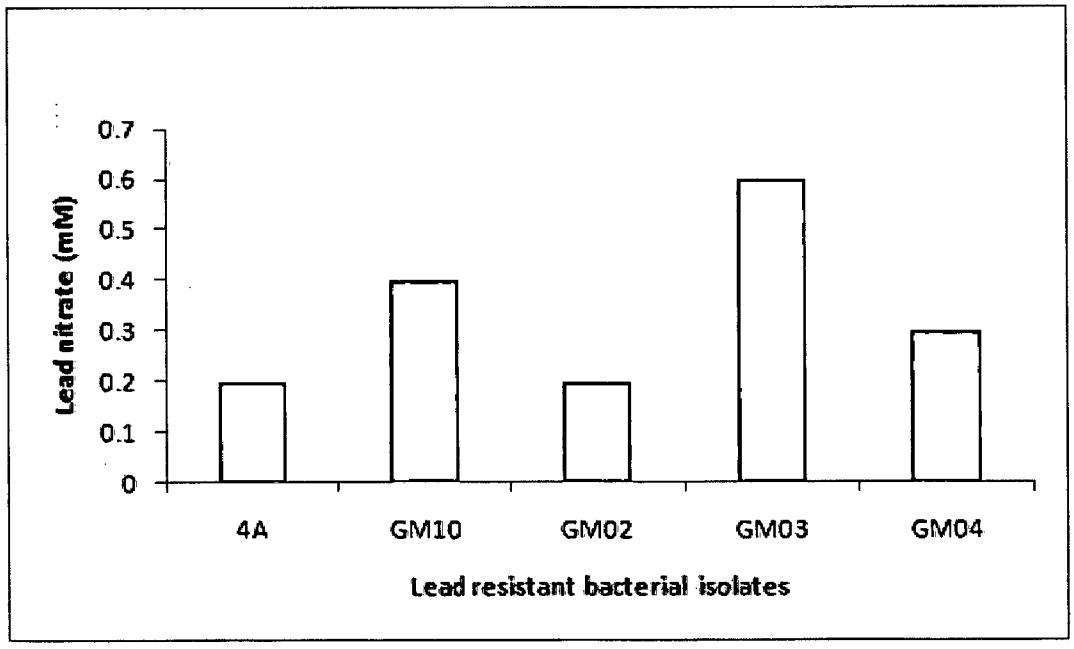


Fig.2.21 Lead tolerance limit of bacterial strains from lead battery manufacturing company

Table.2.7 Biochemical characteristics of lead resistant bacterial strains

TESTS	Strain 4EA	Strain M-9	Strain WI-1	Strain M-11	Strain P2B	Strain 2EA
Gram staining	Gram negative short rods	Gram negative short rods	Gram negative short rods	Gram negative short rods	Gram negative short rods	Gram negative short rods
Motility	motile	motile	motile	motile	motile	motile
HL-test	oxidative	oxidative	oxidative	Oxidative+ fermentative	Oxidative+ fermentative	Oxidative+ fermentative
Indole	+	+	+	+	-	+
MR-test	-	-	-	-	-	+
VP-test	-	-	-	-	+	-
Citrate	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
oxidase	+	+	+	+	-	-
Urease	-	-	-	-	-	-
Nitrate reduction	-	+	+	+	+	+
H2S	-	-	-	-	-	-
Phenylalanine deaminase	-	-	-	-	-	+
Starch hydrolysis	-	+	-	+	-	-
TSI media	Alkaline butt+ alkaline slant	Alkaline butt + alkaline slant	Alkaline butt +Alkaline slant	Acid butt + alkaline slant	Acid butt + acid slant + gas	Acid butt + alkaline slant
Gelatinase	+	-	+	+	+	-
ONPG	-	-	-	+	+	-
Lysine δc	-		-	+	-	-
Ornithine δc	-		-	+	+	-
Luminescence	-	-	-	+	-	-
Fluorescent pigment	+ greenish yellow	-	+ greenish yellow	-	-	-
Sugar fermentation						
Glucose	-	-	-	+	(+)	(+)
sucrose	-	-	-	+	+	+
rhamnose	-	-	-	-	+	+
xylose	-	-	-	-	(+)	(+)
fructose	-	-	-	+	(+)	(+)
mannose	-	-	-	+	(+)	(+)
galactose	-	-	-	+	(+)	(+)
Tentatively Identified as	***Pseudomonas aeruginosa***	***Pseudomonas stutzeri***	***Pseudomonas aeruginosa***	***Vibrio harveyi***	***Enterobacter cloacae***	***Providencia alcalifaciens***

Key : - =Negative result, + = Positive result, (+) = Acid + gas, + = only acid

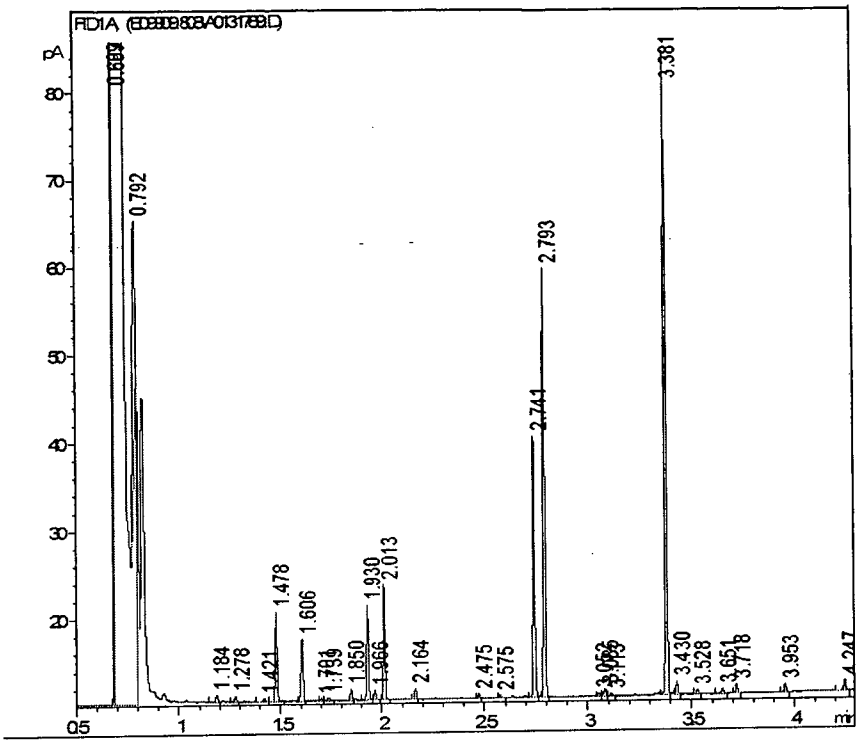


Fig.2.22.a FAME profile of lead resistant bacteria *Pseudomonas aeruginosa* strain 4EA

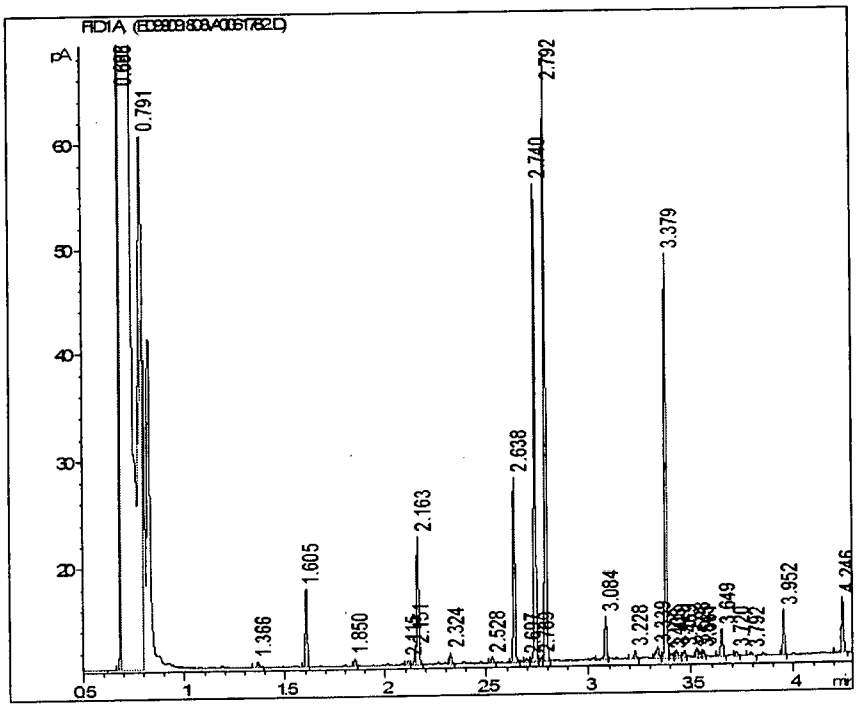
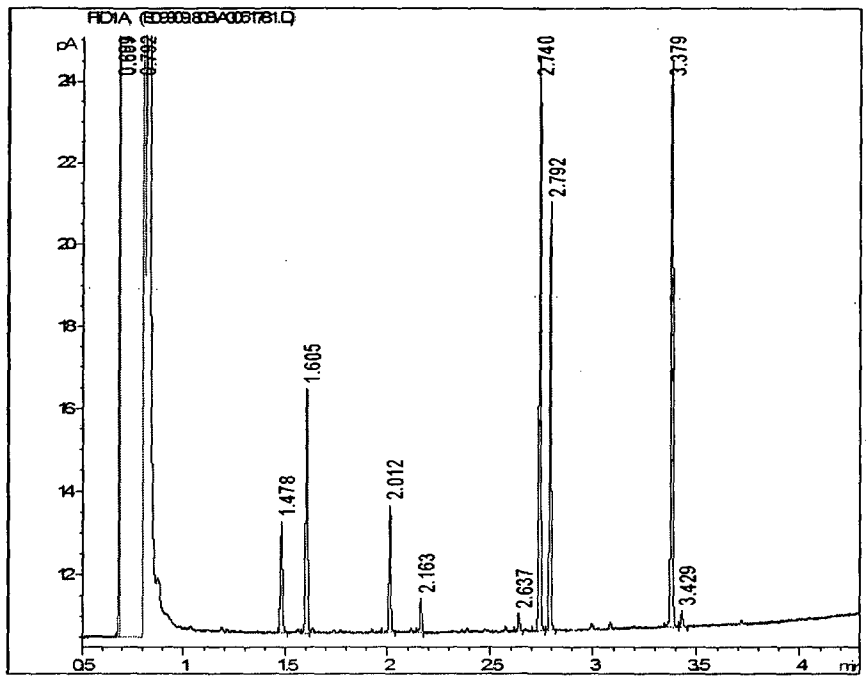
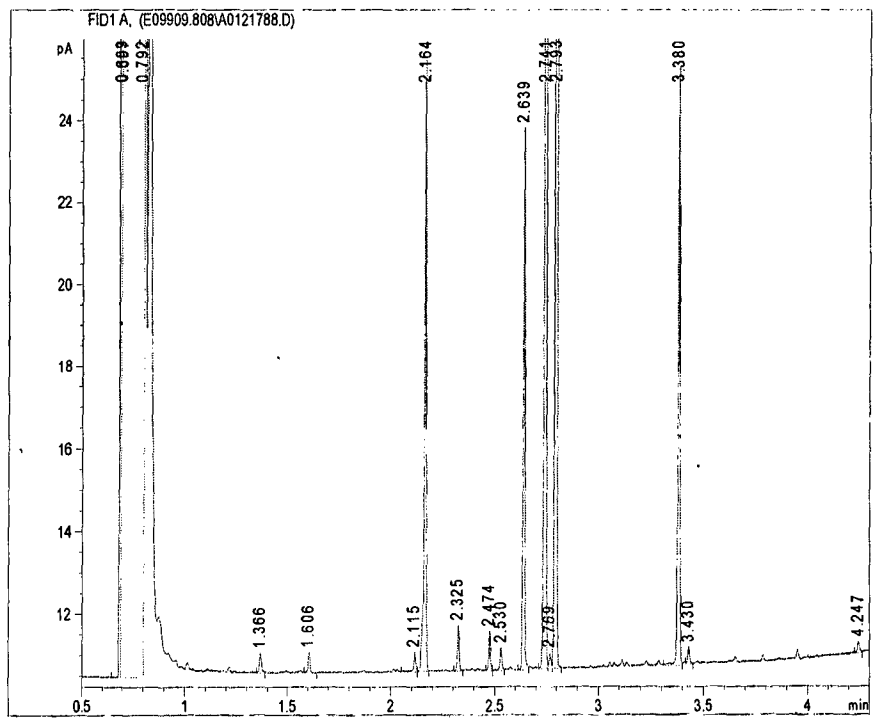


Fig.2.22.b FAME profile of lead resistant bacteria *Enterobacter cloacae* strain P2B



(c)

Fig.2.22.c FAME profile of lead resistant bacteria *Pseudomonas stutzeri* strain M-9



(d)

Fig.2.22.d FAME profile of lead resistant bacteria *Providencia alcalifaciens* strains 2EA

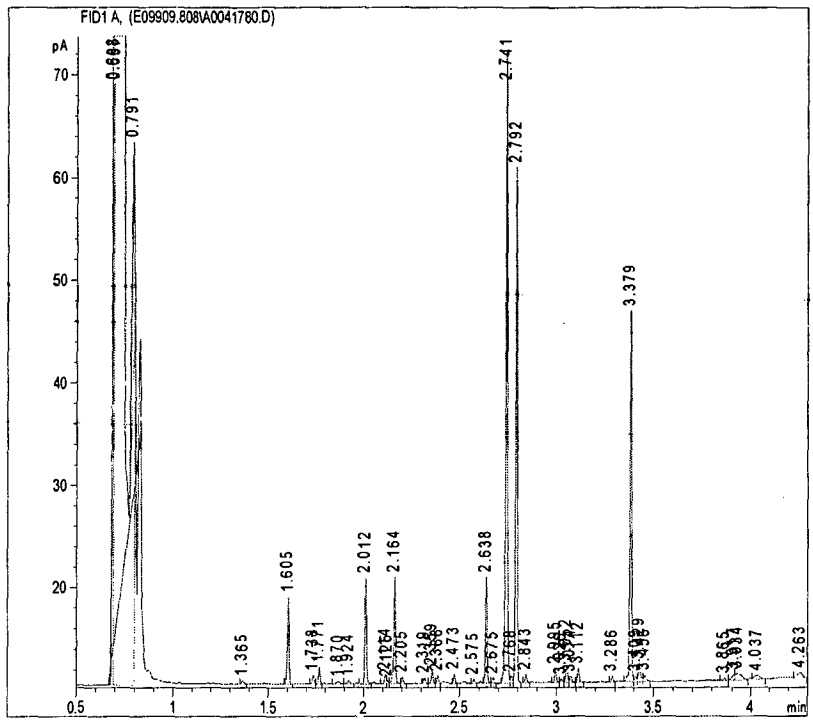


Fig.2.22.e FAME profile of lead resistant bacteria *Vibrio harveyi* strain M-11

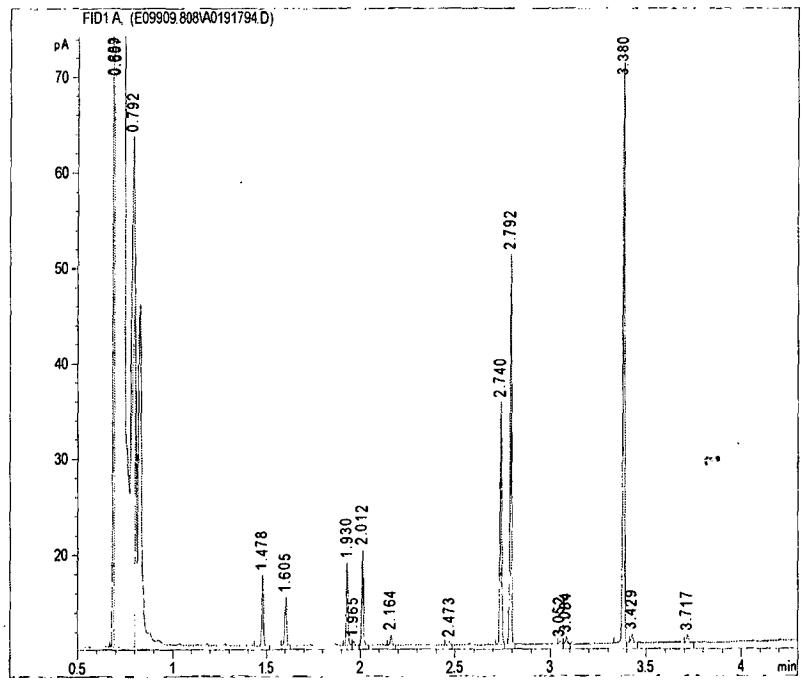


Fig.2.22.f FAME profile of lead resistant bacteria *Pseudomonas aeruginosa* strain WI-1

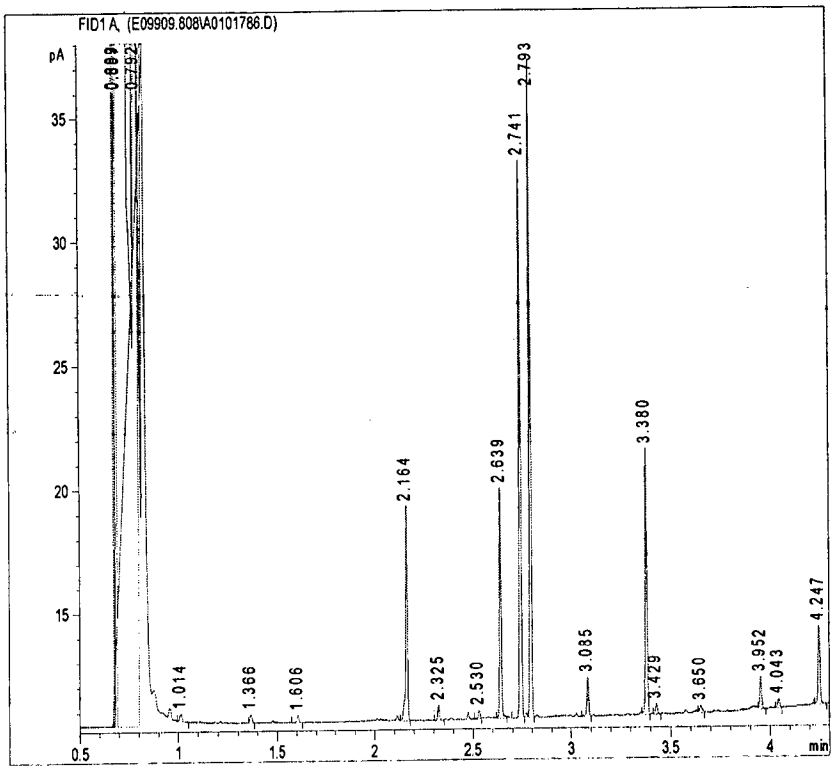
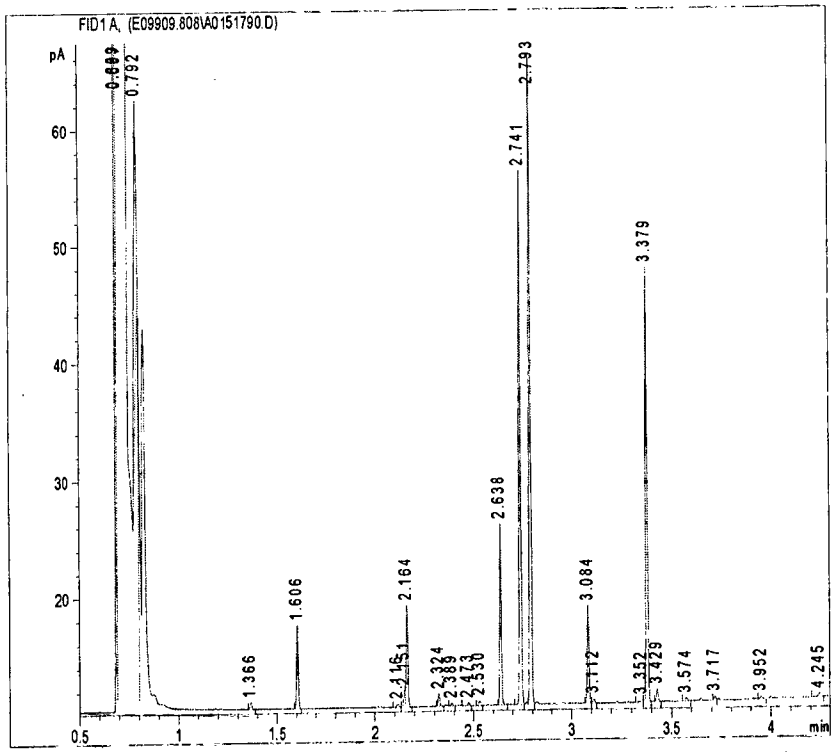


Fig.2.23.a FAME profile of lead resistant *Proteus penneri* strain GM-10 from Lead battery manufacturing industry



b

Fig.2.23.b FAME profile of lead resistant *Salmonella choleraesuis* strain 4A from Lead battery manufacturing industry

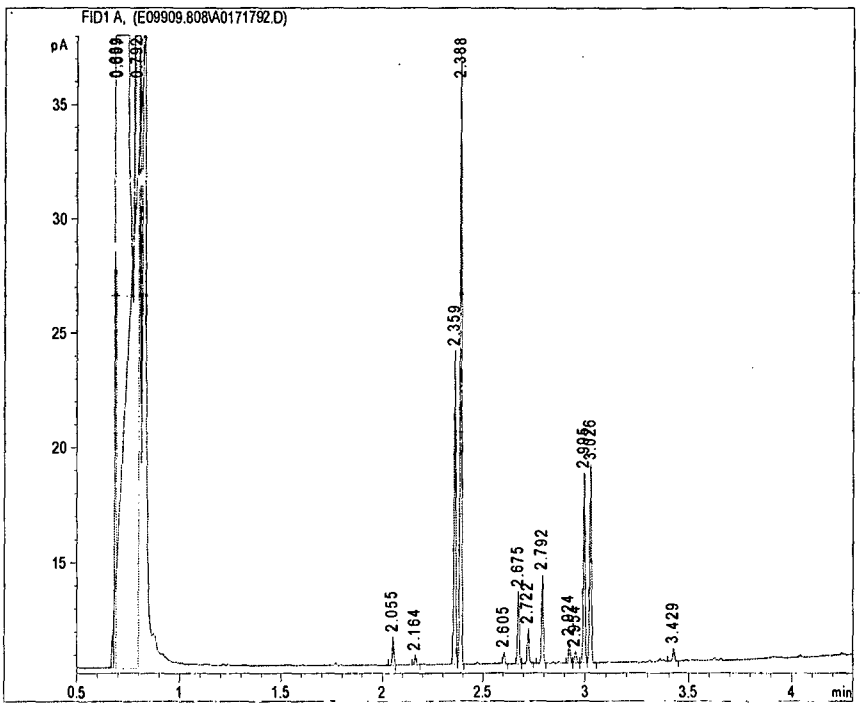


Fig.2.23.c FAME profile of lead resistant *Bacillus subtillis* strain GM02 from Lead battery manufacturing industry

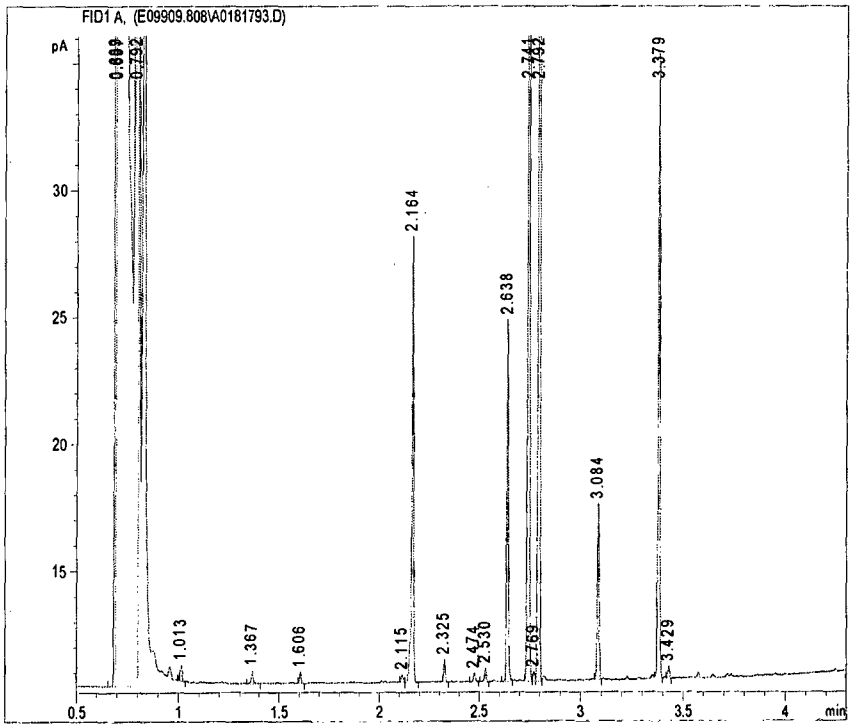


Fig.2.23.d FAME profile of lead resistant *Providencia rettgeri* GM04 from Lead battery manufacturing industry

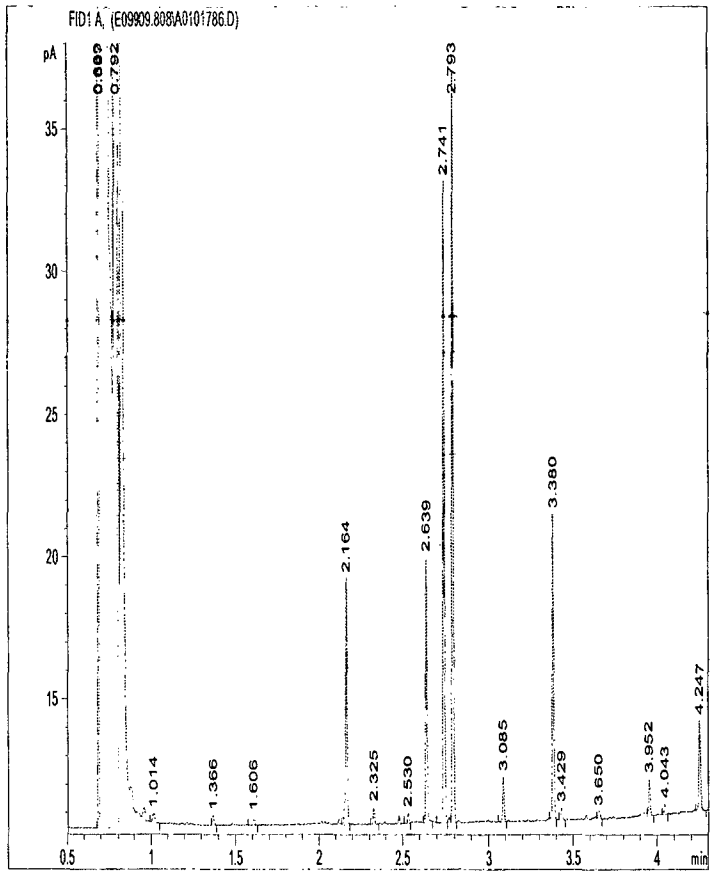


Fig.2.23.e FAME profile of lead resistant *Proteus penneri* strain GM03 from battery manufacturing industry

Table 2.9 Physicochemical characteristics of Industrial waste samples

Sr. No.	Sampling stations	pH	Temperature (°C)
1	Germania batteries Pvt. Ltd.	6.8	28
2	Permalite batteries Pvt. Ltd.	7.2	30
3	Shrine industries Pvt. Ltd.	6.5	32
4	United lead oxide Pvt. Ltd.	6.8	29
5	Bharti electronics Pvt. Ltd.	7.2	28
6	D-Link Pvt. Ltd.	7.0	30

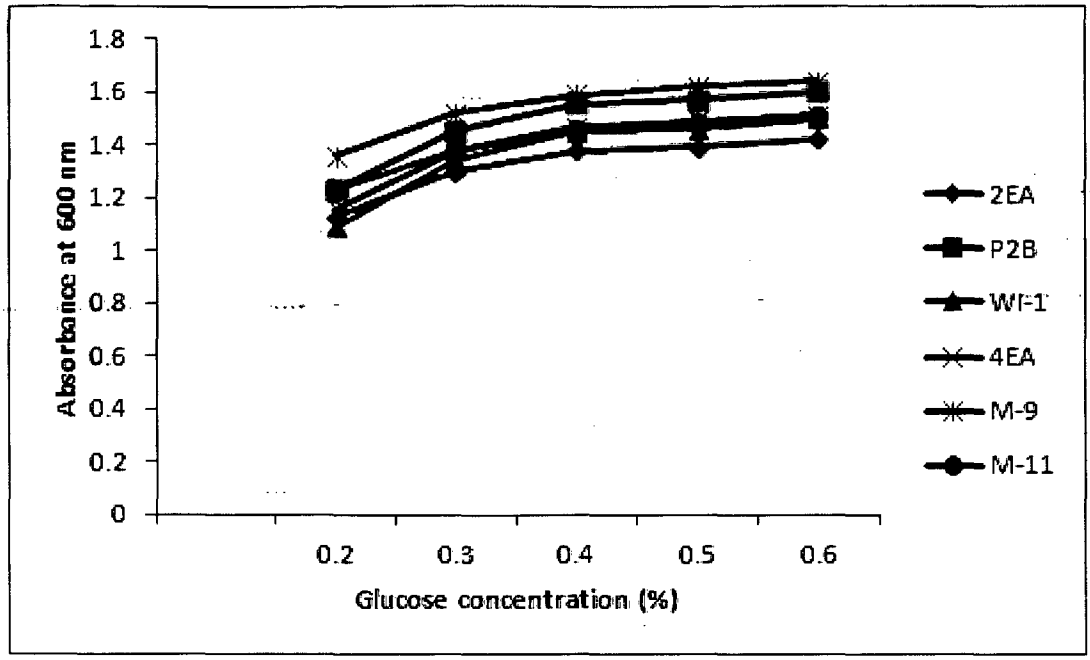
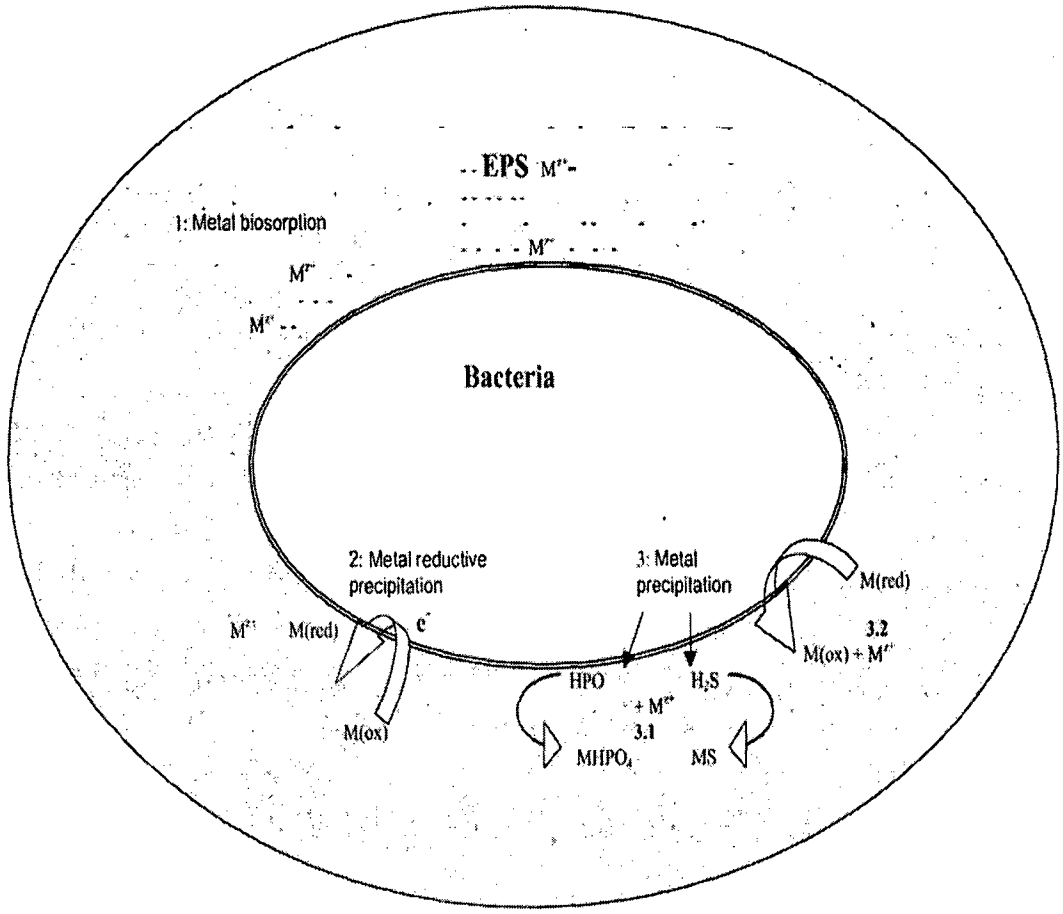


Fig. 2.24 Growth behaviour of lead resistant bacterial strains in TMM supplemented with different concentrations of glucose in the presence of 100 µM lead nitrate.



Role of bacterial exopolysaccharide in heavy metal resistance

(van Hullebusch et al., 2003)

CHAPTER-III

CHAPTER III

Biochemical characterization of lead resistant bacterial strains

MATERIALS AND METHODS

3.1 Characterization of lead resistant, exopolysaccharide producing *Enterobacter cloacae* strain P2B

3.1.1 Purification and characterization of bacterial EPS

Exopolysaccharide (EPS) production by *Enterobacter cloacae* strain P2B was monitored in the TMM medium without (control) and with 1.6 mM lead nitrate in order to study the role of EPS in lead resistance. Purification of extracellular EPS was done using modified ice cold ethanol precipitation method (Bramhachari and Dubey, 2006). Cultures grown without and with 1.6 mM lead nitrate in a total volume of 1 litre TMM were harvested separately at 10,000 rpm for 15 minutes at 4°C. The supernatant was filtered through 0.22 µm cellulose nitrate filter (Millipore filters, Bangalore, India). EPS was precipitated from the final filtrate by addition of three volumes of ice cold ethanol and stored at 4°C overnight. Resulting precipitate was centrifuged and washed with 70 % ethanol. EPS precipitate was suspended in distilled water and dialysed for 24 h at 4°C using dialysis tube with cut off size-13 kDa (Sigma Aldrich, Germany) against distilled water. This extracellular purified EPS was lyophilised and stored.

In order to extract cell bound EPS, bacterial cell pellet of 1 litre culture suspension was resuspended in appropriate volume of EDTA solution (10 mM EDTA + 1.5 mM NaCl , pH 8) and heated at 50°C for 3 minutes (Royan et al., 1999) and purified in the same way as extracellular EPS. Extracellular and cell bound EPS was combined, lyophilized and stored at

room temperature in a sealed bottle until chemical and physical analysis. EPS production was recorded and expressed in terms of dry weight in control and lead exposed conditions. Chemical composition of EPS produced under the stress of 1.6 mM lead nitrate was determined by GC-MS and FTIR analysis along with Alcian blue staining as per the standard procedures.

3.1.2 Rheological characteristic of EPS

Rheological properties of EPS isolated from *Enterobacter cloacae* strain P2B grown without (control) and with 1.6 mM lead nitrate were determined using programmable Rheometer (Model DV-III, Brookfield, USA) at 25° C. EPS (10mg/ml) in distilled water was used for above analysis. We measured the viscosity of the aqueous solution of EPS over a range (3.75-33.8 s^{-1}) of shear rates. Viscosity (cP) values were plotted against corresponding values of shear rate (s^{-1}) and data was analysed (Arias et al., 2003).

3.1.3 SEM-EDX analysis of bacterial EPS

In order to reveal whether EPS is involved in lead sequestration, scanning electron microscopy coupled with energy dispersive X-ray spectrometry (SEM-EDX, JEOL JSM-5800LV, USA) was performed on bacterial culture grown in TMM supplemented with 1.6 mM lead nitrate. Culture smear was prepared on glass slide, air dried and then fixed in 3 % glutaraldehyde overnight with 50 mM Potassium phosphate buffer. The slide was gently washed thrice with phosphate buffer (Appendix-B2) and dehydrated in gradually increasing concentrations of ethanol i.e., 10, 20, 50, 70, 80, 90, 95, and 100% for 15 min each, air dried and finally stored in vacuum chamber prior to SEM-EDX analysis (Neumann et al., 2005).

3.1.4 Fourier-transformed infrared (FTIR) spectroscopic analysis of bacterial EPS

FTIR spectroscopic analysis of lyophilized EPS was done to reveal major functional groups of EPS. A sample containing 2 mg dried and ground EPS was mixed with 200 mg dry KBr, which was followed by pressing the mixture into 16 mm diameter mould (Bramhachari and Dubey, 2006; Bramhachari et al., 2007). FTIR spectrum was recorded in the region 400-4000 cm^{-1} using SHIMADZU FTIR, model 8201PC (Shimadzu, Japan).

3.1.5 GC-MS analysis of EPS

GC-MS analysis of EPS was performed to investigate presence of neutral sugars. The lyophilized EPS was acid hydrolyzed into monosaccharides followed by conversion of their sugars into respective alditol acetate derivatives, which are finally analysed by GC-MS (model GC-MS-QP-2010–plus; Shimadzu, Japan).This instrument was equipped with a fused silica capillary column coated with CP Sil-88 -25 m. i.d. 0.32 mm, ds 0.12(Chrompack, Middleburg, Netherland) and Helium (3 ml/min) was used as a carrier gas. (Albersheim et al., 1967; Fox et al., 1988). EPS was hydrolysed using 6N HCl at 100°C for 1hr into monomers. The sample was evaporated to dryness at 50° C inside a fume hood. To the sample 100 µl of Sodium borohydride ($NaBH_4$) was added (100 mg/ml in H_2O) to reduce monomers into their respective alditols. Sample was evaporated to dryness at 50° C under fuming hood. After cooling at room temperature. 300 µl of acetic anhydride was added to each vial containing the sample followed by heating at 100° C for 15 min (acetalization step) in order to convert alditols into alditol acetates which was analysed by GC-MS. The following temperature gradient program was used: 75°C for 2 minutes followed by an increase from 75°C to 175°C at a rate of 50° C per min and finally 40 minutes at 230°C. The m/z peaks representing mass to charge ratio characteristic of the monosaccharide sugar fractions were compared with those in the mass spectrum library of the corresponding sugars.

3.1.6 Alcian blue staining of bacterial EPS

Alcian blue staining of EPS produced by *Enterobacter cloacae* strain P2B was performed to investigate its acidic or basic nature. Alcian blue is cationic dye used to stain acidic polysaccharides. Bacterial culture grown in presence of 1.6 mM lead nitrate was smeared on a glass slide was air dried and hydrated with distilled water. The slide was flooded with 20 µl of 0.1% Alcian blue dye in acetic acid (pH 2.5) for 5 minute. Destaining was done gently using running water followed by air drying and observation under oil immersion lence of light microscope (Alldredge et al., 1993; Bhaskar and Bhosle, 2005).

3.2 Characterization of lead resistant, *Providencia alcalifaciens* strain 2EA

3.2.1 Purification of extracellular precipitate produced by *Providencia alcalifaciens* strain 2EA

TMM broth (1 liter) containing 1.4 mM lead nitrate and without lead nitrate (control) was inoculated with overnight grown culture of the lead-resistant *Providencia alcalifaciens* strain 2EA. After 20 h incubation at 34 °C the culture suspensions were centrifuged at 2,000 rpm at room temperature to separate any extracellular bioprecipitate from bacterial cells. Since the bioprecipitate was insoluble, it was located in the pellet fraction; no significant amount of a brown solid was present in the supernatant. The precipitate was washed six times using deionized distilled water to completely remove bacterial cells. After the final washing of precipitate using centrifuge, the supernatant was decanted, and the pellet (precipitate) was gently removed from the centrifuge tubes with deionised distilled water into clean, empty plastic petri dishes. Pellet, was dried in desiccator for 48 h.

3.2.2 Chemical analysis and identification of the precipitate

3.2.2.1 XRD analysis of the precipitate

X-ray diffraction analysis of precipitate produced by *Providencia alcalifaciens* strain 2EA was done by grinding and smearing the precipitate on the XRD glass slide pre-coated with ultra-thin layer of petroleum jelly. Scintag X3 diffractometer coupled with Cu K radiation and Li-drifted germanium detector was used and data was collected at a speed of 2 degrees/min. XRD data was analysed to identify the compound using library of compounds for XRD provided by joint commission on powder diffraction software (JCPDS).

3.2.2.2 SEM-EDX analysis of the precipitate

Scanning electron microscopy coupled with energy dispersive X-ray spectrometry (JEOL JSM-5800LV, USA) was done to examine the chemical nature and particle size of the precipitate produced by lead resistant *Providencia alcalifaciens* strain 2EA. The precipitate was placed on a metal stub and coated with gold prior to analysis using SEM-EDX (Nadagouda et al., 2009).

3.3 Morphological characterization of *Pseudomonas aeruginosa* 4EA exposed to lead nitrate

Scanning electron microscopy coupled with energy dispersive X-ray spectrometry (SEM-EDX) (JEOL JSM-5800LV, USA) was employed to examine morphological changes and lead biosorption in terms of weight % as compared to major and minor elements present on the cell surface of lead-resistant *Pseudomonas aeruginosa* strain 4EA exposed to toxic levels of lead nitrate during exponential growth phase. Cells grown in TMM without lead served as control for SEM-EDX analysis. Cells were also grown in TMM with 0.8 mM lead

nitrate and effect of lead on cell morphology was analyzed by fixing bacterial cells in 3% glutaraldehyde overnight with 50 mM phosphate buffer at 4°C. Cells were washed thrice with phosphate buffer, pH 7 and dehydrated in gradually increasing concentrations of ethanol, i.e., 10, 20, 50, 70, 80, 90, 95, and 100% for 15 min each, air dried and stored in vacuum chamber prior to SEM-EDX analysis (Neumann et al., 2005).

3.4 Characterization of siderophore production in *Pseudomonas aeruginosa* strain 4EA exposed to lead nitrate

3.4.1 Characterization of siderophore

Pseudomonas aeruginosa strain 4EA was grown in TMM with different concentrations of lead nitrate; cells in stationary phase were harvested to collect yellow–green supernatant containing siderophore which was filter sterilized (0.22 μm). Siderophore production was monitored by chrome azurol S agar diffusion (CASAD) assay (Shin et al., 2001). In CASAD assay, each well (5 mm) was filled with 40 μl of siderophore containing supernatant and incubated for 2 h at 30°C. After incubation the diameter of orange halo around each well was measured and compared. Desferal mesylate (Sigma, USA), was used as a positive control and sterile TMM as negative control. Characterization of siderophore of lead-resistant *Pseudomonas aeruginosa* strain 4EA was done using UV–Vis spectrophotometer (Shimadzu, UV-2450, Japan) and spectrofluorimeter (Shimadzu, RF-5301-PC, Japan). Siderophore pigments were extracted from culture medium using organic solvents chloroform or ethylacetate for spectrophotometric analysis.

CAS agar plates were prepared using 60.5 mg Chrome azurol - S(CAS) dissolved in 50 ml distilled water and mixed with 10 ml iron (III) solution (1 mM $FeCl_3$. $6H_2O$, 10 mM HCl). While stirring it was added to a solution of Hexadecyltrimethyl ammonium bromide

(HDTMA, 72.9 mg) dissolved in 40 ml distilled water. The resultant dark blue solution was sterilized using autoclave. Tris-minimal agar (900 ml, pH 6.8) was autoclaved and supplemented with filter sterilized 0.4% glucose. The blue dye solution was finally mixed with TMM agar without generating foam and poured into petri plates (Schwyn and Neilands, 1987).

3.5 Intracellular lead bioaccumulation by lead resistant *Pseudomonas aeruginosa* strain WI-1

Pseudomonas aeruginosa strain WI-1 grown in TMM with 0.6 mM lead nitrate was harvested by spinning at 8000 rpm for 10 min and cell pellet was washed with 20 mM EDTA solution to remove cell surface bound lead. Pellets were dried at 100° C and 0.1 g of dried cell pellet was digested with concentrated HNO_3 using microwave digestion system and level of intracellular lead was determined by Atomic absorption spectrophotometer (AAS-Varian AA240 FS, Australia) following standard procedure of Joshi et al., 2008.

3.6 Detection of anti-fungal activity of lead resistant *Pseudomonas aeruginosa* strain WI-1

Antifungal activity of filter sterilised (0.22 µm), 50 µl culture supernatant of *Pseudomonas aeruginosa* strain WI-1 grown in TMM was evaluated on the plant pathogen, *Fusarium oxysporum* NCIM 1008 (NCL, Pune, India) using well diffusion method on Potato dextrose agar. Spore suspension of *Fusarium oxysporum* NCIM 1008 was prepared in sterile Tween80 and spread plated on PDA. Culture medium without inoculum of the test bacterial strain served as negative control. Siderophore production by the test bacterial strain WI-1 was also monitored using chrome azurol-S agar assay, spectrophotometer and spectrofluorimeter (Schwyn and Neilands, 1987, Naik and Dubey, 2011).

3.7 Detection of Indole acetic acid (IAA) production in lead resistant bacteria

Pseudomonas aeruginosa strain WI-1 was inoculated in nutrient broth without (control) and with tryptophan (500 µg/ml) and incubated at 30°C. 5 ml culture suspension was removed from each tube and harvested at 10,000 rpm for 15 min at room temp. An aliquot of 2 ml supernatant was transferred to a fresh tube to which 100 µl of 10 mM ortho-phosphoric acid and 4 ml of a reagent A containing 1ml of 0.5 M $FeCl_3$ in 50 ml of 35% $HClO_4$ was added. The mixture was incubated at room temperature for 25 min and development of intensity of pink colour was recorded (Bano and Musarrat, 2003).

3.8 Detection of HCN production by lead resistant bacteria

In order to monitor HCN production by *Pseudomonas aeruginosa* strain WI-1, bacterial culture was spot inoculated on King's B agar (King and Adler, 1964). A sterilized filter paper saturated with 1% solution of picric acid and 2% sodium carbonate was placed in the lid of this petri plate. The petri plate was sealed with parafilm and incubated at 30°C for 4 days. A change in colour of the filter paper from yellow to redish brown as an indicator of cyanogenic activity (Bano and Musarrat, 2003).

RESULTS AND DISCUSSION

3.9 Characterization of exopolysaccharide produced by *Enterobacter cloacae* strain P2B

Extracellular polymeric substances (EPS) are biosynthetic polymers produced by both prokaryotic and eukaryotic microorganisms growing in natural as well as artificial environments either as single species, in binary association or in heterogenous communities. Irrespective of their origin, EPS are localized at or outside the bacterial cell surface and comprised of a variety of high molecular weight organic macromolecules such as polysaccharides, proteins, nucleic acids, phospholipids along with other non-polymeric constituents of low molecular weight (Wingender et al., 1999; Bhaskar and Bhosle, 2005). During growth under natural environment, bacterial EPS play important role in cell adhesion, formation of microbial aggregates such as biofilms, flocs, sludges and biogranules (Sutherland, 2001; Tay et al., 2001; Comte et al., 2006) and protect cells from hostile environments. They are also involved in the degradation of particulate substances, sorption of dissolved materials including heavy metals, (Gutnick and Bach, 2000; Morillo, et al., 2006) leaching of minerals from sulphide ores as well as biocorrosion (Gehrke et al., 1998).

Over the last few decades studies on the use of microorganisms for environmental restoration have primarily focused attention towards exploiting microbial potential for remediation of heavy metal contamination in both terrestrial and aquatic environments. Bioremediation of toxic metals and radionuclides from polluted sediments and waste stream employ living and / or non-living microbial biomass or isolated biopolymers as agents for biosorption (Gadd, 2000; Morillo, et al., 2008; Braissant et al., 2007; Pal and Paul 2008). These relatively simple and inexpensive technologies try to exploit the cation-binding ability of microbial biomass / biopolymer to form a stable, non-toxic complex. The electrostatic

interactions between the metal ligands and negatively charged biopolymeric substances outside the cells lead to formation of stable complexes. Though availability of cheap biomass and immobilization techniques have made bioremediation process advantageous, the metal-binding capacity depends on the nature of biomass and availability of specific biopolymers. Moreover, microbial EPS differs both in specificity and metal sorption capacity with only few functional groups potentially involved in cation-binding.

In our studies *Enterobacter cloacae* strain P2B which resists lead nitrate up to 1.6 mM showed enhanced production of EPS from 28 mg/L without lead (control) to 108 mg/L with 1.6 mM lead nitrate in TMM. Furthermore, it was revealed that viscosity of EPS produced by *Enterobacter cloacae* strain P2B increased from 33.3 cP to 63 cP when exposed to 1.6 mM lead nitrate (Fig.3.1). Viscosity of EPS produced by bacterial cells with and without lead did not decrease significantly with increase in shear rate thus showed Newtonian fluid like behaviour (Calvo et al., 1998; Kumar and Singhal, 2011). FTIR analysis of EPS interestingly revealed that two different kinds of EPS were produced in absence and presence of 1.6 mM lead nitrate as there was significant shift and change in intensity of functional groups. Significant structural change in constituents of EPS was also noticed in presence of lead which may result in more efficient binding of lead (Fig.3.2.a, b).This clearly suggested involvement of EPS in lead resistance in the test bacterium.

3.9.1 Chemical composition of EPS

It is imperative to know the chemical constituents of microbial EPS to study its metal resistance mechanism. Microbial EPS is composed of sugars, uronic acid and proteins which play an important role in metal binding and sequestration due to presence of hydroxyl, carboxylic, amide, phosphoryl and sulphate groups (Bramhachari et al., 2007; Braissant et al., 2007).

In our studies FTIR spectrum of purified EPS of *Enterobacter cloacae* strain P2B produced in the presence of 1.6 mM lead nitrate revealed characteristic functional groups such as broad stretching hydroxyl group at 3278 cm^{-1} and weak C-H stretching of methyl group at 2927 cm^{-1} (Fig.3.2.b). A broad stretching of C-O-C, C-O at 1076-1213 cm^{-1} corresponds to the presence of carbohydrates. Absorption peak between1076-1122 cm^{-1} ascertained presence of uronic acid and o-acetyl ester bonds. The absoption bands 1662 cm^{-1} (mainly C=O stretch) and 1529 cm^{-1} (mainly N-H stretch) can be attributed to amide I and amide II of amide bond due to peptide bond of protein. Carboxyl group at 1404 cm^{-1} indicates symmetric stretching. Presence of glycosidic bond in sugars was confirmed by a band at 804 cm^{-1}. Similar findings were put forth with reference to EPS produced by marine *Vibrio* spp. (Bramhachari and Dubey 2006; Bramhachari et al., 2007).

GC-MS analysis of EPS produced by test bacterium *Enterobacter cloacae* strain P2B revealed that it is primarily composed of neutral sugars viz. rhamnose, arabinose, xylose, mannose, galactose and glucose with retention time 16.734, 18.174, 18.882, 19.147, 19.492 and 22.256 minutes respectively (Fig.3.3) whereas mass to charge ratio (m/z) matched very well with the standards neutral sugars in the MS library (Fig.3.4.A-F).These results also matched with earlier reports on the sugars of EPS produced by marine *Vibrio furnissi* strain VB0S3 ((Fox et al., 1988; Pithard and Finch 2001; Bramhachari et al., 2007).Glucose and galactose were most abundant monosaccharides of this heteropolymeric EPS. Percent relative abundance of sugars are rhamnose, 2.64%; arabinose, 8.77%; xylose 15.36%; mannose, 8.23%; galactose, 26.53% and glucose, 37.47% respectively. Alcian blue staining resulted in blue colour of EPS whereas bacterial cell appeared pink which confirmed acidic nature of this exopolysaccharide (Fig.3.5).Similar result was demonstrated by Bhaskar and Bhosle (2005) in case of *Phaeocystis* sp and Alldredge, et al., 1993.

3.9.2 Characterization of EPS using SEM-EDX

In order to study involvement of bacterial EPS in lead sequestration at the cell surface of the test bacterium, Scanning electron microscopy coupled with EDX was done which revealed mucoidal nature of the exopolymer surrounding the bacterial cells and interestingly confirmed that EPS of *Enterobacter cloacae* strain P2B can sequester 17 % lead (as weight %) (Fig.3.6). Microbial polymers differ widely both in specificity and in their metal-binding capacity depending on their physicochemical properties therefore chemical composition of the EPS is helpful in order to understand the nature of the metal ion-EPS interactions and its involvement in metal resistance mechanism (Arias et al., 2003; Bhaskar and Bhosle, 2006; Morillo et al., 2006). The heavy metal-binding capacity of polysaccharides is usually attributed to the high hydrophilicity of the polymer due to the presence of hydroxyl groups along with other reactive functional groups and flexible structure of polymer chains (Iyer et al., 2004; Pal and Paul 2008). Bacterial EPSs contain ionizable functional groups, such as carboxyl, amide, sulphate, acetate, and hydroxyl groups which enable these polymers to bind heavy metals. It is interesting to note that sequestration of heavy metals by EPS is a metabolism independent process and mainly depends on interaction between metal cations and negative charged acidic functional groups present on EPS (van Hullebusch et al., 2003; Bhaskar and Bhosle 2006). *Pseudomonas marginalis* was able to resist 2.5 mM lead by sequestering lead in its exopolymer (Roane, 1999). Similarly exopolysaccharide produced by *Paenibacillus jamilae* can biosorb 303.03 mg lead/g EPS from lead solution (Morillo, et al., 2008). *Paenibacillus jamilae*, which could use toxic olive-mill wastes as fermentation substrate for production of exopolysaccharide showed preferential binding of lead using multi-metal sorption system (Morillo, et al., 2006). In marine *Pseudomonas aeruginosa* CH07 lead was entrapped in EPS and EPS served as a possible lead resistance mechanism (De et al., 2007, 2008). Enzymatic activities in bacterial EPS are also involved in

detoxification of heavy metals by transformation and subsequent precipitation resulting in entrapment of metals in these polymeric substances (van Hullebusch et al., 2003; Pal and Paul, 2008; Poli et al., 2010). Therefore understanding the mechanism by which EPS producing bacteria sequester/adsorb toxic heavy metals extracellularly to protect themselves from toxic effects on their physiological and biochemical processes is crucial to the development of microbial processes for concentration, removal and recovery of toxic metals from industrial wastes and effluents.

Bioremediation technology is simple, cost effective, highly efficient and environmentally sustainable as compared to physicochemical methods to remove heavy metals. Our studies have clearly demonstrated that lead ions could interact with carboxyl, hydroxyl and amide groups along with uronic acids from different chains of polyanionic exopolysaccharide produced by *Enterobacter cloacae* strain P2B. This exopolymer acts as an electrostatic bridge between them producing finally a mesh of polymer large enough to sequester 17% lead as weight percent. Thus use of isolated microbial biopolymer in metal bioremediation process is more economically viable and ecofriendly alternative than environmentally risky chemical methods such as precipitation, coagulation, ion exchange and electrochemical processes. Therefore *Enterobacter cloacae* strain P2B may serve as a potential microorganism in bioremediation of lead contaminated environmental sites. Since this exopolysaccharide also exibits good rheological properties it may be used in wide range of other applications viz. stabilizing, thickening, gelling, coagulating, film-forming, detergent, textile, paper and paint industries as well.

3.10 *Providencia alcalifaciens* strain 2EA showing precipitation of lead as lead Phosphate i.e. $Pb_9 (PO_4)_6$

Providencia alcalifaciens strain 2EA isolated from lead contaminated soil sample of car battery manufacturing M/S Permalite industry (Fig.3.7) containing 5,496 ppm lead could resist lead nitrate up to 1.4 mM (MIC of lead : 1.5 mM). No precipitate was produced in control flask but brown precipitate was observed in TMM amended with 1.4 Mm lead nitrate. This result confirmed that *Providencia alcalifaciens* strain 2EA produced brown bioprecipitate when exposed to lead nitrate. X- ray diffraction analysis of purified brown precipitate revealed close resemblance with lead phosphate, $Pb_9(PO_4)_6$ (Fig. 3.8) using the JCPDS library of compounds and JCPDS card no. 33-0768 (Mire et al., 2004; Martinez et al., 2004). Scanning electron microscopy and elemental analysis for lead and phosphorus and oxygen in the brown precipitate further confirmed that the precipitate is a complex lead phosphate (Fig. 3.9.a, b).

Bioprecipitation of toxic heavy metals viz. Cd, U, Zn, Pb into non-bioavailable forms such as metal sulphides and phosphates is one of the important resistant mechanisms present in microorganisms to withstand high levels of toxic heavy metals (Wang et al., 2002; Macaskie, et al., 2000; Mire et al., 2004). In very few cases chemical nature of the lead precipitate produced by bacteria has been determined. It is interesting to note that lead was precipitated on the cell surface of *Citrobacter* sp. as $PbHPO_4$ (Aickin et al., 1979) which was revealed by electron microscopy and X-ray elemental analysis (SEM-EDS). Levinson et al., (1996) have suggested intracellular accumulation of $Pb_3(PO_4)_2$ as precipitate produced by *S. aureus* when grown in the presence of high concentrations of soluble lead nitrate. *Vibrio harveyi* is also capable of precipitating lead as an unusual lead phosphate, $Pb_9(PO_4)_6$ (Mire et al., 2004). Similarly *Klebsiella* sp. cultured in phosphate-limited medium has been reported

to precipitate lead as PbS (Aiking et al., 1985). Phosphate solubilising *Enterobacter cloacae* resists lead by immobilizing lead as insoluble lead phosphate mineral i.e. pyromorphite (Park et al., 2011). X ray spectroscopic data (EXAFS) and Transmission electron microscopy (TEM) observations showed that the enhanced Pb accumulation by *Burkholderia cepacia* biofilms is due to the formation of nanoscale crystals of pyromorphites i.e $Pb_5(PO_4)_3OH$ (Templaton et al., 2003). In case of *Staphylococcus aureus* lead precipitation occurred in both lead-sensitive and lead-resistant strains, however the resistant strains were more effective in precipitation of lead (Levinson and Mahler, 1998). Lead resistant *Bacillus iodinium* GP13 and *Bacillus pumilus* S3 precipitates lead as lead sulphide (PbS) (De et al., 2008).

In our studies with lead resistant *Providencia alcalifaciens* strain 2EA, we have observed production of an insoluble, extracellular brown precipitate when cells were grown in TMM amended with 1.4 mM lead nitrate. XRD and SEM-EDX analysis further confirmed brown precipitate as $Pb_9(PO_4)_6$ (Mire et al., 2004; Martinez et al., 2004). *Providencia alcalifaciens* strain 2EA has developed resistance mechanism in which soluble toxic lead (Pb^{+2}) is transformed into insoluble, nontoxic, biologically unavailable $Pb_9(PO_4)_6$. It appears that this strain has evolved this bioprecipitation mechanism to survive in toxic level of lead nitrate. Bioavailability of toxic metals in the environment is an important factor for their toxicity on natural biota. Insoluble lead phosphate, $Pb_9(PO_4)_6$ is not available freely as lead ions (Pb^{+2}) to the test organism *Providencia alcalifaciens* strain 2EA making it resistant to lead. This study has provided first evidence of lead precipitation as $Pb_9(PO_4)_6$ by naturally occurring *Providencia alcalifaciens* strain 2EA which was isolated from soil contaminated with car battery waste containing lead as there is no report on precipitation of lead by *Providencia alcalifaciens*. Therefore we can employ lead resistant *Providencia alcalifaciens*

strain 2EA for bioremediation of toxic lead through its conversion into insoluble lead phosphate, $Pb_9(PO_4)_6$ extracellularly which can be removed easily by centrifugation.

3.11 *Pseudomonas aeruginosa* 4EA exposed to lead: Morphological characterization and biosorption studies

Pseudomonas aeruginosa strain 4EA isolated from soil sample of Germania company containing 7,767 ppm lead could resist up to 0.8 mM lead nitrate in TMM (MIC:1 mM). The cell morphology of *Pseudomonas aeruginosa* strain 4EA was normal when grown in TMM without lead, but significant change in cell morphology as reduction in cell size due to shrinkage was evidently observed when cells were exposed to 0.8 mM lead nitrate which was revealed by SEM (Fig. 3.10.a, b).

Change in morphology is one of the strategies that bacteria adopt to cope up with environmental stresses. It was observed that the maximum alterations in size occurred when the bacterium *Acidiphilium symbioticum* H8 was exposed to sub-inhibitory concentrations of Cu and Cd. Loosely packed coccobacillus-type normal cells formed characteristic chains of coccoidal, lenticular shape with constrictions at the junctions between them in the presence of Cd, whereas Cu induced their transformation into round cells. Ni caused cell aggregation, but Zn showed no effect (Chakravarty and Banerjee, 2008). Cadmium-exposed *Pseudomonas putida* showed extensive blebbing of the outer membrane along with polyphosphate granules containing Cd^{2+} which was revealed by electron microscopy. Cells from exponential phase cultures of cadmium adapted *P. putida* were found in clusters and were much smaller than control cells grown without cadmium and contained electron dense aggregates also (Higham et al., 1986). Scanning probe atomic force microscopy (AFM) analysis indicated that exposure of *Pseudomonas* sp. G1DM21 to 1 mM Cr (VI) for 24 h, leads to an increase in cell

length and height (Desai et al., 2008). Interestingly *Pseudomonas aeruginosa* strain 4EA along with reduction in cell size also showed biosorption of 11% lead (as weight %) as compared to other elements present on the cell surface when grown in 0.8 mM lead nitrate which was clearly revealed by SEM-EDX analysis. Whereas bacterial cells grown in TMM without lead nitrate demonstrated absence of any signal pertaining to lead (Fig. 3.10.a, b).

3.12 Characterization of siderophore production in *Pseudomonas aeruginosa* strain 4EA exposed to lead nitrate

Lead resistant *Pseudomonas aeruginosa* strain 4EA resists up to 0.8 mM lead nitrate in TMM and produced siderophore as yellow-green, diffusible pigment. UV–Vis spectrophotometric and spectrofluorimetric analysis of this yellow-green pigment clearly revealed presence of two types of siderophores such as pyochelin and pyoverdine. Pyochelin showed absorbance maxima at 247 nm and 310 nm in UV range, whereas pyoverdine showed strong absorbance at 370 nm (Fig. 3.11). Pyoverdine exhibited emission at 460 nm when excited at 405 nm whereas pyochelin emitted at 448 nm when excited at 350 nm (Fig. 3.12. a, b). The earlier UV–Vis spectrophotometric and spectrofluorimetric studies on microbial siderophores (pyochelin and pyoverdine) have also shown similar results (Cox and Adams, 1985; Namiranian et al., 1997; Pandey et al., 2005).

Lead enhanced gradual siderophore production was observed with increase in lead levels in the growth medium which was evidently revealed as increase in the diameter of orange halo on CAS agar plates using CASAD assay (Fig.3.13.). It is interesting to note that siderophore production was enhanced maximally up to 0.5 mM lead nitrate in TMM and was repressed above this level as evident from decrease in diameter of orange halo (Fig. 3.14.). *Pseudomonas aeruginosa* is known to secrete two chemically distinct iron chelators

(siderophore pigment) viz. pyoverdine and pyochelin in iron limited conditions to solubilise Fe^{+3} and transport into the bacterial cells via specific receptors (Cox and Adams, 1985; Namiranian et al., 1997). Besides Fe^{+3} these microbial siderophores also form stable complexes with metals such as Cd^{+2}, Pb^{+2}, and Zn^{+2} (Namiranian et al., 1997; Gilis et al., 1998; Hepinstall et al., 2005). Induction of bacterial siderophore synthesis in response to Cd^{+2}, Zn^{+2} and Cu^{+2} stress has also been reported which is responsible for detoxification of these heavy metals as a consequence of chelation (Clarke et al., 1987; Rossbach et al., 2000; Sinha and Mukherjee 2008) and thus proved to be an important strategy of microorganisms to sequester non- toxic (micronutrients) and toxic metals. In the present investigation, alteration in the cell morphology and enhancement of siderophore production in lead exposed *Pseudomonas aeruginosa* strain 4EA appeared to be the protective response of bacterial cells against toxic lead.

Lead is also known to inhibit chlorophyll biosynthesis; growth and bioproductivity of plants (Sengar and Pandey, 1996).We have clearly demonstrated that lead resistant *Pseudomonas aeruginosa* strain 4EA has potential to biosorb significantly high levels of lead and also to produce lead enhanced siderophores. Thus, this lead resistant bacterial strain may serve as a bioinoculant for crop plants to supplement sufficient amount of iron (Fe^{+3}) by producing lead enhanced siderophores along with biosorptive amelioration of lead present in contaminated crop fields.

3.13 Lead resistant *Pseudomonas aeruginosa* WI-1 also exhibits antifungal activity

Pseudomonas aeruginosa WI-1 resists up to 0.6 mM lead nitrate with MIC of 0.8 mM in TMM and accumulated lead up to 26.5 mg/g of dry cell biomass. Orange zone around bacterial colony on chrome azurol S agar proved the siderophore producing potential of this

strain (Fig.3.15.). This strain also produced a biocidal compound, which was present in the culture supernatant when cells were grown in TMM. The supernatant showed significant zone (2.6 ± 0.4 cm) of inhibition on a potato dextrose agar plate with lawn of *Fusarium oxysporum* NCIM 1008 by agar well diffusion method. Whereas uninoculated sterile growth medium i.e. TMM broth showed no zone of inhibition (Fig.3.16.a, b). This clearly revealed that the lead resistant *Pseudomonas aeruginosa* strain WI-1 also possessed anti-fungal activity against important phytopathogens of crop plants. UV–Vis spectrophotometric and spectrofluorimetric analysis of this yellow-green pigment clearly revealed presence of pyoverdine siderophore. Pyoverdine showed strong absorbance at 370 nm in the UV range. Pyoverdine exhibited fluorescent emission at 460 nm when excited at 405 nm (Fig.3.17). This isolate also produced HCN (Fig. 3.18) and plant growth promoting hormone Indole acetic acid (IAA). A change in colour of the filter paper placed in lid of petriplate from yellow to redish brown indicates cyanogenic activity of *Pseudomonas aeruginosa* strain WI-1. Development of intense pink colour after addition of ortho-phosphoric acid and reagent A to the culture supernatant of *Pseudomonas aeruginosa* strain WI-1grown in Nutrient broth with tryptophan confirmed high amount of IAA production.

Phytopathogens cause approximately 30% loss of agricultural production every year and soils contaminated with heavy metals severely affect crops productivity and also pose serious threat to human health (Sengar and Pandey, 1996; Jiang et al., 2008; Sayyed and Chincholkar, 2009). Therefore there is pressing need to clean up metal contaminated crop fields using biotechnological approach along with eradication of phytopathogens without affecting environmental sustainability. Plant growth promoting rhizobacteria (PGPR) are known to produce variety of secondary metabolites viz. siderophores, antibiotics, growth hormones and HCN which protect plants from phytopathgens and improve plant growth by supplying essential nutrients (Bano and Musarrat, 2003; Sayyed and Chincholkar, 2009).

Pseudomonas aeruginosa is a well known metabolically versatile PGPR which also shows heavy metal resistance (Sinha and Mukherjee, 2008). It is interesting to note that Siderophore (Pyochelin and Pyoverdine) mediated suppression of *Pythium*-induced damping off of tomato by *Pseudomonas aeruginosa* 7NSK2 and involvement of fluorescent siderophore produced by *Pseudomonas* sp. in the biological control of bacterial wilt in tomato suggests the possible role of siderophores in the biocontrol of phytopathogens (Buysens et al., 1996; Jagadeesh et al., 2001). Siderophore-rich culture supernatant culture supernatant and purified siderophore preparation of *Alcaligenes feacalis* has been reported to exert antifungal activity against *Aspergillus niger* NCIM 1025 and *Fusarium oxysporum* NCIM 1008 (Sayyed and Chincholkar, 2009). In our study we have clearly demonstrated that lead resistant *Pseudomonas aeruginosa* strain WI-1 possessed anti-fungal activity against important phytopathogen *Fusarium oxysporum* NCIM 1008 along with plant growth promoting metabolites viz siderophore and IAA. Thus this lead resistant bacterial strain may serve as a bioinoculant for crop plants in lead contaminated fields.

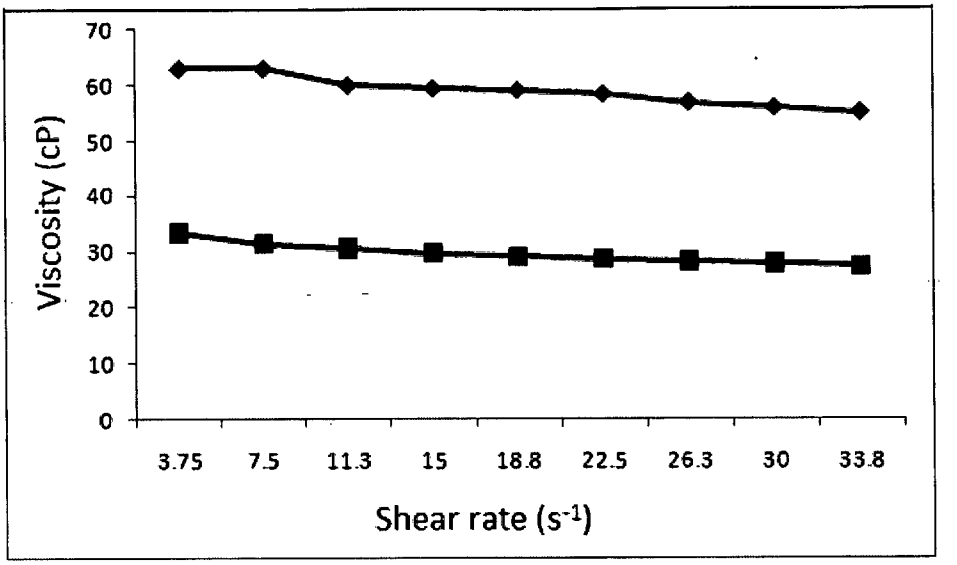


Fig.3.1 Rheological studies on EPS of *Enterobacter cloacae* strain P2B grown in presence of 1.6 mM (Filled diamond ◆) and 0 mM (Filled square ■) lead nitrate (control) in TMM

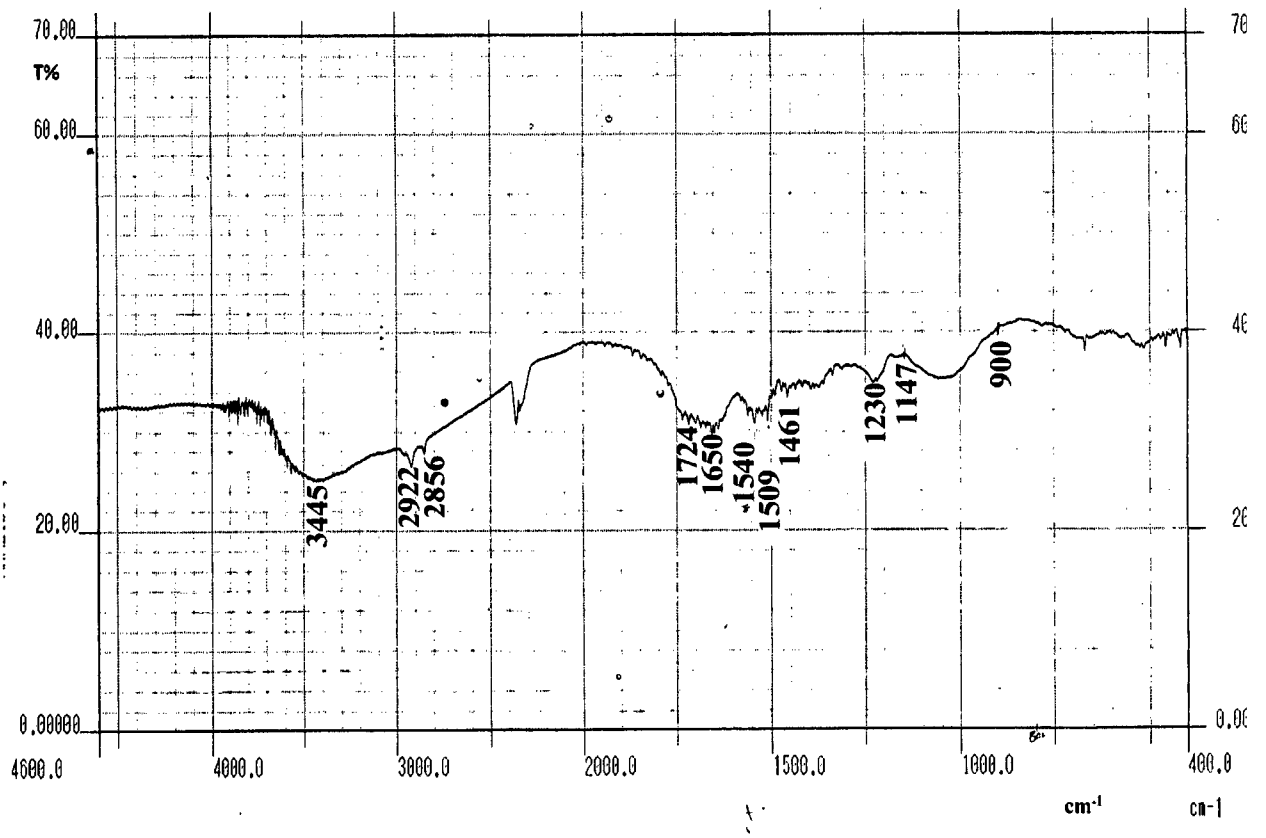


Fig.3.2.a FTIR spectrum of purified EPS produced by *Enterobacter cloacae* strain P2B grown without lead nitrate in TMM

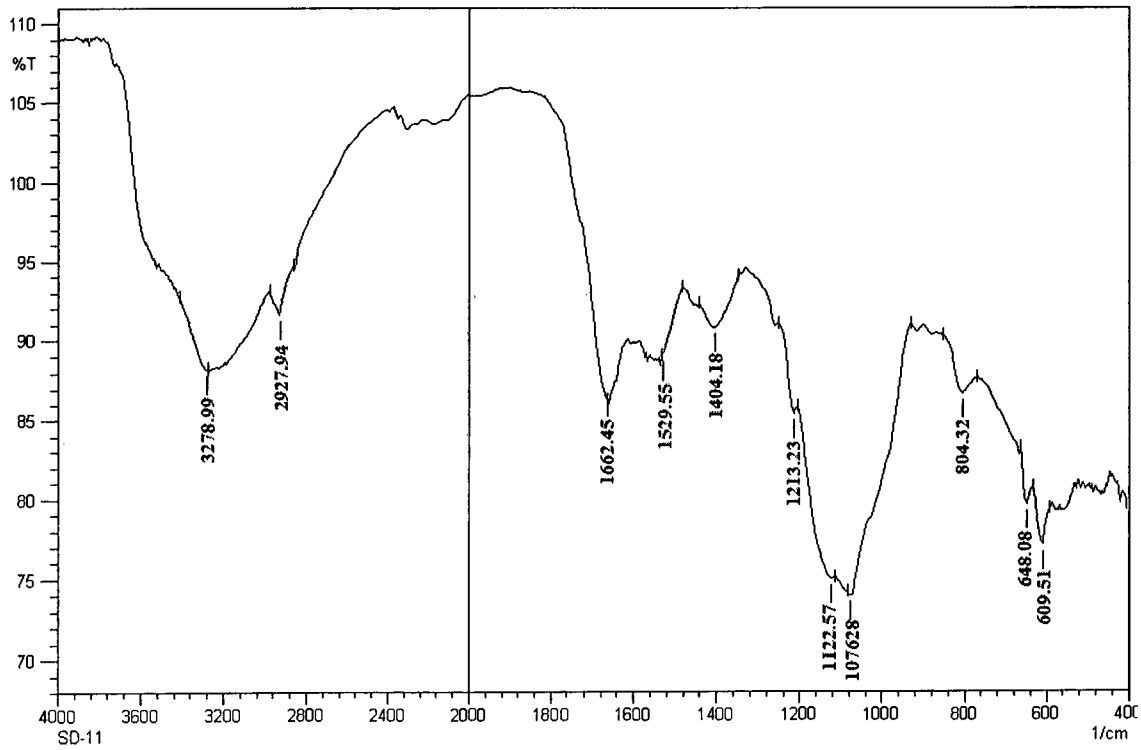


Fig.3.2.b FTIR spectrum of purified EPS produced by *Enterobacter cloacae* strain P2B in the presence of 1.6 mM lead nitrate in TMM

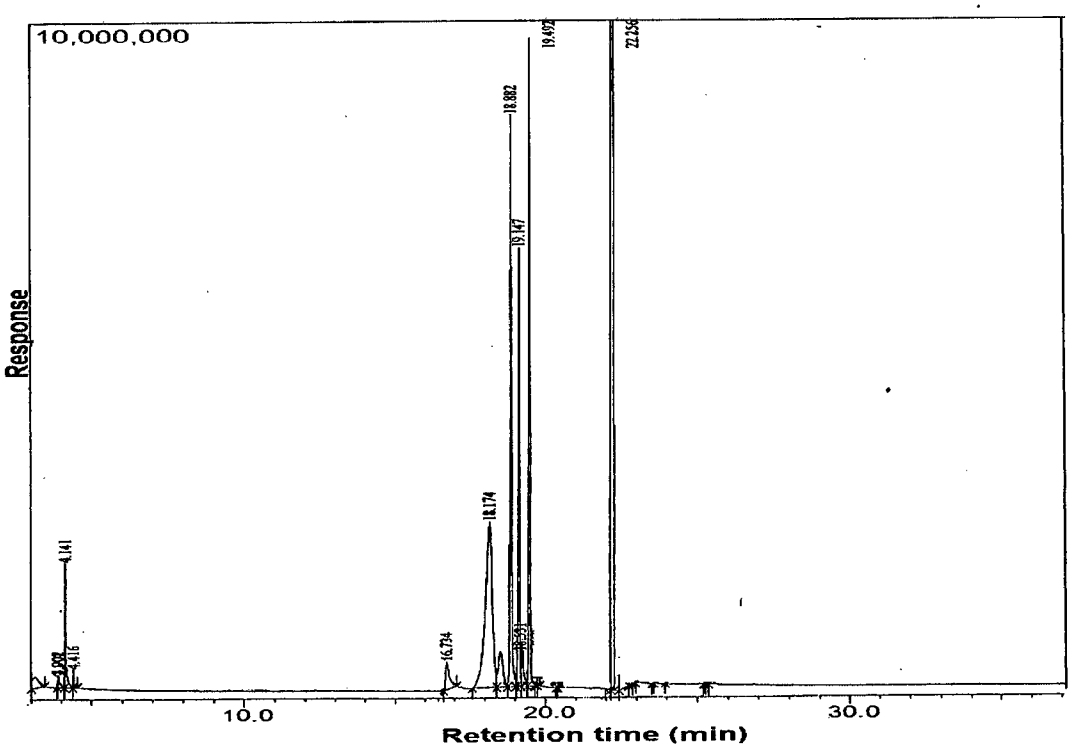
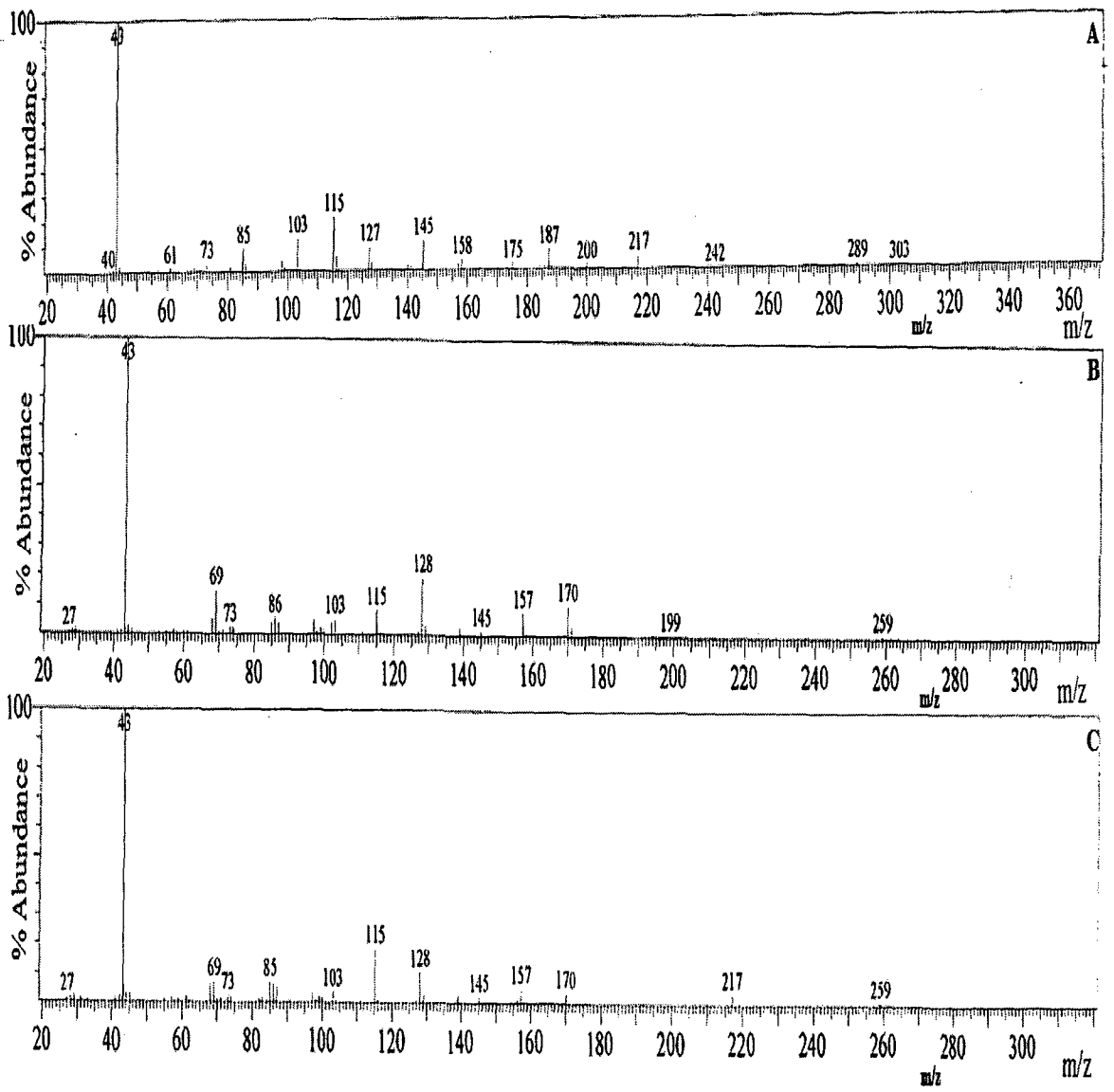


Fig.3.3 Gas Chromatogram of EPS produced by *Enterobacter cloacae* strain P2B grown in presence of 1.6 mM lead nitrate in TMM


A
% Abundance
100
43
40
61
73
85
103
115
127
145
158
175
187
200
217
242
289
303
20 40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360
m/z
B
% Abundance
100
43
27
69
73
86
103
115
128
145
157
170
199
259
20 40 60 80 100 120 140 160 180 200 220 240 260 280 300
m/z
C
% Abundance
100
43
27
69
73
85
103
115
128
145
157
170
217
259
20 40 60 80 100 120 140 160 180 200 220 240 260 280 300
m/z

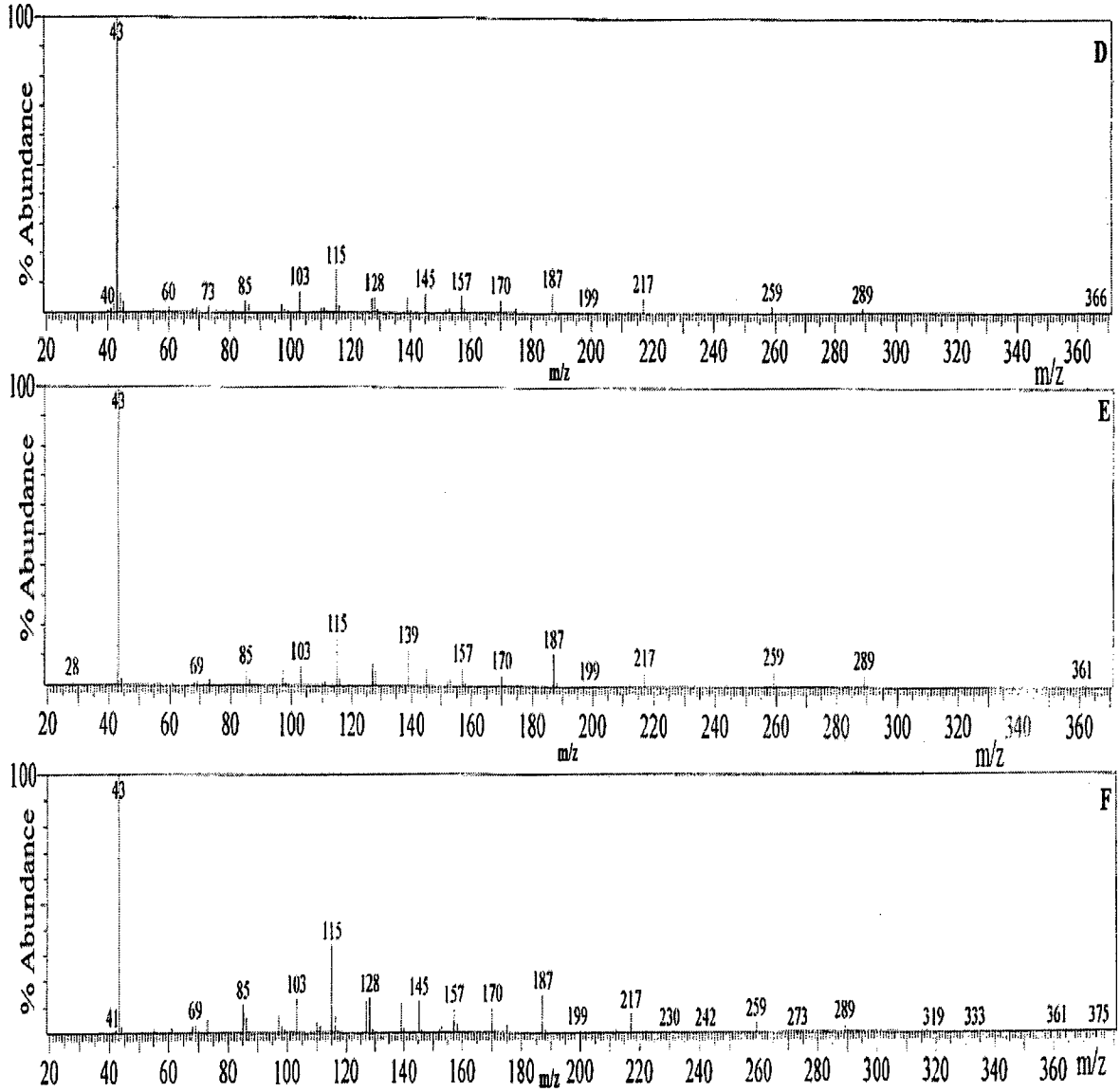


Fig.3.4.A-F Mass Spectra (with m/z ratio) of EPS produced by *Enterobacter cloacae* strain P2B in the presence of 1.6 mM lead nitrate.(A- Rhamnose, B- Arabinose, C-Xylose, D- Mannose, E- Galactose, F- Glucose)

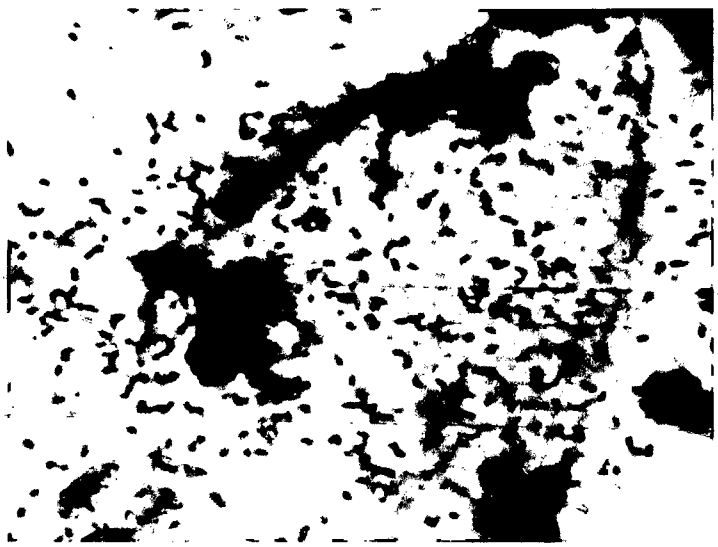


Fig. 3.5 Photomicrograph of EPS of *Enterobacter cloacae* strain P2B stained with alcian blue (X100)

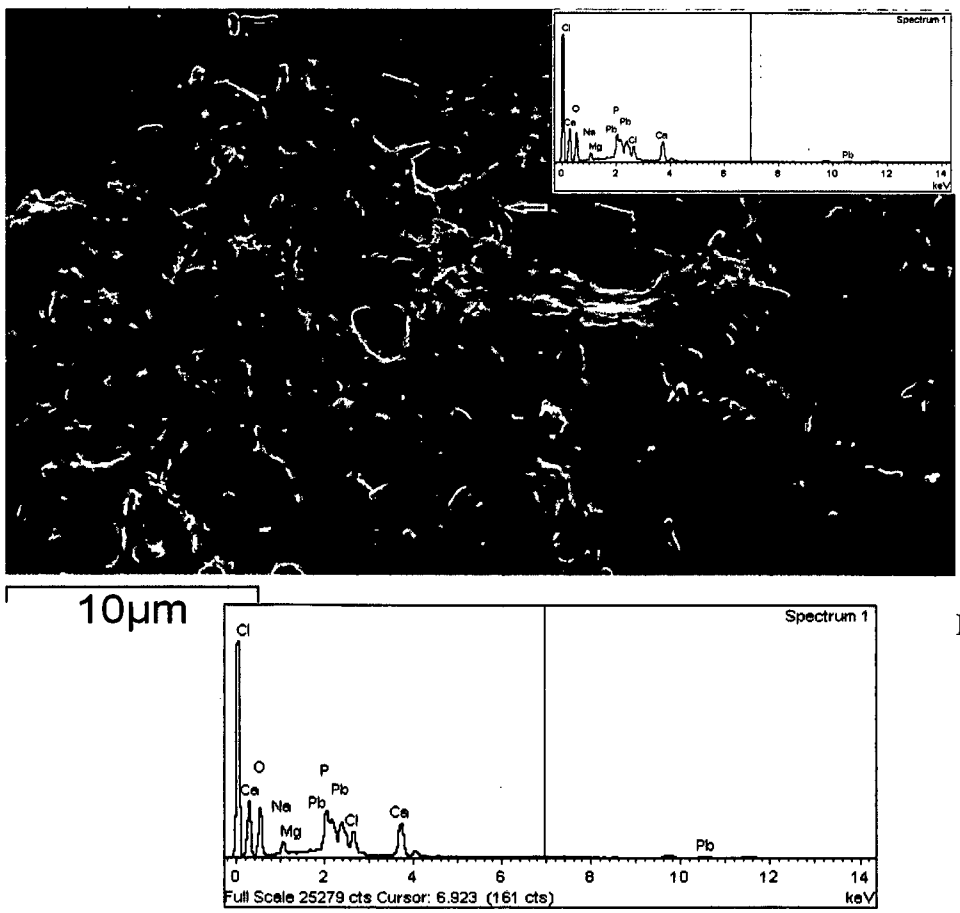


Fig.3.6 SEM - photomicrograph of EPS surrounding the cells of *Enterobacter cloacae* strain P2B grown in 1.6 mM lead nitrate in TMM and SEM - EDX analysis for biosorption of lead (SEM - magnification, X 9000) (Arrow points to the area considered for EDX analysis)

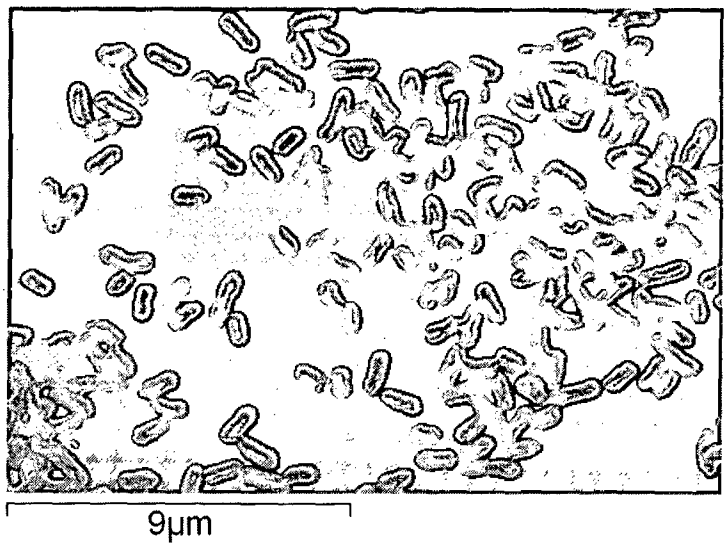


Fig.3.7 SEM-photomicrograph of lead resistant *Providencia alcalifaciens* strain 2EA (magnification: X9000)

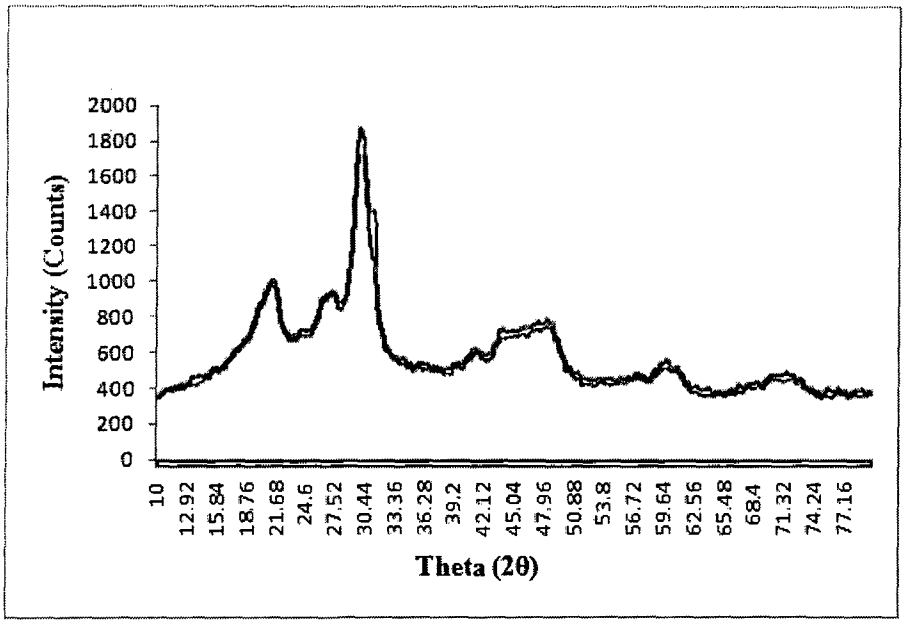
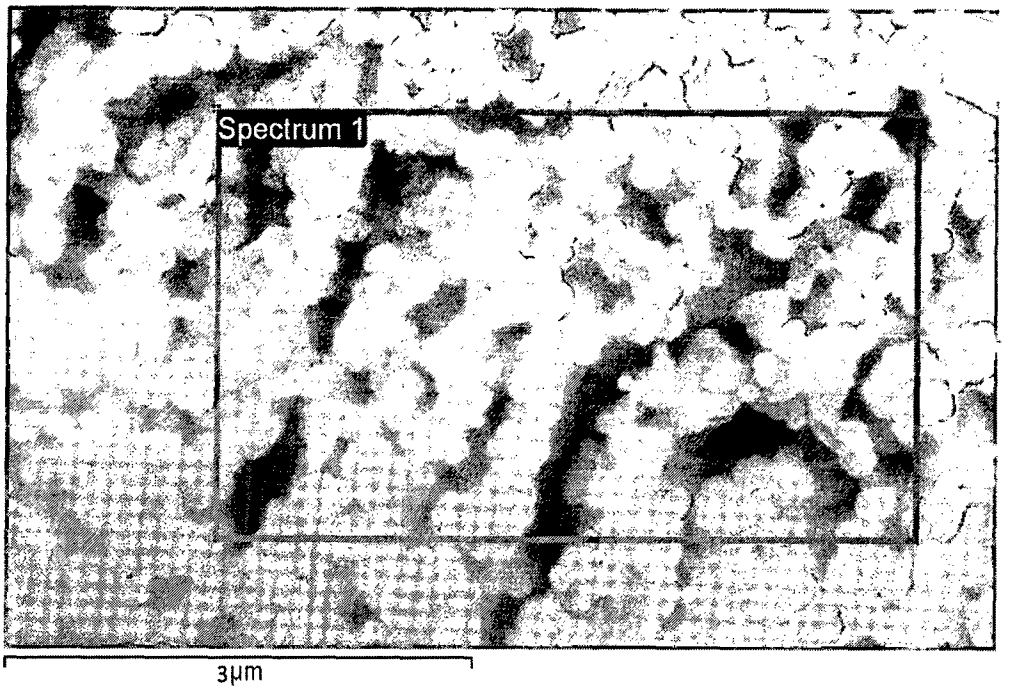
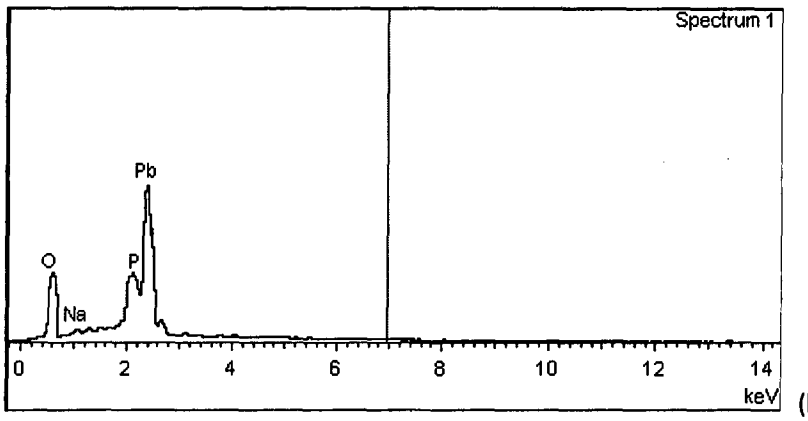


Fig.3.8 X-ray diffraction pattern of purified brown precipitate produced by *Providencia alcalifaciens* strain 2EA in the presence of 1.4 mM lead nitrate in Tris-minimal medium (JCPDS pattern 33-0768)



(a)



(b)

Fig.3.9.a, b SEM- photomicrograph (a) and Energy dispersive X-ray analysis (b) of extracellular brown precipitate produced by *Providencia alcalifaciens* strain 2EA (Area marked with square is used for EDX analysis)

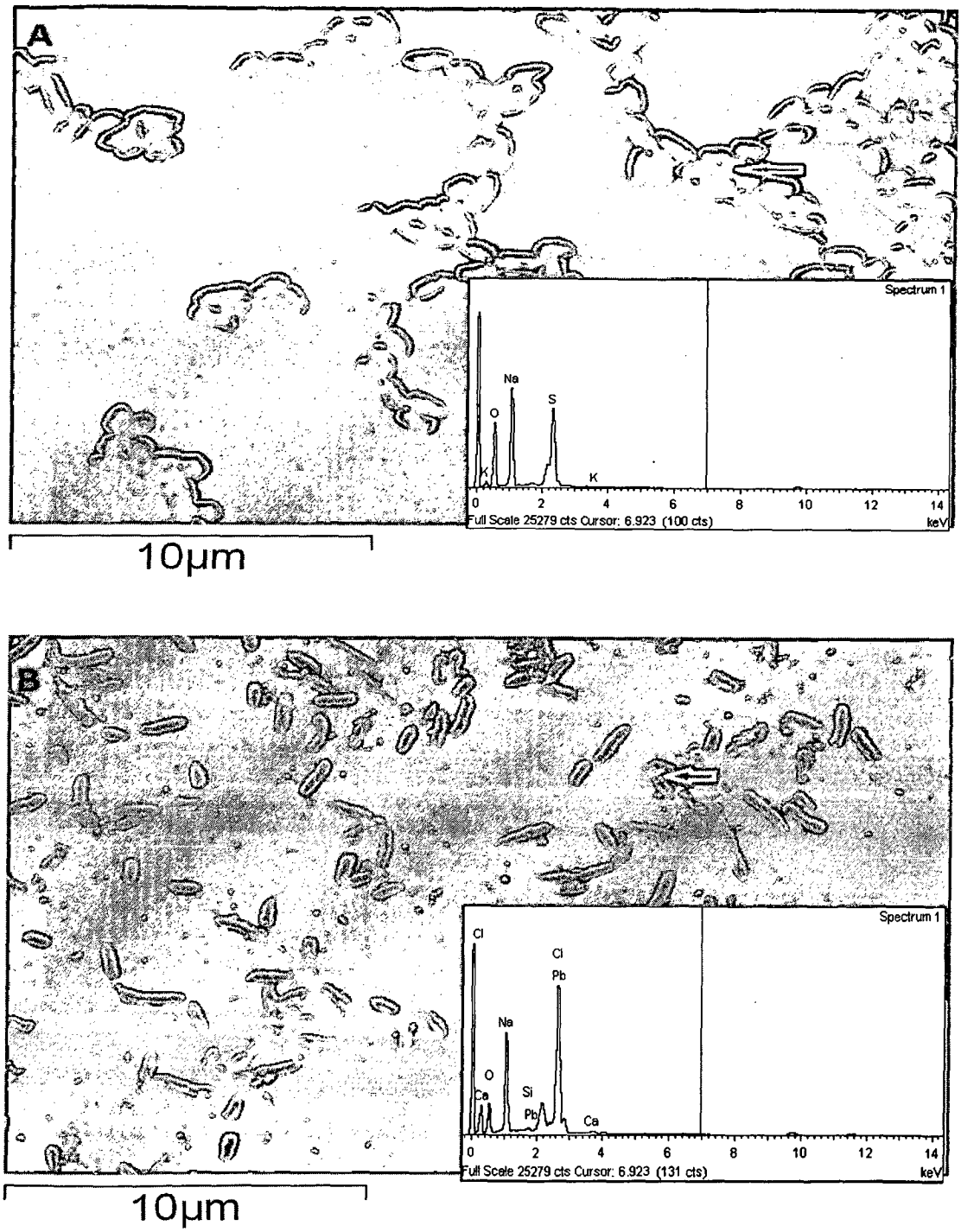


Fig.3.10. A, B SEM photomicrograph of *Pseudomonas aeruginosa* strain 4EA grown with and without exposure to lead nitrate in TMM (SEM-magnification, X9000) and EDX spectrum of a marked area to show biosorption of lead (Arrow is pointing to the area considered for EDX analysis) A- Control cells (no lead exposure); B- Cells exposed to lead nitrate (0.8 mM)

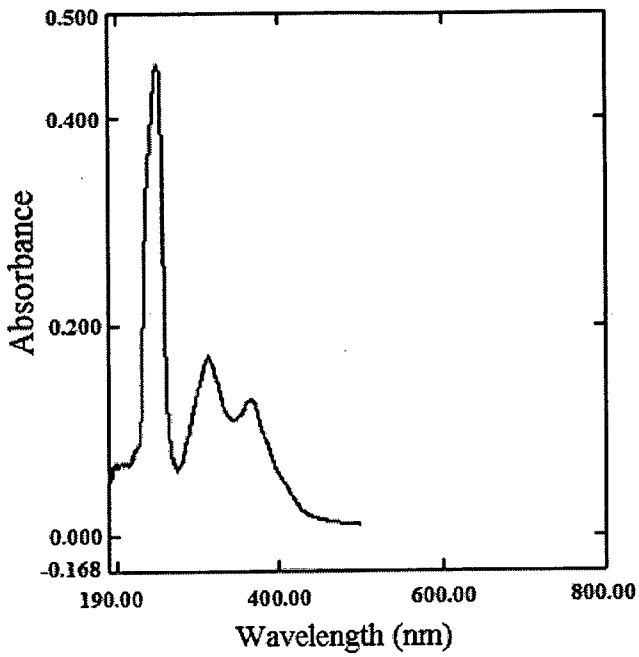
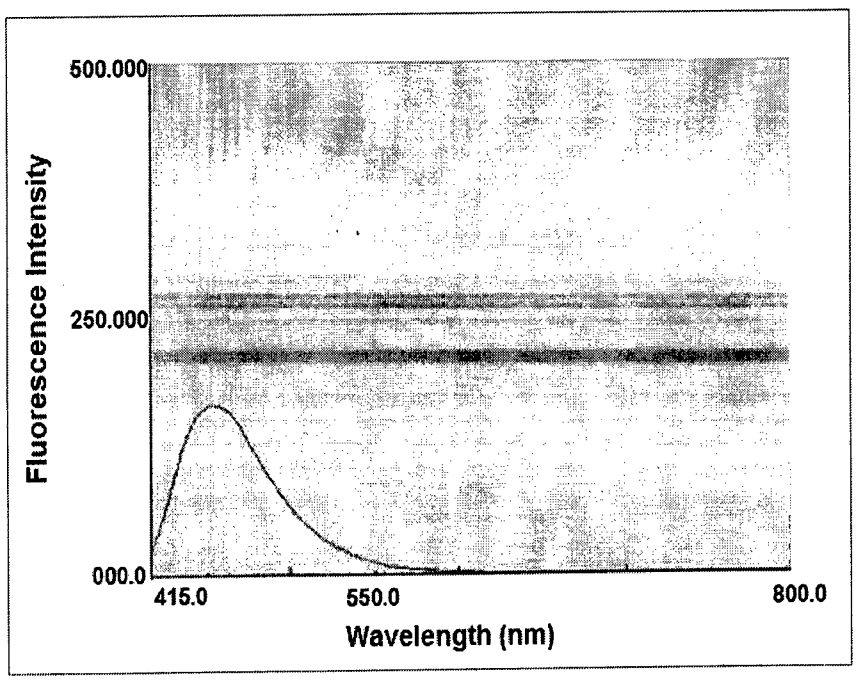
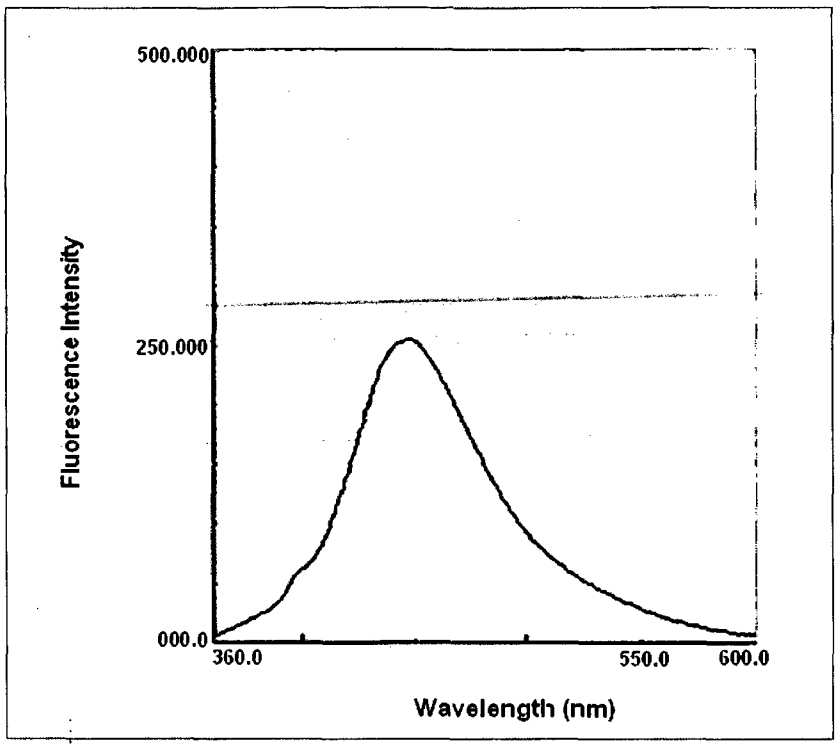


Fig.11. UV–Vis spectrophotometric analysis of siderophore produced by *Pseudomonas aeruginosa* 4EA.



(a)



(b)

Fig. 3.12.a, b Emission spectrums of siderophore -pyoverdine of *Pseudomonas aeruginosa* strain 4EA when excited at 405 nm (a) and siderophore-pyochelin when excited at 350 nm (b)

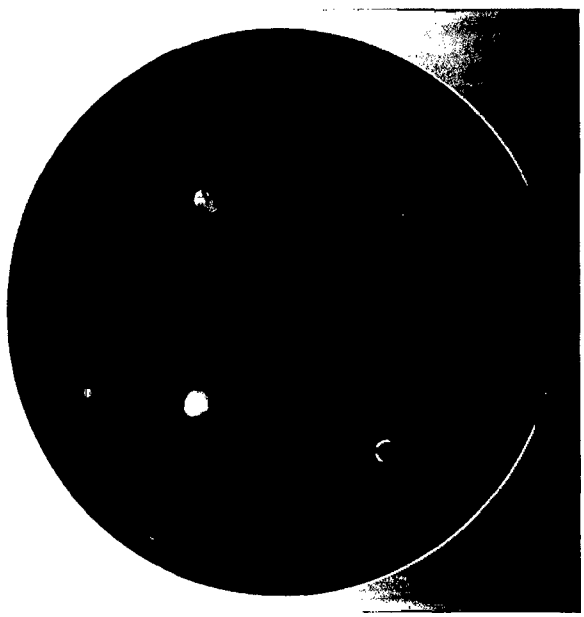


Fig.3.13 CAS agar diffusion (CASAD) assay to demonstrate lead enhanced siderophore production by *Pseudomonas aeruginosa* strain 4EA in terms of diameter of orange halo (cms). B, C and D indicate siderophore production in presence of 0.1, 0.3, and 0.5 mM lead nitrate respectively whereas A indicates basal level of siderophore production(Control)

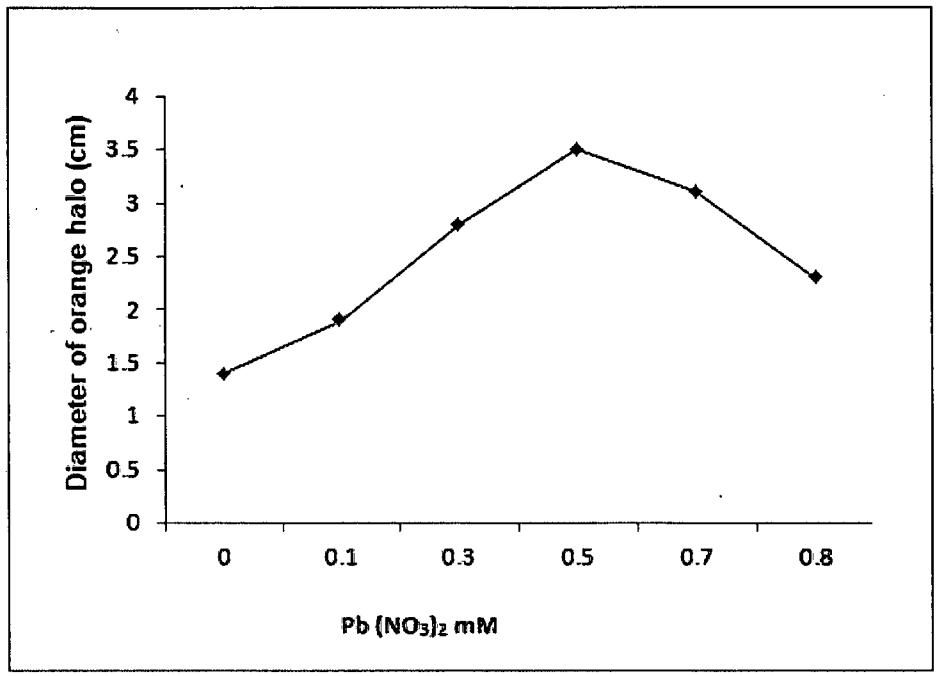


Fig.3.14. Effect of different concentrations of lead nitrate on siderophore production by *Pseudomonas aeruginosa* strain 4EA in TMM as diameter of orange halo (cms) on CAS agar.

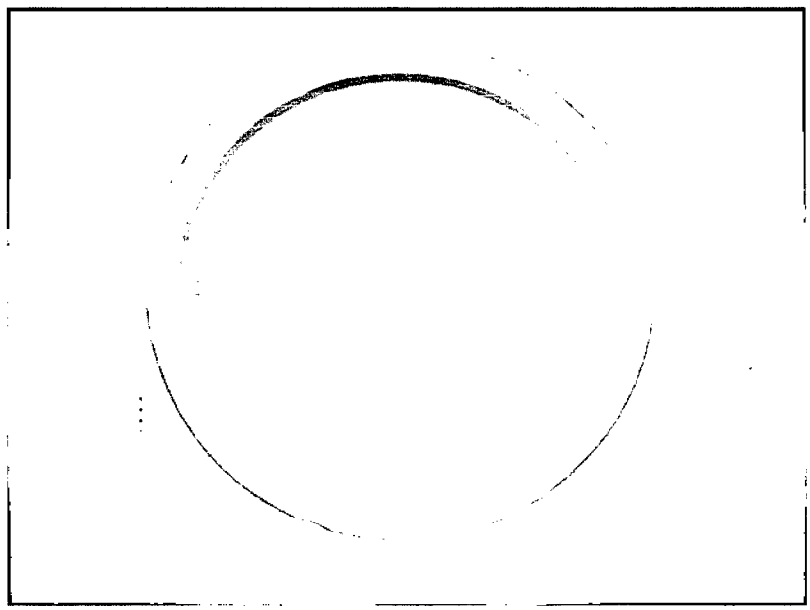
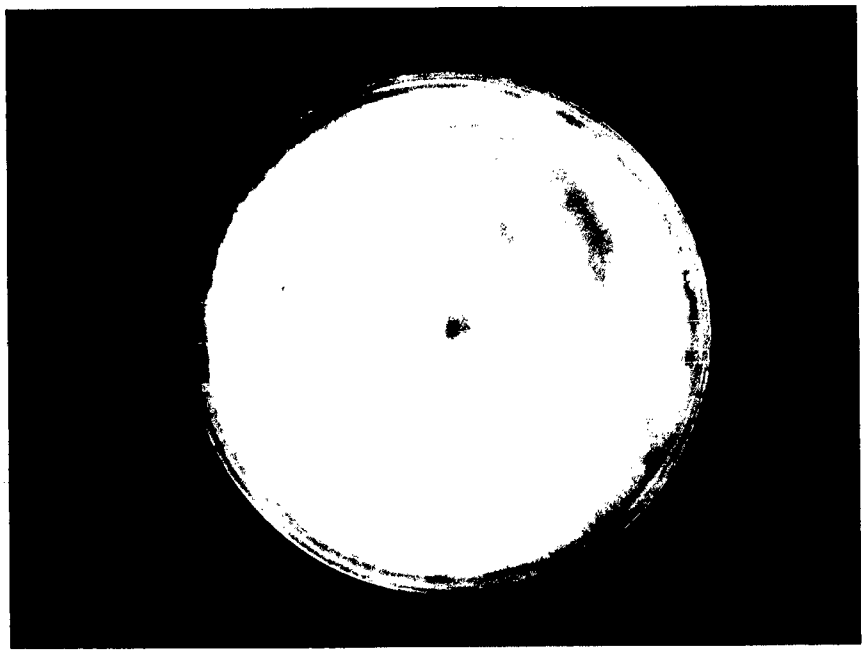
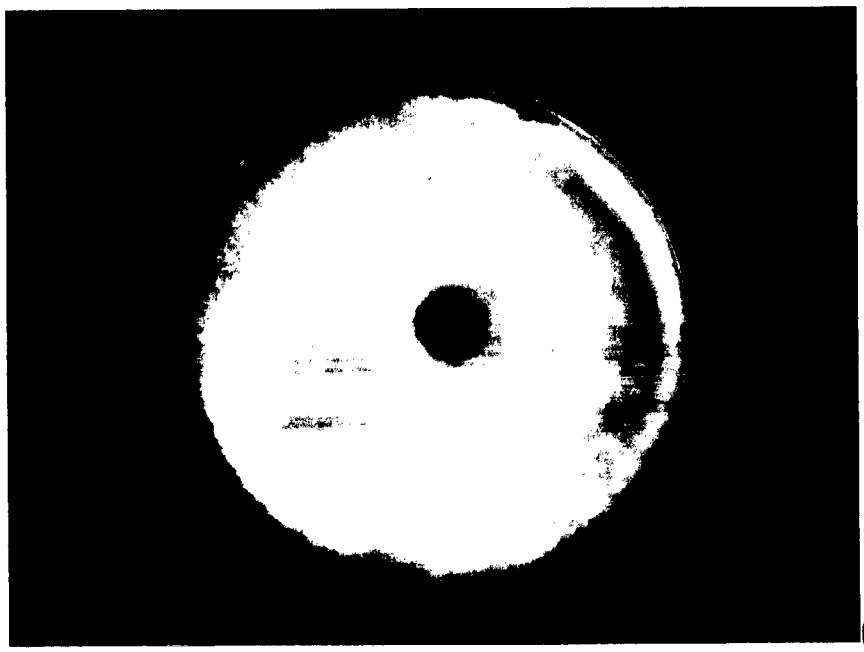


Fig.3.15 Siderophore production by *Pseudomonas aeruginosa* strain WI-1 on CAS agar



(a)



(b)

Fig.3.16.a, b Potato dextrose agar plate showing antifungal activity of *Pseudomonas aeruginosa* WI-1 on the plant pathogen, *Fusarium oxysporum* NCIM 1008 a. Well with sterile TMM only (Control); b. Well with culture filtrate of *Pseudomonas aeruginosa* WI-1 grown in Tris minimal medium (TMM)

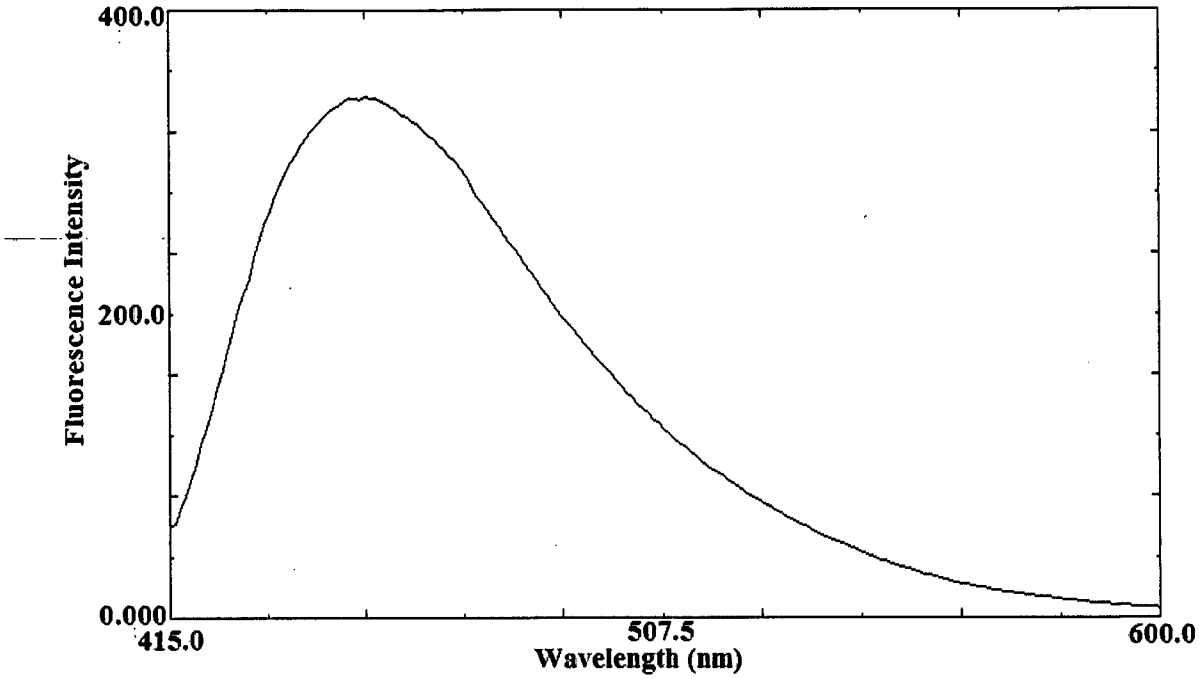


Fig.3.17 Emission spectrum of siderophore -pyoverdine of *Pseudomonas aeruginosa* strain WI-1 when excited at 405 nm

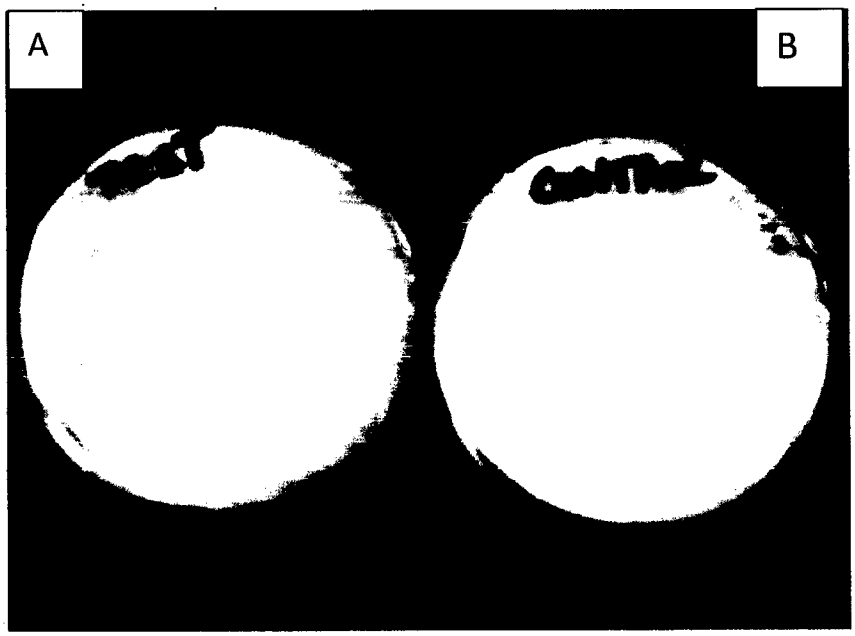
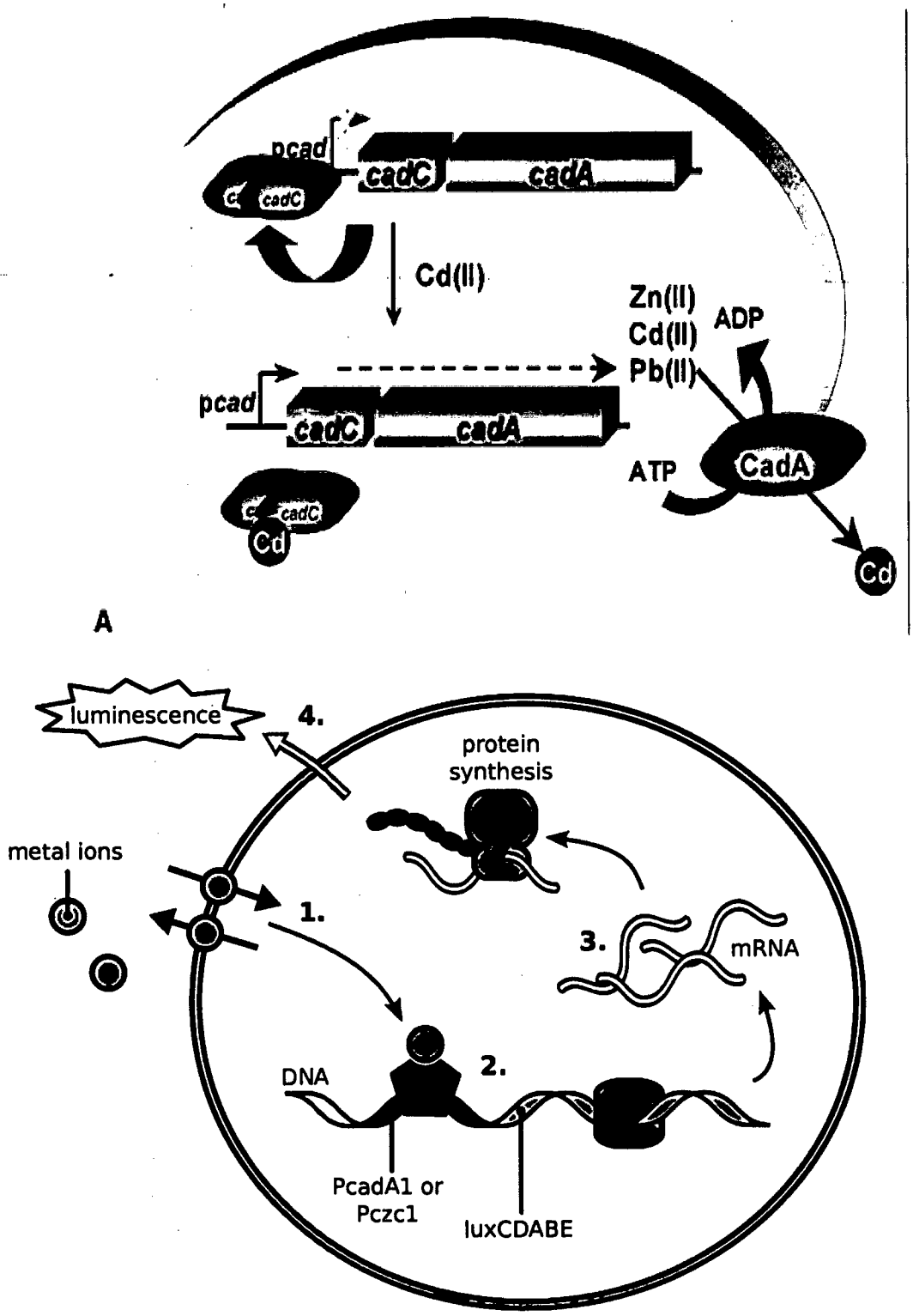


Fig. 3.18 HCN production by *Pseudomonas aeruginosa* strain WI-1. (A) Kings B agar inoculated with *Pseudomonas aeruginosa* strain WI-1; (B) Control



Development of heavy metal biosensor (Hynninen et al., 2009)

CHAPTER-IV

CHAPTER 4

Molecular and genetic characterization of lead resistant bacteria to explore lead and multiple drug (antibiotic) resistance

MATERIALS AND METHODS

4.1 Genomic DNA extraction of lead resistant bacteria (Jones and Barlet, 1990)

Lead resistant bacterial strains were cultured at their optimal growth conditions for 16 hrs in Nutrient broth for terrestrial isolates and Zobell marine broth for estuarine isolates (Appendix A.3, A7). 1.5 ml culture suspension was transferred in a microfuge tube followed by cell harvesting for 2 minutes using eppendorf refrigerated centrifuge (Eppendorf 5417R, Germany). The supernatant was decanted and drained well on a Kim wipe. The cell pellet was resuspended in 467 µl TE buffer by repeated pipetting. 30 µl of 10% SDS and 3 µl of 20 mg/ml Proteinase-K were added and mixed well; suspension was incubated for 1 hr at 37°C. Equal volume of phenol: chloroform (1:1) was added and mixed well by inverting the tube until the phases are completely mixed. This sample was harvested at 9,000 rpm at 4°C for 10 minutes. The upper aqueous phase was transferred to a new microfuge tube and an equal volume of phenol: chloroform was added followed by centrifugation at 9000 rpm at 4°C for 5 minutes. The upper aqueous phase was transferred to a new microfuge tube. 3 M sodium acetate ($1/10^{th}$ volume of aqueous phase) and isopropanol (0.6 volumes of aquous phase) were also added and mixed gently in order to precipitate the DNA. This sample was centrifuged at 9000 rpm at 4°C for 10 mins to get the pellet as DNA (genomic). The DNA pellet was washed with 0.5 ml 70% ethanol and finally resuspended the air dried pellet in 20 µl TE buffer. The DNA concentration was measured by diluting 10 µl of DNA into 1ml TE buffer (1:100 dilution) and measured absorbance at 260 nm. Concentration of Genomic DNA

was determined using following formula: DNA µg/ml =Abs ×100×50 µg/ml. (Appendix E.2.).

4.2 Isolation of Plasmid DNA from lead resistant bacteria

Lead resistant bacterial isolates were screened for the presence of plasmids. Plasmid mini preps were done, using alkaline lysis method (Birnboim and Doly, 1979; Sambrook et al., 1989). A single bacterial colony was transferred into 10 ml of NB in case of terrestrial isolates and ZMB for estuarine isolates and incubated overnight at their respective optimal growth conditions. 1.5 ml of culture suspension was taken in an eppendorf microfuge tube and centrifuged at 11,000 rpm for 5 min at 4°C. The supernatant was discarded leaving the bacterial pellet. The pellet was suspended in 100 µl ice-cold glucose EDTA tris-buffer (solution I) (Appendix E.3) by vortexing and microfuge tube was subsequently kept in ice for 10 mins. 200 µl of freshly prepared solution II (appendix E.3) was added and the contents were mixed by inverting the microfuge tubes rapidly 4-5 times. The microfuge tubes were stored on ice for 10 min. Then 150 µl of ice-cold solution III (appendix E.3) was added and the microfuge tubes were gently vortexed to disperse solution III through the viscous bacterial lysate. The microfuge tubes were kept on ice for 3-5 min, followed by harvesting at 11,000 rpm for 5 mins at 4°C. The clean supernatant was transferred to a fresh microfuge tube. Plasmid DNA was precipitated with two volumes of cold ethanol at room temperature. The contents were gently mixed by inversion followed by incubation on ice for 2 min. The sample in microfuge tube was centrifuged at 11000 rpm for 5 min at 4°C. The supernatant was discarded and the pellet (plasmid DNA) was washed with 70% (v/v) chilled ethanol at 4°C. The supernatant was discarded again and the pellet was allowed to dry in air for 10 min by keeping microfuge tubes inverted on tissue paper. The dry pellet containing plasmid DNA was resuspended in appropriate volume (20-50 µl) of TE buffer (pH 8.0) containing DNase

free RNase (20 µg/ml) and stored at -20°C until needed for agarose gel electrophoresis analysis.

4.3 Agarose gel electrophoresis of DNA

4.3.1 Agarose gel electrophoresis

Agarose gel electrophoretic analysis of DNA (Genomic DNA and plasmid DNA) was performed using horizontal slab gels BG-100, 10×6 cms apparatus (Banglore Genei, Bangalore, India) and 0.8 % agarose (with 0.5 µg/ml ethidium bromide) prepared in 1X TAE buffer. Electrophoresis was performed using 1X TAE buffer (Appendix E.4) as electrode buffer at constant voltage of 80 V for 90 min in the gel. DNA samples (5 µl) containing genomic DNA/ plasmid DNA/ PCR product were mixed with 2 µl 6X DNA loading buffer (Appendix E.4), briefly spun using eppendorf centrifuge and loaded into the wells of agarose gel flooded with 1X TAE buffer. Large agarose gel electrophoresis units (BG-200) were also used (Bangalore, Genei). (Approximately 10×15 cm, capacity 100 ml of agarose gel solution) for 4 hrs (80 V, 1X TAE electrophoresis buffer). The electrophoresis was done usually until the dye front has travelled 2/3rd of the agarose gel. The gel was viewed and photographed using a gel documentation system (Alpha-Innotech, USA). If required, appropriate DNA markers (viz. 1 kb and 100 bps) were also loaded on agarose gels in parallel wells with the DNA samples to determine the size of the DNA fragment (Sambrook et al., 1989).

4.4 Detection of *pbrA* gene mediating efflux of lead in lead resistant bacteria

4.4.1 PCR based detection of *pbrA* gene encoding soft-metal-transporting P_{IB}-type ATPase

Nested PCR was done to detect amplicon of *pbrA* gene using both genomic and plasmid DNA of test bacterial isolates as template and *pbrA* specific forward and reverse

primers. Following three primers were used in the two-step nested PCR (Coomb and Barkay, 2004).

R- 84JC 5'-GGAGCATCGTTAATDCCRTCDCC-3'

F1- (first reaction): 79JC 5'-TGACTGGCGAATCGGTBCCBG-3'

F2- (second reaction): 81JC 5'-GGATGTCCTTGTGCTYTART-3'

Genomic DNA of lead sensitive *E.coli* HB101 was used as negative control for this PCR. PCR was performed using 50 pmoles primers, 50 ng template DNA (plasmid/genomic), dNTPs (0.2 mM each), 0.5 U *Taq* DNA polymerase and 1.5 mM $MgCl_2$. PCR conditions included a 10-min hot start step at 94°C. First reaction was run with 94°C melting step for 1 min, 59 ^{0}C annealing step for 1 min extension at 72 ^{0}C for 1.5 min. Whereas second PCR reactions were performed for 30 cycles, with 94°C melting step for 1 min, 49 ^{0}C annealing step for 1 min and extension at 72 ^{0}C for 1.5 min. Final extension was performed at 72°C for 7 min. for both reactions. PCR product was electrophoresed in a 0.8% agarose gel.

4.4.2 Transformation of plasmid DNA

Transformation of plasmid DNA from *Pseudomonas stutzeri* strains M-9 and *Vibrio harveyi* M-11 and was done separately using lead and ampicillin sensitive *E. coli* HB101 as host (Bangalore Genei, India) by heat shock method (Hanahan, 1983). *E. coli* HB101 host cells transformed with both plasmids separately were plated on LB agar plates with 50 µg/ml ampicillin. Host cells transformed with plasmid pUC18 served as positive control and ampicillin sensitive *E coli* HB101 cells as negative control. Transformation mix was plated on TMM agar plates containing 0.2 mM lead nitrate. Transformants which appeared on ampicillin containing LB agar plates were further checked for presence of plasmids and replica plated on TMM agar containing 0.2 mM lead nitrate.

4.4.2.1 Preparation of competent cells

Single isolated colony of *E. coli* HB101 was inoculated in 10 ml Luria Bertani broth and grown overnight at 30°C.The overnight grown culture was inoculated in 100 ml Luria Bertani broth in 250 ml Erlenmeyer flask. Inoculum density at this point should have absorbance of 0.1 at 600 nm. The culture was incubated at 30°C at 150 rpm and cells were grown till absorbance at 600 nm reached 0.5-0.6. The host cells were harvested by centrifugation at 6000 rpm for 10 min at 4°C, chilled on ice and supernatant was discarded retaining the cell pellets. The cell pellet was washed with 40 ml of 0.01M NaCl (prechilled at 4°C) and incubated on ice for 20 min. Cells were harvested at 6000 rpm for 5 min and supernatant was discarded. Resuspend the pellet in 3 ml of 0.03 M $CaCl_2$ and now competent cells are ready to be used for transformation. It may be stored for 1 week at 4°C as they are good within this period for better transformation results.

4.4.2.2 Transformation experiment

The Competent cells (100 µl) are dispensed into prechilled 15 ml falcon tubes. Added 1.7 µl 1:10 dil β-mercaptoethanol to each 100 µl competent cells and incubated in ice for 10 min while swirling the ice bucket every 2 min. Plasmid DNA (0.1-50 ng) was added to the competent cells and mixed gently by pipetting. Incubate cells with plasmid DNA at 4 °C using ice bucket. Cells in the falcon tubes were heat shocked for 45 sec at 42°C using water bath (Julabo F25, Japan). Ice quenching of cells was performed in ice bucket for 2 min after heat shock. Cells were mixed with 0.9 ml SOC (42 °C) (Appendix A.5), and incubated at 37°C with shaking at 225 rpm for 1 hour. Transformation mix was plated on TMM + lead nitrate (0.2 mM) as well as LB agar + ampicillin plates to check plasmid mediated lead resistance. Positive transformants were also analysed for presence of respective bacterial plasmids using alkaline lysis method (Birnboim and Doly, 1979).

4.5 Detection of *mdrL* gene encoding multi-drug efflux pump

PCR amplification of *mdrL* gene (amplicon) encoding multi-drug efflux pump was carried out using following primers: lltb1 5'-AAATGGATAACAGCGGCAG -3' and lltb2 5'-TGTAAGGTAAAATGTGCTGG -3' using genomic DNA as template (Mereghetti et al., 2000). Antibiotic sensitive *E. coli* HB101 was used as negative control. PCR reaction was performed using 20 pmoles each primer, 25 ng genomic DNA as template, 0.2 mM each dNTPs and 0.5 U *Taq* DNA polymerase. The reaction conditions include an initial denaturation step of 94°C for 2 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extention at 72°C for 1.5 min. Final extention reaction was performed at 72 °C for 10 min. PCR product was analysed on 1% agarose gel by agarose gel electrophoresis and result was recorded using gel doc system (Alpha Innotech, USA).

4.6 PCR mediated detection of bacterial metallothionein encoding gene (*bmtA*) in lead resistant bacteria

In order to investigate involvement of metallothionein gene in lead resistance in *Pseudomonas aeruginosa* strain WI-1, PCR amplification of metallothionein encoding gene (*bmtA*) was carried out using genomic DNA as a template with following primers :

P3 5'-GGTGGATCCCCATGAACAGCGAAACCT-3' and

P4 5'-GGTGAATTCTCAGGGCGAGATCGGGTCGC-3' (Blindauer et al., 2002)

PCR was performed using 30 pmol each primer, 25 ng purified genomic DNA, dNTPs (0.2 mM each), 0.25 U of *Taq* DNA polymerase and 1.5 mM $MgCl_2$. PCR conditions include 10-min hot start step at 94°C and 30 cycles were performed, with 94°C melting step 1min, 62°C annealing step for 1 min, 72°C step for 1.5 min followed by final extension at 72 °C for 10 min (Sambrook et al., 1989). PCR product was analysed on 1% agarose gel by agarose gel electrophoresis and result was recorded using gel doc system (Alpha Innotech, USA).

4.6.1 PCR mediated detection of *Synechococcus* metallothionein encoding gene (*smtA*) in lead resistant bacteria

In order to detect internal fragment of cynobacterial (*Synechococcus* sp.strain PCC 7942) *smtAB* genes, PCR was performed using primers: smt1 (5'- GAT CGA CGT TGC AGA GAC AG- 3') and smt2 (5'- GAT CGA GGG CGT TTT GAT AA- 3') and both plasmid and genomic DNAs of lead resistant bacterial strains 4A, GM-10, GM02, 4EA, GM03 and GM04 as templates (Naz et al., 2005). Total reaction volume was 50 µl which contained 0.2 mmol of each dNTPs, 20 pmol of each primer, 10 ng template DNA, 1.5 mM $MgCl_2$ and 0.25 U *Taq* DNA polymerase. Amplification was carried out for 35 cycles of 1 min 94 °C, 1 min 56 °C, and 1 min 72 °C followed by extension cycle of 5 min at 72 °C. PCR product was analysed on 1% agarose gel by agarose gel electrophoresis and result was recorded using gel doc system (Alpha Innotech, USA).

4.6.1.1 Extraction of cellular proteins for SDS-PAGE and protein estimation

5 ml bacterial culture was harvested during exponential growth phase for 5 minutes at 10,000 rpm and 4°C (Remi cooling centrifuge, C-24 rotor). Cells were suspended in 50 mM Tris-HCl buffer (pH 8.0). In order to extract the cellular proteins cells were disrupted by sonication (Braun Biotech 450, USA) at 45 watts, duty cycle of 10 pulsed of 20 sec for 5 minutes. The cells were kept on ice during sonication and tip was allowed to cool every 30 seconds pulse. Cell debris was removed by centrifugation for 15 minutes at 12,000 rpm, using cooling centrifuge. The supernatant was collected and kept at -20°C until use. Similar procedure was followed for the extraction of proteins from the metal treated bacterial cultures as well as untreated cells (control).

4.6.1.2 Estimation of protein concentration

Standard procedure was followed to estimate protein concentration of bacterial cells (Lowry et al., 1951). To 1 ml of diluted supernatant (protein sample), 5 ml of alkaline copper sulphate solution was added and kept at room temperature in the dark for 10 minutes (Appendix D.1). 0.5 ml Folin and Ciocalteu's Phenol reagent was added and kept in the dark for 20 minutes. Absorbance was measured at 660 nm against blank and concentration of protein in the samples was determined using Bovine serum albumin as standard.

4.6.1.3 One-dimensional gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate - Polyacrylamide gel electrophoresis (SDS-PAGE) was performed to resolve bacterial proteins using 10% resolving gel (Protean II unit, BIO RAD, USA) and 5% stacking gel according to standard method of Laemmli, 1970 (Appendix E.1). The total protein sample (10 µl containing approx. 30 µg of protein per sample) was mixed with equal volume of 2X sample solubilizing buffer containing bromophenol blue (Appendix E.1, tracking dye) and mixture was placed in boiling water bath for 5 minutes followed by centrifugation at 10,000 rpm at 4 °C for 2 minutes. The clear supernatant was loaded on the SDS-PAGE gel (Appendix E.1). Electrophoresis was carried out in 1X Tris-Glycine electrophoresis buffer at room temperature (25°C). Protein samples were initially electrophoresed at 35 mA until tracking dye entered the resolving gel, subsequently current was increased to 70 mA and run was done till the tracking dye reaches bottom of the resolving gel. Broad range molecular weight marker (MWM, Banglore Genei) was used to determine size of the polypeptides/proteins. The SDS-Polyacrylamide gel was visualized after staining with Coomassie brilliant blue followed by destaining (Appendix E.1) (Sambrook et al., 1989). Gel was recorded using gel documentation system. **In all the SDS-PAGE experiments equal amount of protein was loaded in each well and electrophoresed. In all the SDS-PAGEs protein concentration in each well was 30 µg.**

4.6.1.4 Coomassie brilliant blue staining and destaining of SDS-PAGE gel

Coomassie brilliant blue R-250 staining solution (Sigma,USA) was prepared by dissolving the 0.25 gm dye in 25% methanol before adding 10% acetic acid and 65% Milli-Q water (Total volume:100 ml). Gels were stained in a glass tray for 30 minutes at room temperature with gentle shaking on a gel rocker. The gels were rinsed in Milli-Q water and transferred into destaining solution (10% acetic acid: 40 % methanol: 50 % milli-Q water). Destaining was done thoroughly till clear protein bands appear (Appendix E.1).The gel was observed and recorded in gel documentation system (Alpha-Innotech, USA).

4.6.1.5 SDS-PAGE analysis of lead resistant bacterial strains to explore lead induced proteins

Pseudomonas aeruginosa strain WI-1was grown in TMM amended with 0.6 mM lead nitrate and without lead nitrate, bacterial cells were harvested at mid log phase for protein extraction to study protein profile by SDS-PAGE analysis (Sambrook et al., 1989) in order to reveal lead-induced bacterial metallothionein (BmtA) proteins involved in lead resistance. *Providencia alcalifaciens* strain 2EA was also grown in TMM amended with 1.4 mM lead nitrate and without lead, bacterial cells were harvested at mid log phase for protein extraction and protein profile was studied by SDS- PAGE analysis to confirm possible involvement of lead induced proteins in lead resistance. Gel doc was used to record the image of SDS-PAGE gels (Alpha Innotech, USA).

4.7 Curing of plasmid DNA of *Providencia alcalifaciens* strain 2EA using acridine orange

Curing of plasmid DNA of *Providencia alcalifaciens* strain 2EA was performed using acridine orange (Trevors, 1986).Stock solution (1mg/ml) of acridine orange was prepared in deionised water and filter sterilized. This solution was kept in amber coloured bottle in dark under cold conditions. Different concentrations of acridine orange ranging from 25-225 µg/ml were added to Nutrient broth (NB) and overnight grown culture of *Providencia alcalifaciens* strain 2EA was inoculated, cells were incubated in optimum growth conditions for 24 h. After 24 h incubation absorbance of all the culture suspensions was recorded at 600 nm and graph was plotted as concentration of acridine orange (µg/ml) v/s % survival. Cells not exposed to acridine orange served as control (100% survival). LD_{50} value of acridine orange for the bacterial culture was determined from the graph of acridine orange (µg/ml) V/S % survival. The plasmid bearing bacterial strain was subcultured in Nutrient broth with two different levels of acridine orange (AO) below its LD_{50} value separately (Trevors, 1986; Gerhardt et al., 1994). Bacterial cells were subcultured at these AO levels 6 times while keeping one culture without AO as control. After serial dilution of these cultures 100 µl cell suspension was plated on Nutrient agar and incubated at room temperature to get discrete colonies. The discrete colonies were selected and transferred to TMM agar plate containing 1.4 mM lead nitrate to check lead resistance and these selected colonies were also inoculated in Nutrient broth to check presence of plasmid (Birnboim and Doly, 1979).

RESULTS AND DISCUSSION

4.8 Agarose gel analysis of genomic DNA extracted from lead resistant bacterial strains

Agarose gel analysis of genomic DNA samples from lead resistant bacterial strains viz. *Pseudomonas aeruginosa* strain 4EA, *Enterobacter cloacae* strain P2B, *Providencia alcalifaciens* strain 2EA, *Pseudomonas stutzeri* strain M-9, *Vibrio harveyi* M-11 and *Pseudomonas aeruginosa* WI-1 clearly revealed clean and very prominent bands. Genomic DNA of lead sensitive, *E. coli* HB101 was also analysed (Fig.4.1). We will use these genomic DNAs as template for PCR analysis of these isolates to screen lead resistance encoding genes.

4.9 Efflux mediated lead resistance and multi-drug resistance

4.9.1 Identification of lead resistant bacterial strains

Bacterial isolates M-9 and M-11 have been identified as *Pseudomonas stutzeri* and *Vibrio harveyi* on the basis of their biochemical characteristics (Krieg and Holt, 1984) and FAME analysis. These strains were further confirmed by 16S rDNA sequencing followed by NCBI-BLAST search as *Pseudomonas stutzeri* and *Vibrio harveyi* (Altschul et al., 1997). 16S rDNA sequence data has already been submitted to GenBank and we have got their accession numbers as GenBank accession no. HQ268731 and HQ268730 respectively. These test bacterial strains resist lead nitrate up to 0.8 mM and 1.2 mM respectively with MIC value of 1 mM and 1.4 mM respectively.

4.9.2 Soft-metal-transporting P-type ATPases regulating efflux of lead

In order to maintain heavy metal homeostasis, intracellular level of toxic heavy metal ions has to be tightly controlled (Nies, 1999). Soft metal transporting P_{IB}-type ATPases are

group of proteins involved in transport of heavy metals outside the cell membrane and governing bacterial heavy metal resistance (Nies and Silver 1995; Rensing et al., 1999). These transporter proteins prevent over-accumulation of highly toxic and reactive metal ions viz. Pb (II), Cu (I), Ag (I), Zn (II) and Cd (II). P_{IB}-type ATPases can be divided into two subgroups: i) Cu (I)/Ag (I) - translocating ATPases encoded by gene *copA* in *Enterococcus hirae*, *Helicobacter pylori* and *E. coli*; and ii) Zn (II) /Cd (II) / Pb(II) - translocating ATPases encoded by gene *zntA* in *E. coli* and gene *cad A* in *Staphylococcus aureus* plasmid, pI258 (Nies and Silver, 1995; Rensing et al., 1999). *Ralstonia metallidurans* CH34 is th only bacterial strain where complete operon *pbrUTRABCD* conferring efflux mediated lead resistance has already been sequenced (Borremans et al., 2001; Taghavi et al., 2009).

In our studies both *Pseudomonas stutzeri* strain M-9 and *Vibrio harveyi* strain M-11 showed presence of plasmids and these plasmids were transformed into lead and ampicillin sensitive *E. coli* HB101 (Fig.4.2 and 4.3). Transformed *E coli* HB101 with M-9 plasmid DNA separately and transformed *E. coli* HB101 with M-11 plasmid DNA did not grow on TMM agar plates amended with 0.2 mM lead nitrate but these cells showed plasmid mediated ampicillin resistance. Nested PCR using their genomic DNA as template, revealed an amplicon of 750 bps corresponding to genes *pbrA* encoding P_{IB}-type ATPases. But plasmid DNA as a template did not generate any amplicon (Figs.4.4, 4.5). It interesting to note that these 750 bps amplicons interestingly matched with *pbrA* gene amplicons (Coomb and Barkay, 2004). Thus these gene encoding metal ion transporter proteins prevent the over-accumulation of highly reactive soft-metals such as lead, Cd and Zn and play an important role in heavy metal resistance. Since we have got 750 bps amplicons corresponding to *pbrA* gene exclusively from genomic DNAs of *Pseudomonas stutzeri* strain M-9 and *Vibrio harveyi* strain M-11 and not from plasmid template, we can conclude that lead resistance is not plasmid mediated but genomic DNA mediated.

4.9.3 MdrL- efflux pump mediating multidrug resistance

Since its discovery, antibiotics are essential drugs to treat bacteria producing infectious diseases. Many of the available antibiotics are no longer effective because of emerging resistance, which is mainly caused by uncontrolled, haphazard and extensive use of antibiotics and anti-microbial drugs in hospitals, aquaculture farms prawn hatcheries and poultry farms has become a major environmental problem due to their ultimate release and persistence in the natural terrestrial and aquatic environments (Khetan and Collins, 2007). Long term exposure of microbes to these residual antibiotics and anti-microbials leads to evolution of drug resistance which is conferred by genetic determinants on plasmids, transposons or integrons (Okeke et al., 2000; Martinez, 2008). These mobile elements often carry several antibiotic resistance genes, and thus their transfer results in immediate multidrug resistance in recipient strains (Horizontal DNA transfer). *L. monocytogenes* isolated from the environment and food products showed multi-drug efflux pump (MdrL) which governed resistance to multiple antibiotics (Mereghetti et al., 2000). In *Enterobacteriaceae*, the expulsion of antibiotics is currently associated with efflux pump belonging to AcrAB-TolC family. This pump contributes to a multidrug resistance (MDR) phenotype often associated to the modification of the outer membrane permeability (Baucheron, et al., 2004).

PCR studies using both lead resistant *Pseudomonas stutzeri* strain M-9 and *Vibrio harveyi* strain M-11 have clearly demonstrated *mdrL* gene amplicon of 1136 bps from genomic DNA as template. These studies clearly revealed that *mdrL* gene encodes MdrL efflux pump which is responsible for multi-drug (antibiotics) resistance (Fig.4.6). Interestingly we have also observed presence of 467 bps amplicon of *orfA* which is a repressor encoding gene to regulate MdrL efflux pump in both strains. These studies clearly

demonstrated that multiple antibiotic resistance is governed by *mdrL* gene responsible for efflux mediated multidrug resistance.

4.10 Plasmid profile of lead resistant bacterial isolates

Plamid profile of lead resistant bacterial isolates from contaminated soil and liquid wastes of car battery manufacturing company clearly revealed that all the lead resistant bacterial strains except *Pseudomonas aeruginosa* 4EA possessed plasmids (Fig.4.7a, b, c).

4.11 Bacterial metallothionein protein as lead resistance mechanism

Intracellular metal bioaccumulation and homeostasis in cell cytosol involves low molecular weight, cystein-rich metallothioneins which range from 3.5 to 14 kDa (Hamer, 1986). These unique proteins also demonstrate induction in response to specific heavy metals such as Cd, Pb, Zn, and Cu (Gadd, 1990; Turner et al., 1996; Blindauer et al., 2002; Liu et al., 2003). Metallothioneins play an important role in immobilization of toxic heavy metals thereby protecting bacterial metabolic processes catalysed by enzymes (Blindauer et al., 2002; Liu et al.,2003).Several cyanobacterial and bacterial strains have been reported to encode metallothioneins for maintaining cytosolic metal homeostasis viz. *Synechococcus* PCC 7942 (SmtA), *Anabaena* PCC 7120 (SmtA), *Oscillatoria brevis (*BmtA*), Pseudomonas aeruginosa* (BmtA) and *Pseudomonas putida* (BmtA) (Turner et al., 1996; Blindauer et al., 2002; Liu et al., 2003). *Pseudomonas* sp. S8A when exposed to >10 mg/l cadmium showed induction of unidentified 28 kDa protein responsible for cadmium resistance (Kassab and Roane, 2006). Genetically modified *Escherichia coli* expressing the metal binding peptide fusion demonstrated enhanced binding of Cd^{2+} and Hg^{2+} compared to bacterial cells lacking the metal binding peptide (Pazirandeh et al., 1998).

Pseudomonas aeruginosa strain WI-1 resists up to 0.6 mM lead nitrate with MIC value 0.8 mM in TMM and accumulates lead up to 26.5 mg/g dried cell biomass. PCR amplification of 270 bps amplicon clearly revealed presence of bacterial metallothionein gene, *bmtA* (Fig.4.8). Further studies using SDS-PAGE analysis also indicated lead induced 11 kDa protein which corresponds to predicted molecular weight of BmtA protein (Fig.4.9). Metallothionein proteins are reported to maintain intracellular homeostasis of essential metal ions and for acquiring resistance against toxic metals (Blindauer et al., 2002; Liu et al., 2003) Our investigation has clearly demonstrated that *Pseudomonas aeruginosa* WI-1 exhibits metallothionein (11 kDa) mediating lead resistance by accumulating significantly high levels of lead (26.5 mg/g) intra-cellularly. The total protein concentration also increased from 528µg/ml to564 µg/ml when *Pseudomonas aeruginosa* WI-1 was grown in the presence of 0.6 mM lead nitrate. This unique characteristic of hyper-lead accumulating *Pseudomonas aeruginosa* strain WI-1 may be exploited for bioremediation of environmental sites contaminated with considerably high levels of toxic lead.

It is evident from Fig 2.19 that *Pseudomonas aeruginosa* strain WI-1 exposed to 0.6 mM lead nitrate showed less biomass and increased lag but total protein concentration was significantly high as compared to control because of high level expression of lead induced stress proteins (11 kDa) which is clearly evident from SDS-PAGE analysis of lead exposed cells and control cells (Fig. 4.9). I have taken equal density of cells for protein estimation when exposed to 0.6 mM lead nitrate and in control conditions.

4.11.1 PCR mediated detection of *Synechococcus* metallothionein encoding gene (*smtA*) in lead resistant bacteria

PCR amplification of 507-bps internal fragment of *smtAB* genes using genomic DNA of *Salmonella choleraesuis* 4A and *Proteus penneri* GM-10 as template clearly revealed possible involvement of metal binding small metallothionein protein, SmtA in lead resistance (Fig.4.10.). But no *smtAB* amplicon was found using plasmid DNA as template in all bacterial isolates including *Salmonella choleraesuis* 4A and *Proteus penneri* GM-10 (Fig. 4.11). Therefore we can infer that *smtAB* genes encoding small metallothioneins confer lead resistance in *Salmonella choleraesuis* 4A and *Proteus penneri* GM-10 and these genes are located on genomic DNA.

4.11.2 Lead induced proteins

Microorganisms including bacteria express stress induced proteins in response to stress stimuli viz. heavy metal, oxidative, temperature, organic toxicants etc. (Harwood-Sears and Gordon, 1990; Noel-Georis et al., 2004; Sharma et al., 2006; Cheng et al., 2009; Ramachandran and Dubey, 2009; Yildirim et al., 2011). One of the common mechanisms of metal resistance in bacteria is induction of specific metal binding proteins facilitating the sequestration/bioaccumulation of toxic metals inside the cell. Two copper-inducible supernatant proteins viz. CuBP1 and CuBP2 with molecular mass 21 kDa and 19 kDa were identified in marine bacterium, *Vibrio alginolyticus* which were 25-46 times amplified in the supernatant of copper-challenged culture as compared with control. Thus these proteins facilitated copper accumulation and homeostasis (Harwood-Sears and Gordon, 1990). Similarly *Pseudomonas fluorescens* exposed to lead although showed 18 differentially expressed proteins, but only one protein could match significantly to spoVG protein which expressed Pb-induced upregulation (Sharma et al., 2006). These unique proteins also demonstrated induction in response to specific heavy metals such as Cd, Pb, Zn, and Cu (Gadd, 1990; Harwood-Sears and Gordon, 1990 Turner et al., 1996; Blindauer et al., 2002; Liu et al., 2003; Sharma et al., 2006; Yildirim et al., 2011).

In our investigation, lead resistant *Providencia alcalifaciens* strain 2EA grown in TMM amended with 1.4 mM lead nitrate clearly revealed induction of 97 kDa and 147 kDa proteins which may be involved in lead resistance mechanism (Fig.4.12). It interesting to mention that total protein concentration also increased from 548 µg/ml when grown in absence of lead to 582 µg/ml when *Providencia alcalifaciens* strain 2EA was grown in the presence of 1.4 mM lead nitrate.

It is evident from Fig 2.17 that *Providencia alcalifaciens* 2EA exposed to 1.4 mM lead nitrate showed less biomass and increased lag but total protein concentration was significantly high as compared to control because of high level expression of lead induced stress proteins (97 kDa and 147 kDa) which is clearly evident from SDS-PAGE analysis of lead exposed cells and control cells (Fig. 4.12).

4.12 Acridine orange curing of plasmid DNA

It is interesting to note that heavy metal resistant bacteria possess genetic determinants (genes) conferring metal resistance either on chromosomal genome, plasmid or transposons (Silver, 1981; Bopp et al., 1983; Lebrun et al., 1994; Silver and Phung, 1996; Crupper, 1999; Nies 1999; Borremans et al., 2001; Bruind et al., 2003; Coomb and Barkay, 2005; Taghavi et al., 2009;). Earlier reports have confirmed that genome of *Pseudomonas putida* KT1440 contained 61 open reading frames involved in resistance to several metals (Canovas et al.,2003), whereas resistance to multiple metals viz. Pd, Cd, Zn, Sn, Cu, and Hg was found in tributyltin resistant 250 bacterial strains (Pain and Cooney, 1998).

The percentage survival curve of test bacterium *Providencia alcalifaciens* 2EA clearly indicated that the culture showed 24 % and 15% survival in presence of acridine orange concentration of 175 and 200µg/ml respectively (Fig. 4.13). Cells subcultured in 175 and 200 µg/ml acridine orange resulted in complete loss of plasmid after sixth subculture in acridine orange (Fig.4.14).But cells were still resistant to lead nitrate (1.4 mM) even after complete loss of plasmid indicating that lead resistance determinants are not on plasmids. Therefore we can conclude that lead resistance is governed by genes located on chromosomal genome. We used acridinre orange for plasmid curing since it is one of the potential curing agents. It is evident from earlier reports that acridine orange inhibits the replication of bacterial plasmid DNA by causing mutation in the absence of light at the site of semi-conservative DNA replication (Webb and Hass, 1984; Trevors, 1986).

4.13 Molecular mechanisms of lead resistance in bacteria

I tried to isolate plasmid DNA from all the 11 lead resistant bacterial isolates out of which 10 isolates were found positive for plasmid (Fig. 4.2, 4.3, Fig. 4.7a, 4.7b, 4.7c, Fig. 4.14).

There are various heavy metal resistance mechanisms operational in bacteria which regulate the toxic level of biologically available metal ions in order to protect the cells from toxic effects of heavy metals on various vital metabolic processes. I have screened presence of *pbrA*, *smtA* and *BmtA* genes involved in lead resistant mechanism using plasmid/ genomic DNA of selected potential isolates (Figs. 4.2, 4.3, 4.4, 4.5, 4.8, 4.9, 4.10, 4.11, 4.12). Therefore I can conclude that there are multiple possible mechanisms for lead resistance in natural isolates. But I do not have any idea weather all the above lead resistance regulating genes are present in a single lead resistant bacterial strains because I have not done sufficient experiments to prove it. We can't deny with the possibility of multiple resistance mechanism.

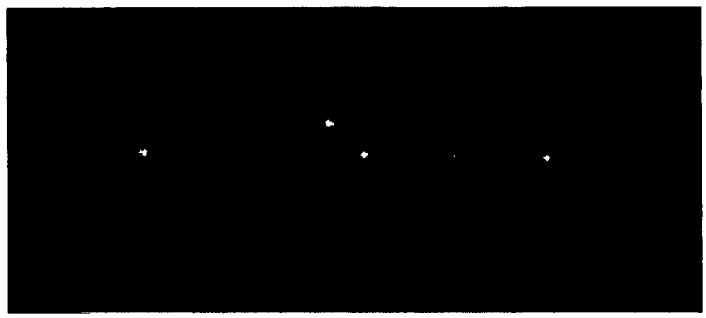


Fig.4.1 Genomic DNA of lead resistant bacteria. Lane.1 *Pseudomonas aeruginosa* strain 4EA; Lane.2 *Enterobacter cloacae* strain P2B; Lane.3 *Providencia alcalifaciens* strain 2EA; Lane.4 *Pseudomonas stutzeri* strain M-9; Lane. 5 *Vibrio harveyi* M-11; Lane.6 *Pseudomonas aeruginosa* WI-1 and Lanes.7, 8 *E. coli* HB101

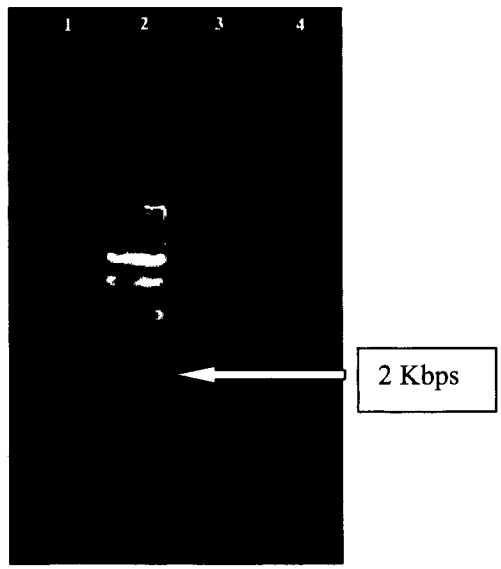


Fig.4.2 Plasmid profile of lead-resistant *Pseudomonas stutzeri* strain M-9. Lane 1 plasmid DNA of strain M-9; lane 2 with 1 Kb DNA marker (ladder); lane 3 plasmid DNA isolated from transformed *E. coli* HB101 and lane 4 *E.coli* HB101 without plasmid (control)

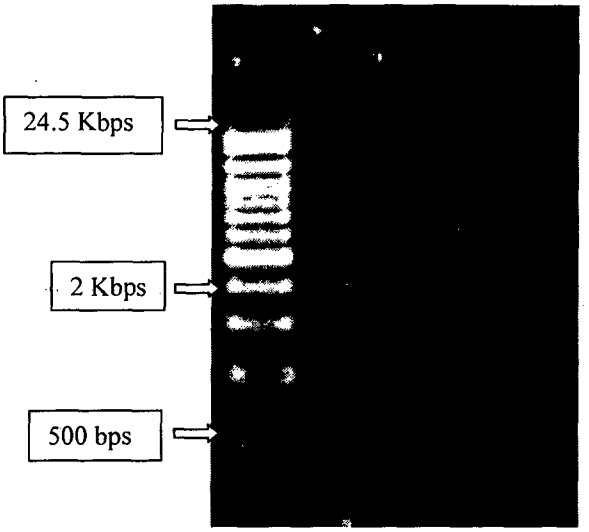


Fig.4.3 Plasmid profile of lead-resistant *Vibrio harveyi* strain M-11. Lane 1 super mix DNA ladder; lane 2 plasmid DNA of strain M-11; lane 3 plasmid DNA isolated from transformed *E. coli* HB101 and lane 4 *E.coli* HB101 without plasmid DNA (control)

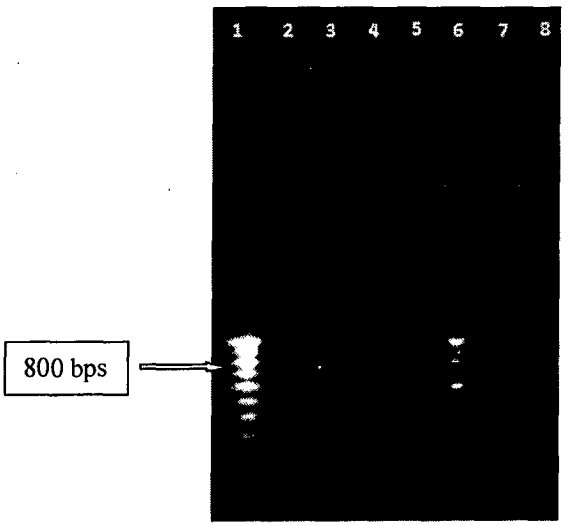


Fig. 4.4 PCR amplification using the *pbrA* specific primer pair. Lanes 1, 6 with 100 bps DNA ladders; lanes 2, 3 and 4, 5 with 750 bps *pbrA* amplicons using genomic DNA as templates of *Pseudomonas stutzeri* stains M-9 and *Vibrio harveyi* strain M-11respectively and lanes 7, 8 negative controls using genomic DNA of lead-sensitive *E. coli* HB101 as template

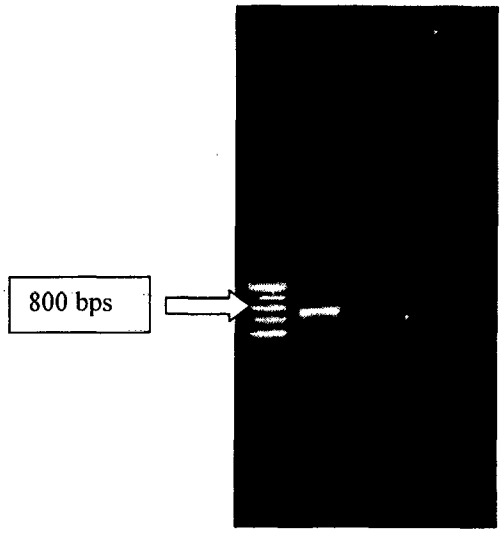


Fig.4.5 PCR amplification using the *pbrA* specific primer pair. Lane 1 with 100 bps DNA ladder; lane 2 with 750 bps *pbrA* amplicon using genomic DNA of *Pseudomonas stutzeri* strain M-9 as template (positive control); lanes 3, 4 PCR amplification using plasmids of strain M-9 and *Vibrio harveyi* strain M-11 as templates and lane 5 genomic DNA of lead-sensitive *E. coli* HB101 as template (negative control)

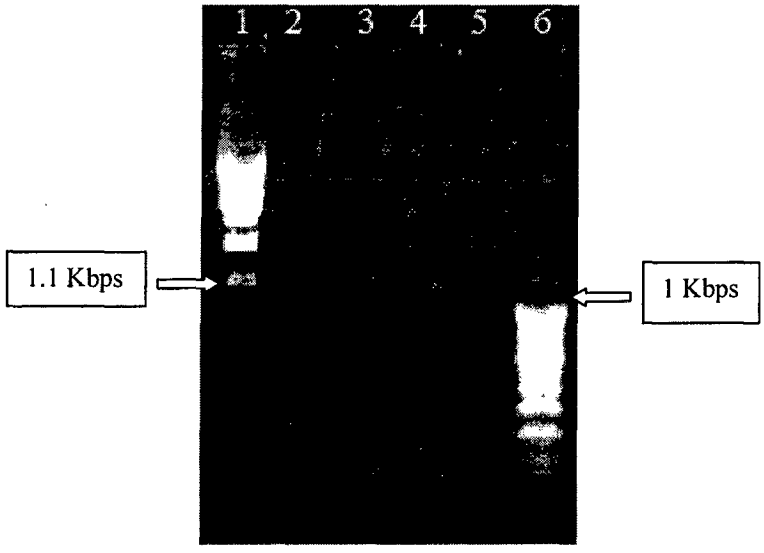
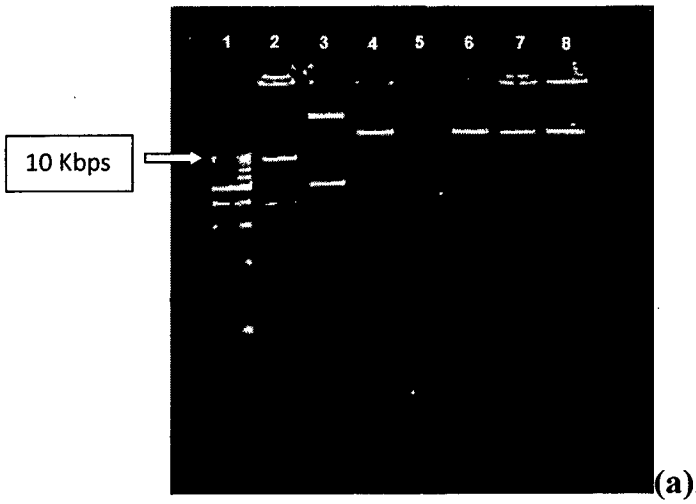


Fig.4.6 PCR amplification using *mdrL* specific primer pair. Lanes 2, 3 and 4 with 1136 bps *mdrL* amplicons using genomic DNA of *Pseudomonas stutzeri* strains M-9 and *Vibrio harveyi* strain M-11 respectively as template; Lane 1, 6 are 500 bps DNA ladder and 100 bps DNA ladder respectively; lane 5 genomic DNA of lead- sensitive *E. coli* HB101 as template (negative control)



(a)

Fig.4.7a Plasmid profile of lead resistant bacterial strains. Lane 1 One Kb DNA marker; Lane 2 *Salmonella choleraesuis* 4A; Lane 3 *Proteus penneri* GM-10; Lane 4 *Bacillus subtillis* GM02; Lane 5 *Pseudomonas aeruginosa* 4EA; Lane 6 *Proteus penneri* GM03; Lane 7, 8 *Providencia rettgeri* GM04

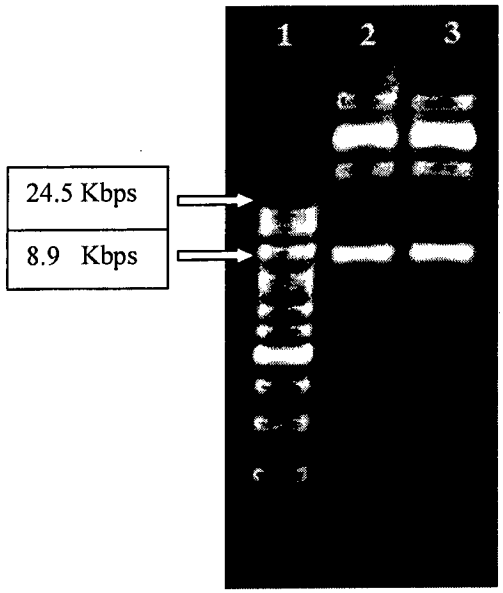


Fig. 4.7b Plasmid profile of lead resistant *Enterobacter cloacae* strain P2B. Lane 1. Supermixed DNA marker; Lane 2, 3 Plasmid isolated from *Enterobacter cloacae* strain P2B

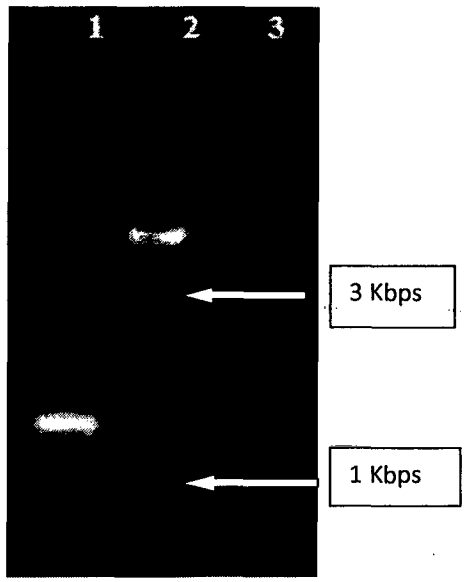


Fig 4.7c Plasmid profile of lead resistant *Pseudomonas aeruginosa* strain WI-1. Lane 1 Plasmid DNA isolated from *Pseudomonas aeruginosa* strain WI-1; Lane 2. 1Kb DNA marker

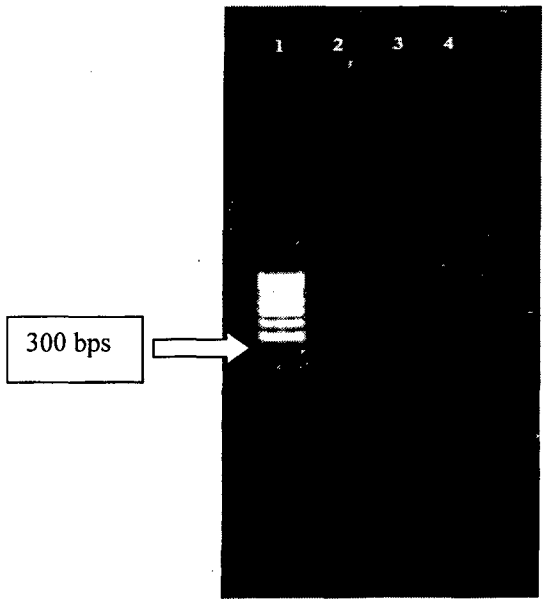


Fig.4.8 PCR amplification of metallothionein encoding gene (*bmtA*) using specific primer pair. Lane 1. 100 bps DNA marker; Lane 2. 270 bps *bmtA* amplicon using genomic DNA of *Pseudomonas aeruginosa* strain WI-1 as template and lane 3, 4 PCR using genomic DNA of *E. Coli* HB101 as template (negative control)

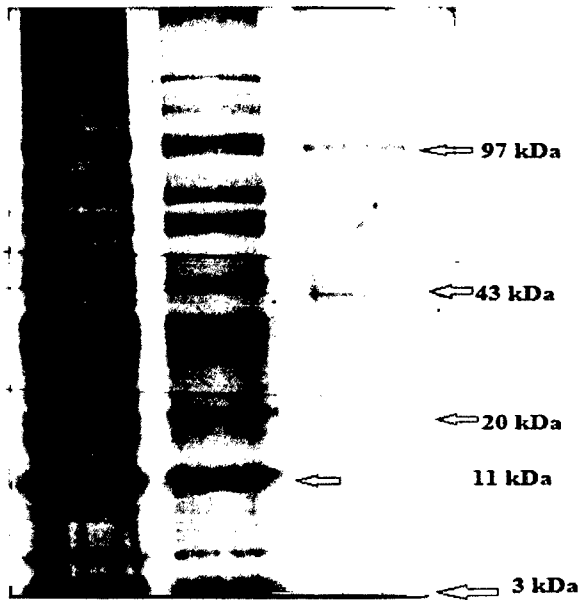


Fig.4.9 SDS-PAGE analysis of whole cell protein of *Pseudomonas aeruginosa* strain WI-1 grown in TMM with, lane 1. zero mM lead nitrate; Lane 2. 0.6 mM lead nitrate and Lane 3. protein marker

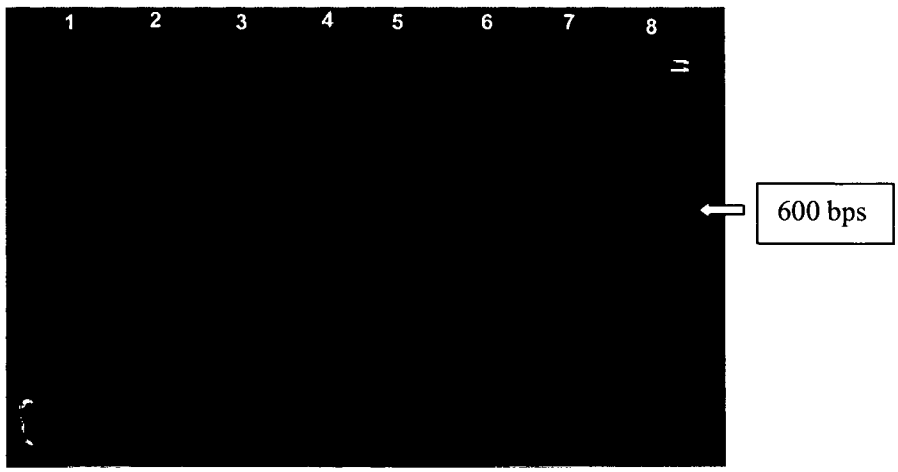


Fig.4.10 PCR amplification of internal fragment of *smtAB* gene using genomic DNA as template. Lane1. *Providencia rettgeri* GM04; Lane.2. *Proteus penneri* GM03; Lane.3. *Pseudomonas aeruginosa* 4EA; Lane.4, 5 *Bacillus subtillis* GM02; Lane.6. *Proteus penneri* GM-10; Lane.7. *Salmonella choleraesuis* 4A and Lane.8. 100 bps DNA ladder

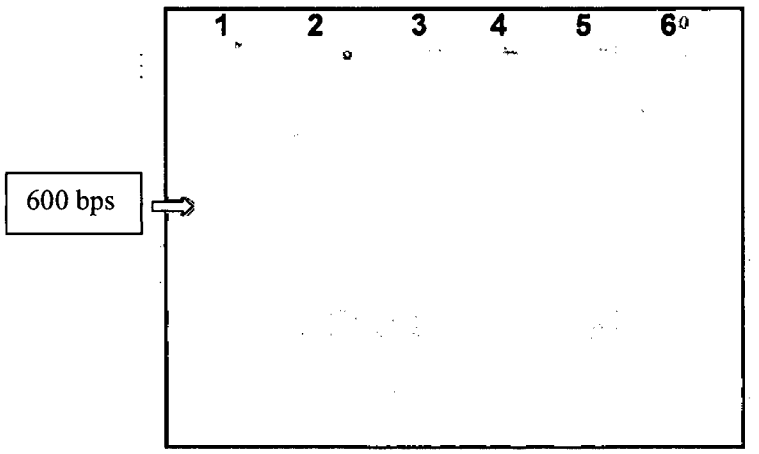


Fig.4.11 PCR amplification of internal fragment of *smtAB* gene using plasmid DNA as template. Lane 1. 100 bps DNA ladder; Lane.2. *Providencia rettgeri* GM04; Lane.3. *Proteus penneri* GM03; Lane.4. *Bacillus subtillis* GM02; Lane.5. *Proteus penneri* GM-10 and Lane.6. *Salmonella choleraesuis* 4A

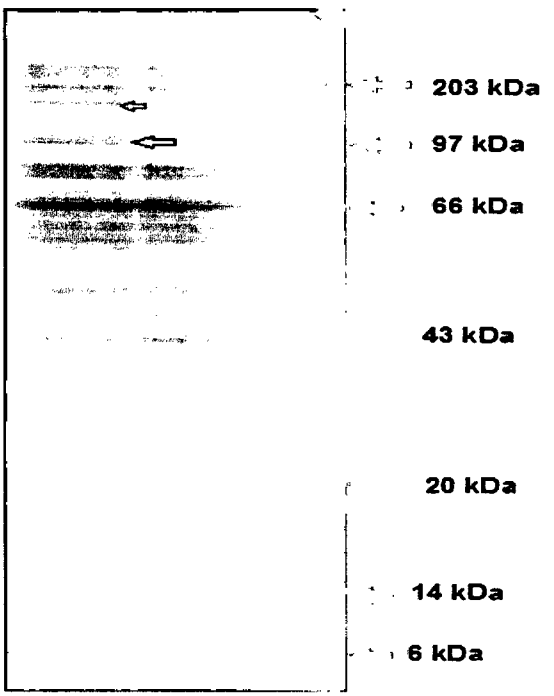


Fig.4.12 SDS-PAGE analysis of whole cell protein of *Providencia alcalifaciens* strain 2EA grown in TMM with, lane 1. 1.4 mM lead nitrate; Lane 2. 0 mM lead nitrate and lane 3. protein marker

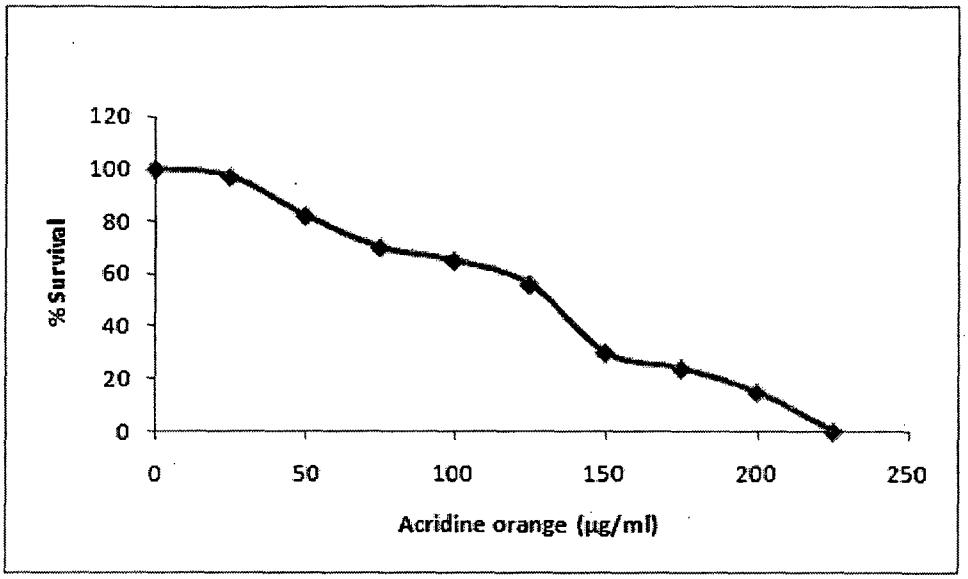


Fig.4.13 Percentage survival curve of *Providencia alcalifaciens* strain 2EA in different concentrations of acridine orange

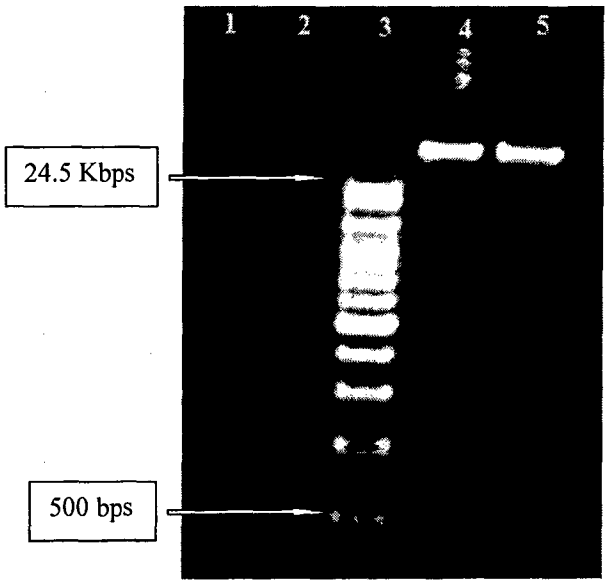


Fig.4.14 Plasmid curing using acridine orange. Lane, 1 and 2 Plasmid DNA preparation from *Providencia alcalifaciens* strain 2EA subcultured 6 times in 175 and 200 µg/ml acridine orange respectively; Lane.3. Supermixed DNA marker; Lane 4 and 5 Plasmid DNA isolated from *Providencia alcalifaciens* strain 2EA not treated with acridine orange (control)

CONCLUSION

1. *Pseudomosas aeruginosa* strain 4EA resists 0.8 mM lead nitrate and exhibits lead enhanced siderophore production and alteration in cell morphology as reduction in cell size when exposed to lead nitrate. Interestingly this bacterial strain also showed 11% (as weights %) lead biosorption as compared to other elements present on the cell surface when grown in 0.8 mM lead nitrate, confirming its potential in bioremediation of lead contaminated environmental sites. Alteration in cell morphology and lead enhanced siderophore production in this isolate also demonstrated its lead biomonitoring potential as a biosensor to detect lead contamination in drinking water as well as in terrestrial environments.

2. *Pseudomonas aeruginosa* strain WI-1 isolated from Mandovi estuary resist 0.6 mM lead nitrate, accumulated 26.5 mg lead/g cell dry weight intracellularly and produced bacterial metallothionein (BmtA), which was evidently demonstrated by AAS, SDS-PAGE and PCR amplification. Significant growth inhibition of phytopathogenic fungi, *Fusarium oxysporum* NCIM 1008 by *Pseudomonas aeruginosa* strain WI-1 and production of plant growth promoting substances such as siderophores and IAA was also observed. Since *Pseudomonas aeruginosa* strain WI-1 can sequester significantly high amount of lead and also produce plant growth promoting substances, we can employ this strain for bioremediation of estuarine environmental sites contaminated with lead and also as plant growth promoting bacteria in the soil heavily contaminated with lead.

3. Similarly *Providentia alcalifaciens* strain 2EA resists 1.4 mM lead nitrate by precipitating lead as insoluble lead phosphate $Pb_9 (PO_4)_6$, which was clearly demonstrated by XRD and SEM-EDS analysis of the light brown precipitate. This bacteria can serve as a biosensor to monitor lead contamination by precipitating lead as insoluble light brown lead phosphate and also as an agent for bioremediation of industrial effluents highly contaminated with lead.

4. *Enterobacter cloacae* strain P2B resists 1.6 mM lead nitrate by entrapping lead in acidic exopolysaccharide (EPS), which was clearly revealed by Alcian blue staining, FTIR and SEM-EDS analysis. Therefore EPS produced by *Enterobacter cloacae* strain P2B can be used for bioremediation of lead contaminated aquatic ecosystems. Since *Enterobacter cloacae* strain P2B showed lead enhanced EPS production, it can also be used for biomonitoring of lead contamination in drinking water.

5. *Pseudomonas stutzeri* strain M-9 and *Vibrio harveyi* strain M-11 isolate from Zuari estuary resist 0.8 mM and 1.2 mM lead nitrate respectively using P_{IB}-type ATPase mediated efflux system, which was demonstrated by PCR mediated amplification of *pbrA* gene (amplicon-750 bps). Genes encoding P_{IB}-type ATPase mediated efflux and repressor (*pbrR*) may be used to develop microbial sensors for environmental biomonitoring of lead using *lux* genes as reporter system and promoter-reporter transcriptional fusion technology.

6. Interestingly, lead resistance in *Salmonella choleraesuis* strain 4A and *Proteus penneri* strain GM-10 was mediated by small metallothionein (SmtA) encoded by *smtA* gene which was clearly revealed by PCR amplification of 507 bps internal fragment of *smtAB* genes and accumulation of significant amount of lead by both lead resistant isolates. This unique characteristic of hyper-lead accumulating bacterial strains 4A and GM-10 may be exploited for bioremediation of environmental sites (industrial waste dump sites) contaminated with considerably high levels of toxic lead. These interesting studies dealing with lead resistant bacteria will definitely facilitate designing strategies for bioremediation of lead contaminated environmental sites.

Summary

Heavy metals viz. lead, cadmium and mercury being non-essential to bacterial cells are toxic even at low concentrations. Generally, both natural and anthropogenic sources are responsible for terrestrial and aquatic contamination with toxic heavy metals. Heavy metals including lead exert toxic effects on living organisms in a variety of ways which include DNA damage, inactivation of proteins, essential metabolic enzymes and lipids. But, interestingly some natural microbial strains employing a variety of protective mechanisms can survive at very high levels of these toxic heavy metals including lead without any impact on their growth and metabolism.Various strategies through which they resist high levels of heavy metals include efflux, reduction, oxidation, extracellular sequestration, biosoption, precipitation and intracellular bioaccumulation It is interesting to note that they possess genetic determinants (genes) conferring metal resistance either on chromosomal genome, plasmid or transposons. This unique characteristic of heavy metal resistant microbes including bacteria makes them an ideal tool for bioremediation of heavy metal contaminated environmental sites.

- In the present study we have screened several lead resistant bacterial strains from terrestrial (waste from car battery manufacturing and electronics industries) and estuarine econiches (Mandovi and Zuari estuaries) of Goa.

- Six highly lead resistant bacterial strains were selected for further characterization.

- Based on morphological, biochemical characteristics, Fatty acid methyl esters profile and molecular characteristics (16S rDNA sequencing) these lead resistant bacterial strains were identified. These include: *Pseudomonas stutzeri* strain M-9, *Vibrio harveyi* strain M-11 and *Pseudomonas aeruginosa* strain WI-1 (estuarine isolates);

Enterobacter cloacae strain P2B, *Providentia alcalifacience* strain 2EA and *Pseudomosas aeruginosa* strain 4EA (terrestrial isolates).

- Lead resistance limit and resistance to multiple antibiotics showed by lead resistant bacterial strains were studied along with tolerance to Cd, Hg and Zn.

- *Pseudomonas stutzeri* strain M-9 and *Vibrio harveyi* strain M-11 resists 0.8 mM and 1.2 mM lead nitrate respectively possibly using P_{IB}-type ATPase mediated efflux mechanism, which was revealed by PCR mediated amplification of *pbrA* gene (750 bps).

- *Enterobacter cloacae* strain P2B resists 1.6 mM lead nitrate by entrapping lead in acidic exopolysaccharide (EPS), which was clearly revealed by Alcian blue staining, FTIR and SEM-EDX analysis.

- Similarly *Providentia alcalifaciens* strain 2EA resists 1.4 mM lead nitrate by precipitating lead as insoluble lead phosphate i.e. $(Pb_9 (PO_4)_6)$, which was clearly demonstrated by XRD analysis and SEM-EDX analysis of the precipitate.

- *Pseudomonas aeruginosa* strain WI-1 resists 0.6 mM lead nitrate and sequestered 26.5 mg lead/g cell dry weight intracellularly and produced bacterial metallothionein (BmtA), which was evidently demonstrated by AAS, SDS-PAGE and PCR amplification.

- *Pseudomosas aeruginosa* strain 4EA resists 0.8 mM lead nitrate and exibits lead enhanced siderophore production and alteration in cell morphology when exposed to lead nitrate. Interestingly this bacterial strain also showed 11% (as weights %) lead

biosorption as compared to other elements present on the cell surface when grown in 0.8 mM lead nitrate.

- Interestingly, lead resistance in *Salmonella choleraesuis* strain 4A and *Proteus penneri* strain GM-10 was mediated by small metallothionein (SmtA) encoded by *smtA* gene which was clearly revealed by PCR amplification of 507 bps internal fragment of *smtAB* genes.

- These interesting studies dealing with lead resistant bacteria will definitely facilitate designing strategies for bioremediation of lead contaminated environmental sites.

FUTURE PROSPECTS OF THE RESEARCH

There are several important prospects of my Ph.D. research which will be evaluated in future.

- Designing and development of packed bed reactor using microbial biomass or exopolysaccharide (EPS) to remove lead from industrial effluents.

- Identification and characterization of lead induced stress proteins using 2D-gel electrophoresis followed by MALDI-TOF-MS.

- Genes encoding lead-induced stress proteins may be used to develop microbial sensors for environmental biomonitoring of lead using *lux* genes as reporter system and promoter-reporter transcriptional fusion technology.

- Molecular biological characterization of lead resistant bacterial strains will be carried out in depth to explore various other mechanisms of lead resistance.

- Several genetic engineering approaches have been developed recently which proved useful in introducing the desired traits in potentially bioremediating estuarine bacteria for designing genetically engineered bacteria with novel remedial characteristics.

- Valuable characteristics already present in certain bacterial strains may be combined or improved using standard genetic engineering tools, but practical impact and delivery of these genetically modified strains under field conditions needs to be studied extensively.

- Terrestrial and estuarine bacterial strains may be genetically engineered to express high levels of metal binding ligands viz. carboxyl, hydroxyl, sulphate, phosphate and amine for biosorption of high levels of toxic metals either on cell surface or microbial products such as EPS and biosurfactants. Modification of these bacterial isolates over producing EPS and biosurfactant will be a good strategy for bioremediation of significant amount of heavy metals including lead.

APPENDIX

APPENDIX

A. Media composition

A.1 Tris minimal medium (single strength) for 1L: (Mergeay et al., 1985)

Tris-HCl	6.06 gm
NaCl	4.68 gm
KCl	1.49 gm
NH_4Cl	1.07 gm
$MgCl_2.\ 6H_2O$	0.2 gm
Na_2SO_4	0.43 gm
$CaCl_2$	0.03 gm
β-glycerol PO_4	0.5 gm
Fe (NH_4) citrate	0.005 g

Add 1 ml of trace element solution SL-7 (Biebl and Pfenning, 1981) for 1000 ml distilled water. Sterilized for 10 min at 120°C and 15 lbs pressure. To prepare TMM agar, TMM broth is mixed with 1.5% agar and autoclaved accordingly.

SL-7 trace element solution (Biebl and Pfenning, 1981)

25% HCl (7.7 M)	10 ml
$FeCl_2.\ 4H_2O$	1.5 mg
$CoCl_2.\ 6H_2O$	190 mg
$MnCl_2.\ 4H_2O$	100 mg
$ZnCl_2$	70 mg
H_3BO_3	62 mg
$Na_2MoO_4.\ 2H_2O$	36 mg
$NiCl_2.\ 6H_2O$	24 mg
$CuCl_2.\ 2H_2O$	17 mg

d/w	1 L

Dissolve FeCl2. $4H_2O$ in 25% HCl and then add d/w followed by the other components.

A.2 Luria Bertani (L.B) Broth (Gerhardt et al., 1994)

Tryptone	10.0 gm
Yeast extract	5.0 gm
Sodium chloride	10.0 gm
Double distilled water	1000 ml

Adjust to pH 7.0 with 0.1 N NaOH

For LB agar, 1.5 gm of agar is added to 100 ml L.B broth. Digest in water-bath and sterilized accordingly.

A.3 Zobell marine broth (Zobell, 1941)

Peptic digest of animal tissue	5 gm
Yeast extract	1 gm
Ferric citrate	0.1 gm
Sodium chloride	19.45 gm
Magnesium chloride	8.8 gm
Calcium chloride	1.8 gm
Pottasium chloride	0.55 gm
Sodium sulphate	3.24 gm
Sodium biocarbonate	0.16 gm
Potassium bromide	0.08 gm
Strontium chloride	0.034 gm
Boric acid	0.22 gm
Sodium silicate	0.004 gm
Sodium fluorate	0.0024 gm
Ammonium nitrate	0.0016 gm

Disodium phosphate	0.008 gm
Double distilled water	1 L

For agar plates add 1.5 % agar to Zobell marine broth and sterilized (autoclaved)

A.4 PYT80 broth (Konopka et al., 1999)

Peptone	80 mg
Yeast extract	80 mg
Tryptone	80 mg
4-morpholineethanesulfonic acid	10 mM
Double distilled water	1L

Adjust pH to 6.8 using 0.1N NaOH

For PYT80 agar add 1.5 gm per 100 ml PYT80 broth. Digest in water bath and sterilized accordingly.

A.5 SOC

Deionized water	950 ml
Tryotone	20 gm
Yeast extract	8 gm
NaCl	0.5 gm

Shake until the solutes have dissolved. Add 1ml of $MgCl_2$-$MgSO_4$ solution (12 gm $MgSO_4$ + 9.5 gm $MgCl_2$) and 1ml of 2M glucose or 20 ml of 20% glucose. Adjust volume of the solution to 1000 ml with deionised water and sterilize by autoclaving for 20 minutes at 15 psi.

A.6 Muller Hinton agar

Commercially available Muller Hinton agar (Himedia, India) was used

A.7 Nutrient Broth

Peptone	10.0 gm
Beef extract	3.0 gm
NaCl	5.0 gm
Double distilled water	1000 ml

pH was adjusted to 7.0 with 0.1 N NaOH

Appendix-B

B. Composition of stains, buffers and reagents

B.1. Stains

i) Gram stain reagents

Crystal violet

Solution A- 2 gm of crystal violet on 20 ml ethanol

Solution B- 0.8 gm ammonium oxalate dissolved in 80 ml d/w

Mixed solution A and B and filter through Whatman filter paper No. 1.

Gram's Iodine

Dissolve 1 gm iodine and 2 gm Potassium iodide in 300 ml d/w. Filtered through Whatman filter paper No. 1 (diameter =12.5 cm).

Safranine

2.5 gm Safranine was dissolved in 10 ml ethanol made the volume to 100 ml with d/w and filter through Whatman filter No.1.

Procedure for gram staining

Prepare smear of the organism on a clean glass slide and heat fixed it. Flood the smear with crystal violet for 1.5 min and gently wash with running tap water, then flood smear with Gram's iodine for a minute. Gently washed with tap water and decolourized with 90% ethanol prepared in d/w till colour oozes out. Counter stained with saffranine for 1 minute. Washed with tap water, air dry the slide and then put a drop of oil on smear to examined under oil immersion lens of microscope.

B.2 Buffers

i) Phosphate buffer (0.05 M)

Solution A (0.05 M monobasic di-hydrogen phosphate): 6.0 gm NaH_2PO_4 dissolve in 1000 ml d/w

Solution B (0.05 M dibasic di-hydrogen phosphate): 7.1 gm of Na_2HPO_4 dissolved in 1000 ml d/w.

X ml of A + Y ml of B mixed to obtain buffer of the desire pH.

X	Y	pH
88.7	12.3	6.0
39.0	61.0	7.0
5.3	97.4	8.0
2	98	9.5

APPENDIX C

C.1. Biochemical media used for identification.

Peptone	5.0 gm
Sugar	1 gm
NaCl	5.0 gm
Phenol red	0.01 gm
pH	7.0
D/W	To make 1 L

Media with Durhams tube was autoclaved at 15 psi for 20 minutes. Tubes inoculated and incubated at R.T. for 24-48 hrs. Change in colour from pink to yellow indicated sugar fermentation and presence or absence of gas was noted. Uninoculated tube served as control.

Nitrate reduction test

Nitrate broth

Peptone	5 gm
Beef extract	3 gm
KNO_3	1 gm
NaCl	5 gm
pH	7.0
d/w	Make volume to 1L

Nitrate broth was inoculated and incubated at R.T. for 24 hrs. After incubation, 5 drop of sulfanilic acid and 5 drop of α-naphthylamine were added. Red coloration indicated a positive test while in a negative test, red coloration is observed after addition of 5 mg of zinc. Uninoculated tubes served as the control.

Citrate utilization test

Simon's citrate agar

Ammonium dihydrogen phosphate	1 gm
Diammonium phosphate	1 gm
Sodium chloride	5 gm
Magnesium Sulphate	0.2 gm
Sodium citrate	5 gm
Bromothymol blue	0.3 gm
d/w	Make volume to 1L
Agar	20 gm
pH	7.0

Inoculate Simon's citrate slant and incubate for 24-48 hrs at R.T. Citrate utilization is indicated by presence of growth and media become dark blue colour.

Catalase test

Three or 4 drops of 3% (v/v) hydrogen peroxide was mixed with a loop full of culture in a plate. Evolution of gas bubbles caused by liberation of free oxygen was indicative of catalase positive organisms.

Oxidase test

A filter paper strip was soaked in Tetramethyl paraphynylene diamine (TMPD) dye. A loop full of fresh bacterial culture was smeared on the moist filter paper. Production of deep purple colour in 5-10 seconds indicated a positive oxidase test. This dye act as electron accepter and get reduced.

Gelatin liquefaction

Nutrient Gelatin

Peptone	5 gm
Beef extract	3 gm
Gelatin	20 gm
pH	7.0
d/w	To make 1 L

Inoculate tubes and incubate at R.T. for 24 hrs, the tubes were refrigerated for 30 min and the medium were observed. Liquid medium after refrigeration showed positive test.

Starch hydrolysis

Starch agar medium

Peptone	5 gm
Beef extract	3 gm
Soluble starch	2 gm
Agar	20 gm
pH	7.0
d/w	Make volume to 1L

Inoculate starch agar plates by spot inoculation. Incubate the plates at R.T. for 24-48 hrs then flood the plates with Gram's iodine for 1 min and pour off the excess stain. Clear zone around the colony indicated positive test and rest of plate appear blue.

Casein hydrolysis

Inoculate milk agar plates (milk added to Nutrient agar) and incubate at R.T. for 24-48 hrs then examine the plates for the presence of a clear area around the colony. A zone of clearance around the bacterial colony indicates positive proteolytic activity.

Hugh and Leifson's test

Peptone	2 gm
NaCl	5 gm
K_2HPO_4	0.3 gm
Glucose	10 gm
Bromothymol blue	0.01gm
pH	7.0
d/w	Make volume to 1 L

Sterilized media and cool immediately to avoid diffusion of air in medium. Inoculate in two tubes and overlay one tube with sterile paraffin oil and incubate. Facultative anaerobes showed change in colour in both tubes from green to yellow whereas aerobic (oxidative) organism showed yellow colour only in tube which is not overlaid with paraffin. Strict anaerobes show yellow colour in tube overlaid with paraffin.

MR-VP tests

MR-VP Medium (Glucose phosphate broth) is recommended for the performance of the Methyl Red and Voges-Proskauer tests in differentiation of the coli-aerogenes group.

peptone	7 gm
Dextrose	5 gm
Dipotassium phosphate	5 gm
Final pH	7 gm
d/w	1L

Methyl red test is employed to detect the production of sufficient acid during the fermentation of glucose phosphate in the medium. In Voges-Proskauer test organisms which produce acetyl methyl carbinol or its reduction product 2, 3 butanediol from glucose are detected. O'Meara's reagent is added and incubated at 37°C for 2 hrs appearance of pink colour indicate positive test.

Indole test

The medium used is tryptone water which is rich in amino acid tryptophan. Some organisms produce an enzyme tryptophanase which breakdown tryptophan into indole and pyruvic acid. Xylene is added in medium to concentrate indole and then 2-3 drops of Kovac's reagent slowly to give pink colour in the xylene layer.

C.2 Reagents for biochemical tests

Reagents for nitrate reduction

Solution A (Sulphanilic acid)

Sulphanilic acid	8 gm
Acetic acid (5N)	One part of acetic acid added to 2.5 parts of d/w
d/w	make final volume 1 L

Solution B (α-naphthylamine)

α-naphthylamine	5 gm
Acetic acid (5N)	1 L

APPENDIX-D

Chemical estimation and standard graphs

D.1 Folin Lowry's method for Protein estimation (Lowry et al. 1951)

Reagent A: 2 % Na_2CO_3 in 0.1 N NaOH

Reagent B: 0.5% $CuSO_4$ in 1% potassium sodium tartarate.

Reagent C: Alkaline Copper solution- Mixed 50 ml A and 1ml B prior to use.

Reagent D: Folin and Ciocalteau's phenol reagent

Commercially available reagent diluted with equal volume of d/w on the day of use. This reagent is a solution of sodium tungstate and sodium molybdate in phosphoric and hydrochloric acids.

Standard bovine serum albumin solutiom: 0.1 mg of BSA dissolve in 1 ml distilled water

Procedure: To 1 ml of the sample, 5 ml of copper sulphate solution was added and kept at room temperature in the dark for 10 minutes. 0.5 ml Folin and Ciocalteu's Phenol reagent was then added and kept in the dark for 20 minutes. Absorbance was measured at 660 nm against blank and concentration of the samples determined from standard graph using Bovine serum albumin as the standard (0-100 µg/ml).

APPENDIX-E

E.1. Stock solution for SDS-PAGE

Acrylamide-bis-acrylamide solution (Monomer solution): 29 gm acrylamide and 1 gm (w/v) N, N-methylene bis-acrylamide was dissolved in 70 ml d/w. pH was adjusted to 7.0 and volume was made up to 100 ml with d/w. Stored in amber coloured bottles at 4°C.

Resolving gel buffer (1.5 M Tris, pH 8.8): Prepared by dissolving 18.615 gm Tris base, in 70 ml d/w. The pH of the solution was adjusted to 8.8 using 6N HCl and volume was made upto 100 ml with d/w. The solution was stored at 4°C.

Stacking gel buffer (1.0 M Tris, pH 6.8): Prepared by dissolving 12.11 gm Tris base, in 70 ml d/w. The pH of the solution was adjusted to 6.8 using concentrated HCl and the volume was made up to 100 ml with d/w. The solution stored at 4°C.

Ammonium per sulphate (APS, 10%): Prepared by dissolving 0.1 gm of APS in 1.0 ml d/w. The solution was prepared fresh each time.

Electrophoresis buffer:

Composition of 1X buffer is as follows:

Tris base	3.0 gm
Glycine	14.4 gm
SDS (10%):	10 ml
d/w	to make total volume 1000 ml
pH	8.3

Sample solubilising buffer

Composition of 4X buffer is as follows:

Tris-HCl (1M, pH 6.8)	0.04 ml
Glycine	0.04 gm
SDS	0.004 gm
β-mercaptoethanol	0.004 ml
d/w	to make total volume 10 ml

Tracking dye:

50% sucrose	10 ml
Bromophenol blue	10 mg

Staining solution:

Coomassie Brillient Blue G-250 solution was prepared by dissolving 0.25 gm Coomassie Brillient Blue G-250 in 100 ml of 25% methanol, 10% glacial acetic acid and 65% d/w.

Destaining Solution

Methanol 40 ml

Acetic acid 10 ml

d/w 50 ml

Preparation of gel monomer

The composition of the resolving and tracking gels is as follows:

Solution	Resolving gel (10%) ml	Stacking gel (5%) ml
Monomer	2.5	0.33
1.5 M Tris, pH 8.8	1.875	-
1.0 M Tris, pH 6.8	-	0.625
10% SDS	0.075	0.025
10 % APS	0.0375	0.025
d/w	3	1.525
TEMED	0.005	0.005

E.2. Reagents for Genomic DNA isolation (Jones and Barlet 1990)

TE buffer: (10 mM Tris HCl, 1mM EDTA, pH 8.0)

10% w/v Sodium dodecyl sulphate

20 mg/ml Proteinase K

Phenol : Chloroform (1:1)

Isopropanol

70% ethanol

3 M Sodium acetate (pH-5.2)

E.3. Reagents for plasmid DNA isolation (Sambrook et al. 1989)

Alkaline Lysis Method (Birnboim and Doly, 1979)

i) Solution I (pH 8.0)

Glucose 0.9 g

Tris-HCl	0.394 gm
EDTA	0.292 gm
d/w	100 ml

ii) Solution II

SDS	1.0 gm
0.2 N NaOH	100 ml

iii) Solution III (pH 5.2)

5 M Potassium acetate	60 ml
Glacial acetic acid	11.5 ml
d/w	28.5 ml
pH	8.0

Tris acetate EDTA (TAE) buffer (pH 8.0)

50X

Tris base	: 2.42 gm
0.5 M EDTA	: 1 ml

Tris base and 0.5 M EDTA was dissolved in 10 ml of double distilled water and pH was adjusted to 8.0 with glacial acetic acid (0.57 ml) and final volume was made to 500 ml.

TE buffer (pH 8.0)

10 ml 0.1 M Tris-HCl (pH 8) was diluted with 90 ml of distilled water and 0.0372 gm of EDTA was added and pH was adjusted to 8.0.

E.4. Agarose gel Electrophoresis

Agarose	0.8 gm

1X TAE buffer 100 ml

Ethidium bromide solution

Ethidium bromide 10 mg

Distilled water 1 ml

Stock solution was prepared and kept cool and dark places. The final concentration used for agarose gel was 5µg/ml

Loading dye

Bromophenol blue	0.25 gm
Sucrose	40 gm
0.1 M EDTA	10 ml
1% SDS	10 ml
d/w	100 ml

Tracking dye was stored at 4°C

E.5. Plasmid curing agent

Acridine orange 10 mg/ml

Stock solution was then filter sterilized

APPENDIX-F

F.1 Preparation of stock solutions

F.1. Metal stock solution

1. Mercuric chloride ($HgCl_2$) (M.W- 271.52)

Stock solution (1 M) - $HgCl_2$ (271.52 gm) was dissolved in 1 Litre of de-ionized double distilled water. The solution was filter sterilized and stored at 4°C in dark places.

2. Cadmium chloride ($CdCl_2$) (M.W- 183.31)

Stock solution (1 M) - $CdCl_2$ (183.31 gm) was dissolved in 1 Litre of de-ionized double distilled water. The solution was filter sterilized and stored at 4°C in dark places.

3. Zinc chloride ($ZnCl_2$) (M.W-136.31)

Stock solution (1 M) - $ZnCl_2$ (136.31 gm) was dissolved in 1 Litre of de-ionized double distilled water. The solution was filter sterilized and stored at 4°C in dark places.

4. Lead nitrate ($Pb(NO_3)_2$ (M.W-331.20)

Stock solution (1 M) - ($Pb(NO_3)_2$) (331.20 gm) was dissolved in 1 Litre of De-ionized double distilled water. The solution was filter sterilized and stored at 4°C in dark places

F.2 Tributyltin chloride (TBTC) (M.W 325.49 g/mole)

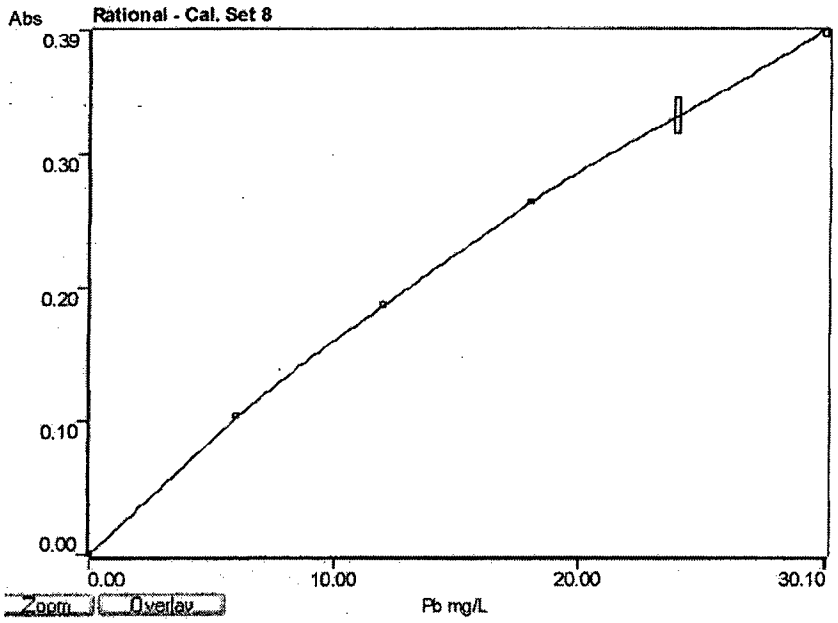
Preparation of 1 M stock of TBTC

Absolute ethanol (72.2 ml) + TBTC (27.8 ml) (3.7 M) was mixed make upto final volume of 100 ml. The solution was kept in amber coloured bottle in cold and dark conditions. The experiment ware performed by removing the required amount stock solution in sterile condition.

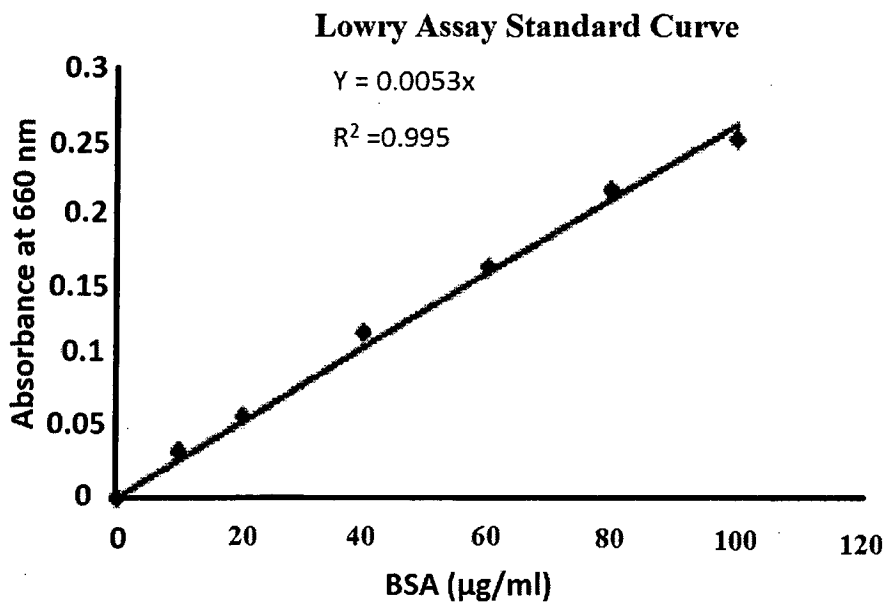
F.3. Ampicillin: 100 µg was weighed and dissolved in 1 ml of double distilled water and filter sterilized. The solution was kept at 4°C.

G. Standard graphs

a) Standard graph of lead using AAS



b) Standard graph of protein (Bovine serum albumin)



BIBLIOGRAPHY

REFERENCES

Asmub, M., Mullenders, L.H.F. and Hartwig, A. 2000. Interference by toxic metal compounds with isolated zinc finger DNA repair proteins. Toxicol. Lett. 112–113:227–231.

Aickin, R.M. and Dean, A.C.R. 1977. Lead accumulation by microorganisms. Microbios Lett. 5: 129-133.

Aickin, R.M., Dean, A.C.R., Cheetham, A.K. and Skarnulis, A.J. 1979. Electron microscope studies on the uptake of lead by a *Citrobacter* species. Microbios Lett. 9: 7–15.

Aiking, H., Govers, H. and van't Riet, J. 1985. Detoxification of mercury, cadmium, and lead in *Klebsiella aerogenes* NCTC 418 growing in continuous culture. Appl. Environ. Microbiol. 50:1262–1267.

Albersheim, P., Nevins, D.J., English, P.D. and Karr, A. 1967. A method for the analysis of sugars in plant cell-wall polysaccharides by gas-liquid chromatography. Carbohyd. Res. 5: 340-345.

Al-Aoukaty, A., Appanna, V.D. and Huang, J. 1991. Exocellular and intracellular accumulation of lead in *Pseudomonas fluorescens* ATCC 13525 is mediated by the phosphate content of the growth medium. FEMS Microbiol. Lett. 83: 283–290.

Alagarsamy, R. 2006. Distribution and seasonal variation of trace metals in surface sediments of the Mandovi estuary, west coast of India. Estuarine Coastal Shelf Sci.67: 333-339.

Alldredge, A. L., Passow, U. and Logan, B. E. 1993. The abundance and significance of a class of transparent organic particles in the ocean. Deep-Sea Res. Part I. 40: 1131–1140.

Altchul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. 1997. Gapped BLAST and PSIBLAST: a new generation of protein database search programs. Nucleic Acids Res. 25: 3389-3402.

Arias, S., del Moral, A., Ferrer, M.R., Quesada, R.T.E. and Bejar, V. 2003. Mauran, an exopolysaccharide produced by the halophilic bacterium *Halomonas maura*, with a novel composition and interesting properties for biotechnology. Extremophiles 7:319–326.

Attri, K. and Kerkar, S. 2011. Seasonal assessment of heavy metal pollution in tropical mangro diments (Goa, India). J. Ecobiotechnol. 3: 9-15.ve se

Babich, H. and Stotzk, G. 1978. Toxicity of zinc to fungi, bacteria and coliphages: Influence of chloride ions. Appl. Environ. Microbiol. 36: 906-914.

Babich, H. and Stotzky, G. 1979. Abiotic factors affecting the toxicity of lead to fungi. Appl. Environ. Microbiol. 38: 506-513.

Bargar, J.R., Brown, G. E. and Parks, G. A . 1998. "Surface complexation of Pb(II) at oxide-water interface: III. XAFS determination of Pb(II) and Pb(II)-chloro adsorption complexes on goethite and alumina." Geochimica Cosmochimica Acta, 62:193-207.

Bano, N. and Musarrat, J. 2003. Characterization of a new *Pseudomonas aeruginosa* strain NJ-15 as a potential biocontrol agent. Cur. Microbiol. 46: 324-328.

Barkay, T., Miller, S.M. and Summer, A.O. 2003. Bacterial mercury resistance from atoms to ecosystems. FEMS Microbiol. Rev. 27: 355-384.

Bauer, A.W., Kirby, W.M.M., Sherris, J.C. and Turck, M. 1966. Antibiotic susceptibility testing by a standardized single disc method. Am. J. Clin Pathol. 45:493–496.

Banjerdkij, P., Vattanaviboon, P. and Mongkolsuk, S. 2003. Cadmium-induced adaptive resistance and cross-resistance to zinc in *Xanthomonas campestris*. Curr. Microbiol. 47:260–262.

Baucheron, S., Tyler, S., Boyd, D., Mulvey, M.R., Chaslus-Dancla, E. and Cloeckaert, A. 2004. AcrAB-TolC directs efflux mediated multidrug resistance in *Salmonella enterica* serovar typhimurium DT104. Antimicrob. Agents Chemother. 48: 3729-3735.

Begum, A., Ramaiah, M., Harikrishna., Khan, I. and Veena, K. 2009. Heavy metal pollution and chemical profile of Cauvery river water. Elec. J. Chemistry. 6: 47-52.

Benes, P., Cejchanova, M. and Havlik, B. 1985. Migration and speciation of lead in a river system heavily polluted from a smelter. Water Res. 19:1-6.

Bhaskar, P.V. and Bhosle, N.B. 2005. Microbial extracellular polymeric substances in marine biogeochemical processes. Curr. Sci. 88: 45-53.

Bhaskar, P.V. and Bhosle, N.B. 2006. Bacterial extracellular polymeric substances (EPS) a carrier of heavy metals in the marine food-chain. Environment International. 32: 192-198.

Bhosle, N.B. 2007. Distribution of tributyltin (TBT) in the Mandovi estuary. NIO, Goa.10:105-113.

Biebl, H. and Pfenning, N. 1981. Isolation of members of the family *Rhodospirillaceae*. In: Starr, M.P (ed.), The prokaryotes, a handbook on habitats, isolation and identification of bacteria, vol. 1. Springer-Verlag, Berlin. pp. 267-273.

Birnboim, H.C. and Doly, J. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7: 1513-1523.

Blindauer, C.A., Harrison, M.D., Robinson, A.K., Parkinson, J.A., Bowness, P.W., Sadler, P.J. and Robinson, N.J. 2002. Multiple bacteria encode metallothioneins and SmtA- like fingers. Mol. Microbiol. 45: 1421-1432.

Bopp, L.H., Chakrabarthy, A.M. and Ehrlich, H.L. 1983. Plasmid determined resistance to Cr (VI) and reduction of Cr (VI) to Cr (III). J. Bacteriol. 155: 1105-1109.

Borremans, B., Hobman, J.L., Provoost A., Brown, N.L. and van der Lelie, D. 2001. Cloning and functional analysis of the pbr lead resistance determinant of *Ralstonia metallidurans* CH34. J. Bacteriol. 183:5651–5658.

Bonnevie, N.L., Gunster, D.G. and Wenning, R.J. 1992. Lead contamination in surficial sediments from Newark bay, New Jersey. Environment International. 18 : 497-508.

Bramhachari, P. and Dubey, S.K. 2006. Isolation and characterization of exopolysaccharide produced by *Vibrio harveyi* VB23. Lett. Appl. Microbiol. 43: 571-577.

Bramhachari, P.V. 2006. Biological characterization of marine bioluminescent bacteria under the stress of metallic and organometallic environmental pollutants. Ph.D. thesis, Goa University, Goa, India.

Bramhachari, P.V., Kavi Kishor, P.B., Ramadevi, R., Kumar, R., Rao, B.R. and Dubey, S.K. 2007. Isolation and characterization of mucous exopolysaccharide produced by *Vibrio furnissii* VB0S3. J. Microbiol. Biotechnol. 17: 44-51.

Braissant, O., Decho, A.W., Dupraz, C., Glunk, C., Przekop, K.M. and Visscher, P.T. 2007. Exopolymeric substances of sulfate-reducing bacteria: Interactions with calcium at alkaline pH and implication for formation of carbonate minerals. Geobiology.5: 401-411.

Bruins, M.R., Kapil, S. and Oehme, F.W. 2003. Characterization of small plasmid (pMBCP) from bovine *Pseudomonas pickettii* that confers cadmium resistance. Ecotoxicol. Environ. Saf. 54:241-248.

Brynhildsen, L., Lundgren, B.V., Allard, B. and Rosswall, T. 1988. Effects of glucose concentrations on cadmium, copper, mercury, and zinc toxicity to a *Klebsiella* sp. Appl. Environ. Microbiol. 54: 1689-1693.

Buysens, S., Heungens, K., Poppe, J. and Hofte, M. 1996. Involvement of pyochelin and pyoverdine in suppression of *pythium*-induced damping-off of Tomato by *Pseudomonas aeruginosa* 7NSK2. Appl. Environ. Microbiol. 62:865-871.

Calomiris, J.J., Armstrong, J.L. and Seidler, R.J. 1984. Association of metal tolerance with multiple antibiotic resistance of bacteria isolated from drinking water. Appl. Environ. Microbiol. 47: 1238-1242.

Cameron, R.E. 1992. Guide to site and soil description for hazardous waste characterization, vol I: Metals. Environmental Protection Agency. EPA/600/4-91/029. p 250.

Canovas, D., Cases, I. and de Lorenzo, V. 2003. Heavy metal tolerance and metal homeostasis in *Pseudomonas putida* as revealed by complete genome analysis. Env. Microb. 5:1242-1256.

Cavlo, C., Martinez-Checa, F., Mota, A., Bejar, V. and Quesada, E. 1998. Effect of cations, pH and sulfate content on the viscosity and emulsifying activity of the *Halomonas eurihalina* exopolysaccharide. J. Ind. Microbiol. Biotechnol. 20: 205–209.

Chakravarty, R., Manna, S., Ghosh, A.K. and Banerjee, P.C. 2007. Morphological changes in an *Acidocella* strain in responce to heavy metal stress. Res. J. Microbiol. 2: 742-748.

Chakravarty, R. and Banerjee, P.C. 2008. Morphological changes in acidophilic bacterium induced by heavy metals. Extremophiles. 12:279–284.

Chang, J.S., Law, R. and Chang, C.C. 1997. Biosorbtion of lead, copper and cadmium by biomass of *Pseudomonas aeruginosa* PU21. Wat. Res. 31: 1651-1658.

Chen, P., Greenberg, B., Taghavi, S., Romano, C., van der Lelie, D. and He, C. 2005. An exceptionally selective lead (II)-regulatory protein from *Ralstonia metallidurans*: development of a fluorescent lead (II) probe. Angew. Chem. Int. Ed. 44: 2715 –2719.

Chen, P.R., Wasinger, E.C., Zhao, J., van der Lelie, D., Chen, L.X. and He, C. 2007. Spectroscopic insights into lead (II) coordination by the selective lead (II)-binding protein PbrR691. J. Am. Chem. Soc. 129: 12350-12351.

Chen, W., Bruhlmann, F., Richins, R.D. and Mulchandani, A. 1999. Engineering of improved microbes and enzymes for bioremediation. Current Opinion Biotechnology. 10:137-141.

Cheng, Z., Wei, Y-Y.C., Sung, W.W., Glick, W.R. and McConkey, B.J. 2009. Proteomic analysis of the response of the plant growth-promoting bacterium *Pseudomonas putida* UW4 to nickel stress. Proteome Science. doi:10.1186/1477-5956-7-18.

Clark, D.L., Weis, A.A. and Silver, S. 1977. Mercury and organomercurial resistances determined by plasmids in *Pseudomonas*. J. Bacteriol. 132: 186-196.

Clarke, S.E., Stuar,t J. and Sanders-Loehr, J. 1987. Induction of siderophore activity in *Anabaena* sp. and its moderation of copper toxicity. Appl. Environ. Microbiol. 53:917–922.

Coombs, J.M. and Barkay, T. 2004. Molecular evidence for the evolution of metal homeostasis genes by lateral gene transfer in bacteria from the deep terrestrial subsurface. Appl Environ Microbiol. 70: 1698-1707.

Coombs, J.M. and Barkay, T. 2005. New findings on evolution of metal homeostasis genes: Evidence from comparative genome analysis of bacteria and archaea. Appl. Environ. Microbiol. 71: 7083–7091.

Comte, S., Guibaud, G. and Baudu, M. 2006. Relations between extraction protocols for activated sludge extracellular polymeric substances (EPS) and EPS complexation properties Part I. Comparison of the efficiency of eight EPS extraction methods. Enz. Microbial. Technol. 38: 237–245.

Cox, C.D. and Adams, P. 1985. Siderophore activity of pyoverdine for *Pseudomonas aeruginosa*. Infect. Immun. 48:130–138.

Crupper, S.S., Worrel, V., Stewart, G.C. and Londo, J.J. 1999. Cloning and expression of *cadD*, a new cadmium resistance gene of *Staphylococcus aureus*. J. Bacteriol. 181: 4071-4075.

Cunningham, T.M., Koehl, J.L., Summers, J.H. and Haydel, S.E. 2010. pH-dependent metal ion toxicity influences the antibacterial activity of two natural mineral mixtures. PLoS ONE. 5: e9456.

Dauvin, J-C. 2008. Effects of heavy metal contamination on the macrobenthic fauna in estuaries: The case of the Seine estuary. Mar. Poll. Bullet. 57: 160-167.

Decho, A.W. 1990. Microbial exopolymer secretion in ocean environment, their roles in food webs and marine processes. In: Barnes, M (ed). Ocean. Mar. Biol. Annu. Rev. 28: 73-153.

Dermatas, D., Dadachov, M., Dutko, P., Menounou, N., Arienti, P. and Shen, G. 2004. Weathering of lead in fort Irwin firing range soils. Global Nest: the Int. J. 6: 167-175.

Dessai, D.V.G. and Nayak, G.N. 2009. Distribution and speciation of selected metals in surface sediments, from the tropical Zuari estuary, central west coast of India. Environ. Monit. Assess. 158:117–137.

Desai, C., Jain, K. and Madamwar, D. 2008. Hexavalent chromate reductase activity in cytosolic fractions of *Pseudomonas* sp. G1DM21 isolated from Cr (VI) contaminated industrial landfill. Process Biochem. 4:713-721.

De Souza, M-J., Nair, S., Loka Bharati, P.A. and Chandramohan, D. 2006. Metal and antibiotic-resistance in psychrotrophic bacteria from Antarctic marine waters. Ecotoxicol. 15:379-384.

De, J., Ramaiah, N., Bhosle, N.B., Garg, A., Vardanyan, L., Nagle, V. L. and Fukami, K. 2007. Potential of mercury resistant marine bacteria for detoxification of chemicals of environmental concern. Microbes. Environ. 22: 336-345.

De, J., Ramaiah, N. and Vardanyan, L. 2008. Detoxification of toxic heavy metals by marine bacteria highly resistant to mercury. Mar. Biotechnol. 10:471-477.

Dubey, S.K. and Rai, L.C. 1990. Impact of chromium and tin on a nitrogen-fixing cyanobacterium *Anabaena doliolum*: Regulation of toxicity by pH, salinity, extracellular metabolites and organic acids. Biomed. Environ. Scien.3: 240-249.

Dubey, S.K. and Roy, U. 2003. Biodegradation of tributyltins (organotins) by marine bacteria. Appl. Organometallic Chem. 17: 1-6.

Dubey, S.K., Tokashiki, T. and Suzuki, S. 2006. Microarray-mediated transcriptome analysis of the tributyltin (TBT)-resistant bacterium *Pseudomonas aeruginosa* 25W in the presence of TBT. The J. Microbiol. 44: 200-205.

Eisler, R. 1988. Lead hazards to fish, wildlife, and invertebrates: a synoptic review. U.S. Fish Wildl. Serv. Biol. Rep. 85: 1-14.

El-Naggar, A.H. and El-Sheekh, M.M. 1998. Abolishing cadmium toxicity in *Chlorella vulgaris* by ascorbic acid, calcium, glucose and reduced glutathione. Environmental Pollution. 101: 169-174.

Facchinelli, A., Sacchi, E. and Mallen, I. 2001. Multivariate statistical and GIS-based approach to identify heavy metal sources in soils. Environ. Pollut. 114: 245–276.

Flora, S.J.S., Mittal, M. and Mehta, A. 2008. Heavy metal induced oxidative stress and its possible reversal by chelation therapy. Indian J. Med. Res. 128: 501-523.

Fowler, B.A. 1998. Roles of lead-binding proteins in mediating lead bioavailability. Environ. Health. Perspect.106: 1585-1588.

Fox, A., Morgan, S.L. and Gilbart, J. 1988. Preparation of alditol acetates and their analysis by gas chromatography and mass spectrometry. In: Analysis of carbohydrates by GLC and MS. (Bierman, C.J. and McGinnis, G., eds). CRC Press, Boca Raton. pp. 87-117.

Francis, C. A. and Tebo, B. M. 1999. Marine *Bacillus* spores as catalysts for oxidative precipitation and sorption of metals. J. Mol. Microbiol. Biotechnol. 1: 71-78.

Gadd, G.M. 1990. Heavy metal accumulation by bacteria and other microorganisms. Experientia. 46: 834-840.

Gadd, G.M. and White, C. 1993. Microbial treatment of metal pollution-a working biotechnology. Trends biotechnol. 11: 353-359.

Gadd, G.M. 2000. Bioremedial potential of microbial mechanisms of metal mobilization and immobilization. Curr. Opin. Biotechnol. 11: 271–279.

Gehardt, P., Murray, R.G.E., Wood, W.A. and Krieg, N.R. 1994. Methods for general and molecular bacteriology. American society for Microbiology. ASM press, Washington, DC. pp791.

Gehrke, T., Telegdi, J., Thierry, D. and Sand, W. 1998. Importance of extracellular polymeric substances from *Thiobacillus ferroxidans* for bioleaching. Appl Environ Microbiol 64: 2743–2747.

Gilis, A., Corbisier, P., Baeyens, W., Taghavi, S., Mergeay, M. and van der Lelie D. 1998. Effect of the siderophore alcaligin E on the bioavailability of Cd to *Alcaligenes eutrophus* CH34. J. Ind. Microbiol. Biotechnol. 20: 61–68.

Gikas, P., Sengor, S.S., Ginn, T., Moberly, J. and Peyton, B. 2009. The effects of heavy metals and temperature on microbial growth and lag. Global NEST Journal. 11: 325-332.

Grant, T., Bonomo, J., Block, M., Hanrahan, D. and Spiegler, J. 2006. The world's worst polluted places-The top ten. Report: The Blacksmith Institute New York City. 1-57.

Griffin, R. A. and Shimp, N. F. 1976. "Effect of pH on exchange-adsorption or precipitation of lead from landfill leachates by clay minerals." Environ. Sci. Technol. 10: 1256-1261.

Gummersheimer, B. and Giblin, T. 2003. Identification of lead resistant bacteria from a heavily contaminated site. Bios.74: 48-54.

Gutnick, D.L. and Bach, H. 2000. Engineering bacterial biopolymers for the biosorption of heavy metals; new products and novel formulations. Appl. Microbiol. Biotechnol. 54: 451–460.

Hamer, D.H. 1986. Metallothioneins. Ann. Rev. Biochem. 55: 913-951.

Hanahan, D. 1983. Studies on transformation of *E. coli* with plasmids. J. Mol. Biol. 166: 557-580.

Harrison, R.M. and Laxen D.P.H. 1981. Lead pollution. Causes and control. Chapman and Hall, New York. pp168.

Hartwig, A., Asmuss, M., Ehleben, I., Herzer, U., Kostelac, D., Pelzer, A., Schwerdtle, T. and Burkle, A. 2002. Interference by toxic metal ions with DNA repair processes and cell cycle control: molecular mechanisms. Environ. Health. Perspect. 110: 797–799.

Harwood-Sears, V. and Gordo, A. S. 1990. Copper-induced production of copper- binding supernatant proteins by the marine bacterium *Vibrio alginolyticus*. Appl. Environ. Microbiol. 56: 1327-1332.

Hassan, M-e-T., van der Lelie, D., Springael, D., Romling, U., Ahmed, N. and Mergeay, M. 1999. Identification of a gene cluster, *czr*, involved in cadmium and zinc resistance in *Pseudomonas aeruginosa*. Gene. 238: 417–425.

Hepinstall, S.E., Turner, B.F. and Maurice, P.A. 2005. Effect of siderophores on Pb and Cd adsorption to kaolinite. Clays Clay Miner. 53: 557–563.

Hernandez, A., Mellado, R.P. and Martinez, J.L. 1998. Metal accumulation and vanadium-induced multidrug resistance by environmental isolate of *Escherichia hermannii* and *Enterobacter cloacae*. Appl. Environ. Microbiol. 64: 4317-20.

Hermansson, M., Jones, G.W. and Kjelleberg, S. 1987. Frequency of antibiotic and heavy metal resistance, pigmentation and plasmids in bacteria of the marine air-water interface. Appl. Environ. Microbiol. 53: 2338-2342.

Hejabi, A.T., Basavarajappa, H.T., Karbassi, A. R. and Monavari, S.M. 2011. Heavy metal pollution in water and sediments in the Kabini river, Karnataka, India. Environ Monit. Assess. DOI 10.1007/s10661-010-1854-0.

Higham, D.P., Sadler, P.J. and Scawen, M.D. 1986. Effect of cadmium on the morphology, membrane integrity and permeability of *Pseudomonas putida.* J. Gen. Microbiol. 132: 1475-1482.

Hildebrand, E.E. and W. E. Blum. 1974. "Lead fixation by clay minerals." Nature wissenschaften. 61:169-170.

Hynninen, A., Touze, T., Pitkanen, L., Mengin-Lecreulx, D. and Virta, M. 2009. An efflux transporter PbrA and a phosphatase PbrB cooperate in a lead-resistance mechanism in bacteria. Molecular Microbiology.74: 384–394.

Hynninen, A., Tonismann, K. and Virta, M. 2010. Improving the sensitivity of bacterial bioreporters for heavy metals. Bioengineered Bugs. 1: 132-138.

Ivask, A., Rolova, T. and Kahru, A. 2009. A suite of recombinant luminescent bacterial strains for the quantification of bioavailable heavy metals and toxicity testing. BMC Biotechnol. 9: 41.

Iyengar, G.V. and Nair, P.P. 2000. Global outlook on nutrition and the environment: meeting the challenges of the next millennium. Sci. Total Environ. 249: 331–346.

Iyer, A., Mody, K. and Jha, B. 2004. Accumulation of hexavalent chromium by an exopolysaccharide producing marine *Enterobacter cloaceae*. Mar. Poll. Bullet. 49: 974-977.

Jagadeesh, K.S., Kulkarni, J.H. and Krishnaraj, P.U. 2001. Evaluation of the role of fluorescent siderophore in the biological control of bacterial wilt in tomato using Tn5 mutants of fluorescent *Pseudomonas* sp. Cur. Sci. 81: 882-883.

Jarvie, A.W. 1998. Organoleads in the environment: a review. Sci. Total Environ. 73:121-126.

Jayabarath, J., Shyam Sundar, S., Arulmurugan, R. and Giridhar, R. 2009. Bioremediation of heavy metals using biosurfactants. Inter. J. Biotechnol. Appl. 1: 50-54.

Jaworsky, J.F., Nriagu, J., Denny, P., Hart, B.T., Lasheen, M.R., Subramanian, V. and Wong, M.H. 1987. Group Report: Lead. Lead, mercury, cadmium and arsenic in the environment. John Wiley & Sons Ltd. pp 3-16.

Jayaraju, N., Reddy, B.C.S. and Reddy, K. R. 2011. Anthropogenic impact on Andaman coast monitoring with benthic foraminifera, Andaman Sea, India. Environ. Earth. Sci. 62:821-829.

Jiang, C-y., Sheng, X-f., Qian, M. and Wang, Q-y. 2008. Isolation and characterization of a heavy metal-resistant *Burkholderia* sp. from heavy metal-contaminated paddy field soil and its potential in promoting plant growth and heavy metal accumulation in metal-polluted soil. Chemosphere. 72: 157-164.

Jones and Barlet. 1990. "Preparation of Genomic DNA from Bacteria". Modified from Experimental Techniques in Bacterial Genetics. pp 20-23.

Johnson, C.E., Siccama, T.G., Driscoll, C.T., Likens, G.E. and Moller, R.E. 1995. Change in lead biochemistry in response to decreasing atmospheric inputs. Ecol. Appl. 5: 813-820.

Joshi, D.M., Patel, J.S., Flora, S.J.S. and Kalia, K. 2008. Arsenic accumulation by *Pseudomonas stutzeri* and its response to some thiol chelators. Environ. Health. Prev. Med. 13: 257-263.

Jude, F., Aprin, C., Brachet-Castang, C., Capdepuy, M., Caumette, P. and Quentin, C. 2004. TbtABM, a multidrug efflux pump associated with tributyltinresistance in *Pseudomonas stutzeri*. FEMS Microbiol. Lett. 232: 7-14.

Kashem, M.D.A., Singh, B.R. and Kawai, S. 2006. Mobility and distribution of cadmium, nickel and zinc in contaminated soil profiles from Bangladesh. Nutr. Cycl. Agroecosyst. 77: 187–198.

Kassab, D.M. and Roane, T.M. 2006. Differential responses of a mine tailings *Pseudomonas* isolate to cadmium and lead exposures. Biodegradation. 17: 379–387.

Khan, S., Lin, A., Zhang, S., Hu, Q. and Zhu, Y.G. 2008. Accumulation of polycyclic aromatic hydrocarbons and heavy metal in lettuce grown in the soils contaminated with long-term waste water irrigation. J. Hazard. Mat. 152: 506–515.

Khan, S., Hesham, A.E., Quiao, M., Rehman, S. and He, J-Z. 2010. Effect of Cd and Pb on soil microbial community structure and activities. Environ. Sci. Pollut. Res. 17: 288-296.

Khan, S., Rehman, S., Khan, A.Z., Khan, M.A. and Shah, M.T. 2010. Soil and vegetables enrichment with heavy metals from geological sources in Gilgit, northern Pakistan. Ecotoxicol. Environ. Safety .73: 1820–1827.

Khetan, S.K. and Collins, T.J. 2007. Human pharmaceuticals in the aquatic environment: A challenge to green chemistry. Chem. Rev. 107: 2319-2364.

Kimani, N.G. 2010. Environmental pollution and impacts on public health. Report: Dandora municipal dumping site in Nairobi, Kenya. United Nations Environment Programme and the St. John Catholic Church, Korogocho.

King, B.M. and Adler, D.L. 1964. A previously undescribed group of *Enterobacteriacea.* Amer. J. Clin. Pathol. 41:230-232.

Konopka, A., Zakharova, T., Bischoff, M., Oliver, L., Nakatsu, C. and Turco, R.F. 1999. Microbial biomass and activity in lead contaminated soil. Appl. Environ. Microbiol. 65: 2256–2259.

Krieg, N.R and Holt, J.G. 1984. Bergey's manual of systematic bacteriology, vol 1. The Williams and Wilkins, Baltimore, USA, pp 140–309.

Krishnamurthy, R., Cabral, L., Vidya, R. and Dubey, S.K. 2007. Isolation and biological characterization of a tributyltin chloride degrading marine bacterium, *Vibrio* sp. from Bombay high oil field, India. Curr. Sci. 93:1073-1074.

Kumar, M.S. and Singhal, R.S. 2011. Rheological behaviour of Schizophyllan in fermentation system. Am. J. Food Technol. 6: 781-789.

Lam, T.V., P. Agovino, X. N. and L. Roche. 2007. Linkage study of cancer risk among lead exposed workers in New Jersey. Sci. Total Environ. 372: 455-462.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227: 680-685.

Lacerda, C.M.R., Choe, L.E. and Reardon, K.F. 2007. Metaproteomic analysis of a bacterial community response to cadmium exposure. J. Proteome Res. 6: 1145-1152.

Larsen, T.S., Kristensen, J.A., Asmund, G. and Bjerregaard, P. 2001. Lead and zinc in sediments and biota from Maarmorilik, west Greenland: An assessment of the environmental impact of mining wastes on an Arctic fjord system. Environ. Pollut. 114: 275–283.

Laplace, J. M., Hartke, A., Giard, J. C. and Auffray, Y. 2000.Cloning, characterization and expression of an *Enterococcus faecalis* gene responsive to heavy metals. Appl. Microbiol. Biotechnol. 53: 685–689.

Lebrun, M., Audurier, A. and Cossart, P. 1994. Plasmid-borne cadmium resistance genes in *Listeria monocytogenes* are present on Tn5422, a novel transposon closely related to Tn917. J. Bacteriol.176: 3049-3061.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275.

Levinson, H.S., Mahler, I., Blackwelder, P. and Hood, T. 1996. Lead resistance and sensitivity in *Staphylococcus aureus.* FEMS Microbiol. Lett. 145: 421-425.

Levinson, H.S. and Mahler, I. 1998. Phosphatase activity and lead resistance in *Citrobacter freundii* and *Staphylococcus aureus.* FEMS Microbiol. Lett. 161: 135–138.

Liu, T., Nakashima, S., Hirose, K., Uemura, Y., Shibasaka, M., Katsuhara, M. and Kasamo, K. 2003. A metallothionein and CPx-ATPase handle heavy-metal tolerance in the filamentous cyanobacteria *Oscillatoria brevis.* FEBS Letters. 542: 159-163.

Li, X.D., Lee, S.L., Wong, S.C., Shi, W.Z. and Thornton, I. 2004. The study of metal contamination in urban soil of Hong Kong using a GIS base approach. Environ. Pollut. 129: 113–124.

Lombardi, P.E., Peri, S.I. and Verrengia Guerrero, N.R. 2010. ALA-D and ALA-D reactivated as biomarkers of lead contamination in the fish *Prochilodus lineatus.* Ecotoxicol.

Environ. Safety. 73: 1704-1711.

Macaskie, L.E., Bonthrone, K.M., Yong, P. and Goddard, D.T. 2000. Enzymically mediated bioprecipitation of uranium by a *Citrobactor* sp.: a concerted role for exocellular lipopolysaccharide and associated phosphatase in biomineral formation. 146: 1855-1867.

Maldonado, J., Diestra, E., Huang, L., Domènech, A.M., Villagrasa, E., Puyen, Z.M., Duran, R., Esteve, I. and Sole, A. 2010. Isolation and identification of a bacterium with high tolerance to lead and copper from a marine microbial mat in Spain. Ann. Microbiol. 60: 113–120.

Martinez, C.E., Jacobson, A.R. and Mcbridge, M.B. 2004. Lead phosphate minerals: solubility and dissolution by model and natural ligands. Environ. Sci. Technol. 38: 5584-5590.

Martinez, R.J., Wang, Y., Raimondo, M.A., Coomb, J.M., Barkey, T. and Sobecky, P.A. 2006. Horizontal gene transfer of P_{IB}-type ATPases among bacteria isolated from radionuclide- and metal-contaminated subsurface soils. Appl. Environ. Microbiol. 72: 3111-3118.

Martinez, J.L. 2008. Antibiotics and antibiotic resistance genes in natural environments. Science. 321: 365-367.

Matyar, F., Akkan, T., Ucak, Y. and Eraslan, B. 2010. *Aeromonas* and *Pseudomonas*: antibiotic and heavy metal resistance species from Iskenderun Bay, Turkey (northeast Mediterranean Sea). Environ. Monit. Assess. 167: 309–320.

Mandelstam, J. 1962. The repression of constitutive p-galactosidase in *Escherichia coli* by glucose and other carbon Sources. Biochem. J. 82: 489-493.

Meng, W., Qin, Y., Zheng, B. and Zhang, L. 2008. Heavy metal pollution in Tianjin Bohai Bay. China. J. Environ. Sci. 20: 814-819.

Mereghetti, L., Quentin, R., Marquet-van der mee, N. and Audurier, A. 2000. Low sensitivity of *Listeria monocytogens* to quarternary ammonium compounds. Appl. Environ. Microbiol. 66: 5083-5086.

McArthur, J.V. and Tuckfield, R.C. 2000. Spacial pattern in antibiotic resistance among stream bacteria: Effects of industrial pollution. Appl. Environ. Microbiol. 66: 3722-3726.

Meena, R.M., Garg, A. and Jadhav, S. 2009. Seasonal variation in organotins in the waters of the Dona Paula bay, west coast of india. Bull. Environ. Contam. Toxicol. 82: 586–58.

Mergeay, M., Nies D.H., Schlegel, H.G., Gerits, J., Charles, P. and Van Gijsegem, F. 1985. *Alcaligenes eutrophus* CH34 is a facultative chemolithotroph with plasmid-bound resistance to heavy metals. J. Bacteriol. 162: 328–334.

Mire, C.E., Tourjee, J.A., O'Brien, W.F., Ramanujachary, K.V. and Hecht, G.B. 2004. Lead precipitation by *Vibrio harveyi*: Evidence for novel quorum-sensing interactions. Appl. Environ. Microbiol. 70: 855-864.

Morillo, J.A., Aguilera, M., Ramoz-Cormenzana, A. and Monteoliva, S.M. 2006. Production of metal-binding exopolysaccharide by *Paenibacillus jamilae* using two-phase olive-mill waste as fermentation substrate. Curr. Microbiol. 53: 189-193.

Morillo, J.A., Garcia-Ribera, R., Quesada, T., Aguilera, M., Ramos-Cormenzana, A. and Monteoliva-Sanchez, M. 2008. Biosorption of heavy metals by the exopolysaccharide produced by *Paenibacillus jamilae*. World J. Microbiol. Biotechnol. 24: 2699–2704.

Nadagouda, M.N., Schock, M., Metz, D.H., Desantis, M.K., Lytle, D. and Welch, M. 2009. Effect of phosphate inhibitors on the formation of lead phosphate/carbonate nanorods, microrods and dendritic structures. Crystall Growth Design. 9: 1798-1805.

Naik, M.M. and Dubey, S.K. 2011. Lead-enhanced siderophore production and alteration in cell morphology in a Pb-resistant *Pseudomonas aeruginosa* strain 4EA. Curr. Microbiol. 62: 409–414.

Nair, S., Chandramohan, D. and Loka Bharathi, P.A. 1992. Differential sensitivity of pigmented and non-pigmented marine bacteria to metals and antibiotics. Water Research. 26: 431-434.

Namiranian, S., Richardson, D.J., Russell, D.A. and Sodeau, J.R. 1997. Excited state properties of siderophore pyochelin and its complex with zinc ions. Photochem. Photobiol. 65: 777-782.

Naz, N., Young, H.K., Ahmed N. and Gadd, G.M. 2005. Cadmium accumulation and DNA homology with metal resistance genes in sulphate-reducing bacteria. Appl. Environ. Microbiol. 71: 4610-4618.

Neumann, G., Veeranagauda, Y., Karegoudar, T.B., Sahin O, Mausezahl, I., Kabelitz, N., Kappelmeyer, U. and Heipieper, H.J. 2005.Cells of *Pseudomonas putida* and *Enterobacter sp.* adapt to toxic organic compounds by increasing their size. Extremophiles. 9: 163–168.

Neto, J.A.B., Smith, B.J. and McAllister, J.J. 2000. Heavy metal concentrations in surface sediments in a nearshore environment, Jurujuba Sound, Southeast Brazil. Environ. Pollut. 109: 1–9.

Nies, D.H. and Silver, S. 1995. Ion efflux systems involved in bacterial metal resistance. J. Ind. Microbiol. 14: 186–199.

Nies, D.H. 1999. Microbial heavy-metal resistance. Appl. Microbiol. Biotechnol. 51: 730–750.

Nies, D.H. 2003. Efflux-mediated heavy metal resistance in prokaryotes. FEMS Microbiol. Reviews. 27: 313-339.

Nilgiriwala, K.S., Alahari, A., Rao, A.S. and Apte, S.K. 2008. Cloning and overexpression of alkaline phosphatase *PhoK* from *Sphingomonas* sp. strain BSAR-1 for bioprecipitation of uranium from alkaline solutions. Appl. Environ. Microbiol. 74: 5516-5523.

Noël-Georis, I., Vallaeys, T., Chauvaux, R., Monchy, S., Falmagne, P., Mergeay, M. and Wattiez, R. 2004. Global analysis of the *Ralstonia metallidurans* proteome: Prelude for the large-scale study of heavy metal response. Proteomics . 4: 151–179.

Nriagu, J.O. 1978. The biogeochemistry of lead in the environment. Part B. Biological effects. Elsevier/North Holland Biomedical Press, Amsterdam. pp 397.

Okeke, I.N., Fayinki, S.T. and Lamikarna, A. 2000. Antibiotic resistance in *Escherichia coli* from Nigerian students, 1986-1998. Emerg. Infect. Dis. 6: 393-396.

Osborn, A.M., Bruce, K.D., Strike, P. and Ritchie, D.A. 1997. Distribution, diversity and evolution of the bacterial mercury resistance (mer) operon. FEMS Microbiol. Rev.19: 239-262.

Pain, A. and Cooney, J.J. 1998. Characterization of organotin resistant bacteria from Boston harbour sediment. Arch. Environ. Contam. Toxicol. 35:291-297.

Pal, A. and Paul, A.K. 2008. Microbial extracellular polymeric substances: central elements in heavy metal bioremediation. Indian J. Microbiol. 48: 49-64.

Pandey, P., Kang, S.C., Gupta, C.P. and Maheshwari, D.K. 2005. Rhizosphere competent *Pseudomonas aeruginosa* GRC1 produces characteristic siderophore and enhances growth of Indian mustard (Brassica campestris). Curr. Microbiol. 51:303–309.

Pandey, J., Shubhashish, K. and Pandey, R. 2010. Heavy metal contamination of Ganga river at Varanasi in relation to atmospheric deposition. Tropical Ecology. 51: 365-373.

Park, J.H., Bolan, N., Meghraj, M. and Naidu, R. 2011. Concomitant rock phosphate dissolution and lead immobilization by phosphate solubilising bacteria (*Enterobacter* sp.). J Environ. Manag. 92: 1115-1120.

Patel, K.S., Shrivas, K., Hoffmann, P. and Jakubowski, N. 2006. A survey of lead pollution in Chhattisgarh state, central India. Environ. Geochem. Health, 28: 11–17.

Pazirandeh, M., Wells, B.M. and Ryan, R.L. 1998. Development of bacterium-based heavy metal biosorbents: Enhanced uptake of cadmium and mercury by *Escherichia coli* expressing a metal binding motif. Appl. Environ. Microbiol. 64: 4068–4072.

Permina, E.A., Kazakov, A.E., Kalinina, O.V. and Gelfand, M.S. 2006. Comparative genomics of regulation of heavy metal resistance in eubacteria. BMC Microbiol. doi:10.1186/1471-2180-6-49.

Pickett, A. W. and Dean, A. C. R. 1976. Cadmium and zinc sensitivity and tolerance in *Klebsiella* (*Aerobacter*) *aerogenes*. Microbios. 15: 79-91.

Pike, R., Stapleton, P., Lucas, V., Roberts, G., Rowbury, R., Richards, H., Mullany, P. and Wilson, M. 2002. Effect of medium composition on the susceptibility of oral *streptococci* to mercuric chloride. Curr. Microbiol. 45: 272-276.

Pitthard, V. and Finch, P. 2001. GC-MS analysis of monosaccharide mixtures as their diethyldithioacetal derivatives: Application to plant gums used in art works. Cromatographia. 53: 317-321.

Poli, A., Anzelmo, G. and Nicolaus, B. 2010. Bacterial exopolysaccharides from extreme marine habitats: production, characterization and biological activities. Mar. Drugs. Doi: 10.3390/md8061779.

Prapagdee, B. and Watcharamusik, A. 2009. Adaptive and cross-protective responce against cadmium and zinc toxicity in cadmium-resistant bacterium isolated from a zinc mine. Braz. J. Microbiol. 40: 838-845.

Rai, L.C. and Dubey, S.K. 1988. Chromium toxicity in cyanobacteria: Possible role of carbon sources in toxicity amelioration. Microbios. 55: 193-2003.

Ramessur, R. 2004. Statistical comparison and correlation of zinc and lead in estuarine sediments along the western coast of Mauritius. Environment International. 30: 1039-1044.

Ramachandran, V. 2009. Molecular biology and biochemistry of Tributyltin chloride resistant marine bacterium *Alcaligenes* sp. Ph.D thesis, Goa University, Goa, India.

Ramachandran, V. and Dubey, S.K. 2009. Expression of TBTCl-induced periplasmic proteins in a tributyltin chloride resistant marine sediment bacterium *Alcaligenes* sp. Curr. Sci. 97: 1717-1718.

Raghunath, R., Tripathi, R.M., Kumar, A.V., Sathe, A.P., Khandekar, R.N. and Nambi, K.S.V. 1999. Assessment of Pb, Cd, Cu and Zn exposures of 6 to 10 year-old children in Mumbai. Environ. Res. 80: 215–221.

Raja, C.E., Sasikumar, S. and Selvam, S. 2008. Adaptive and cross resistance to cadmium (II) and zinc (II) by *Pseudomonas aeruginosa* BC15. Biologia. 63: 461-465.

Rathgeber, C., Yurkova, N., Stackbrandt, E., Beatty, J. M. and Yurkov V. 2002. Isolation of tellurite- and selenite-resistant bacteria from hydrothermal vents of the Juan de Fuca Ridge in the Pacific Ocean. Appl. Environ. Microbiol. 68: 4613-4622.

Rensing, C., Ghosh, M. and Rosen, B.P. 1999. Families of Soft-metal-ion-transporting ATPases. J. Bacteriol. 181: 5891–5897.

Rickard, D. T. and. Nriagu, J. E. 1978. "Aqueous environmental chemistry of lead." In the biogeochemistry of lead in the environment. Part A. Ecological Cycles, J. O. Nriagu (ed.), Elsevier/North Holland, New York, New York. pp. 291-284.

Roane, T.M. 1999. Lead resistance in two bacterial isolates from heavy metal-contaminated soils. Microb. Ecol. 37: 218-224.

Roane, T.M. and Kellogg, S.T. 1996. Characterization of bacterial communities in metal contaminated soil. Can. J. Microbiol. 42: 593-603.

Roane, T.M. and Pepper, I.L. 2000.Microbial responses to environmentally toxic cadmium. Microb. Ecol. 38: 358-64.

Romic, M. and Romic, D. 2003. Heavy metals distribution in agricultural top soils in urban area. Environ. Geol. 43: 795–805.

Rossbach, S., Wilson, T.L., Kukuk, M.L. and Carty, H.A. 2000. Elevated zinc induces siderophore biosynthesis genes and a zntA-like gene in *Pseudomonas fluorescens*. FEMS Microbiol. Lett. 191: 61–70.

Royan, S., Parulekar, C. and Mavinkurve, S. 1999. Exopolysaccharides of *Pseudomonas mendocina* P2d. Lett. Appl. Microbiol. 29: 342–346.

Roy, U., Dubey, S.K. and Bhosle, S. 2004. Tributyltin chloride utilizing bacteria from marine ecosystem of west coast of India. Curr. Sci. 86: 702-705.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory, New York.

Sanchez-Bayo, F. and Goka, K. 2005. Unexpected effect of zinc pyrithione and imidacloprid on Japanese medaka fish (*Oryzias latipes*). Aqat. Toxicol. 74: 285-293.

Sayyed, R.Z. and Chincholkar, S.B. 2009. Siderophore-producing *Alcaligenes faecalis* exhibited more biocontrol potential vis-a-vis chemical fungicides. Cur. Microbiol. 58: 47-51.

Schwyn, B. and Neilands, J.B. 1987. Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. 160: 47-56.

Scoullos, M.J. 1986. Lead in coastal sediments: the case of the Elefsis Gulf, Greece. Sci. Total Environ. 49:199-219.

Scrudato, R. J. and Estes, E. L. 1975. "Clay-lead sorption studies." Environmental Geology, 1: 167-170.

Sengar, R.S. and Pandey, M. 1996. Inhibition of chlorophyll biosynthesis by lead in greening *Pisum sativum* leaf segment. Biol. Plant. 38: 459–462.

Sasser, M. 1990. Identification of bacteria by gas chromatography of cellular fatty acids. Newark, DE, USA: Microbial ID.

Shah, M.T., Begum, S. and Khan, S. 2010. Pedo and biogeochemical studies of mafic and ultramfic rocks in the Mingora and Kabal areas, Swat, Pakistan. Environ. Earth Sci. 60: 1091–1102.

Sharma, S., Sundaram, C.S., Luthra, P.M., Singh,Y., Sirdeshmukh, R. and Gade, W.N. 2006. Role of proteins in resistance mechanism of *Pseudomonas fluorescens* against heavy metal induced stress with proteomics approach. J. Biotechnol. 126: 374-382.

Shin, S.H., Lim, Y., Lee, S.E., Yang, N.W. and Rhee, J.H. 2001. CAS agar diffusion assay for the measurement of siderophores in biological fluids. J. Microbiol. Method. 44: 89–95.

Silver, S. 1981. Mechanisms of plasmid determined heavy metal resistance. In: Molecular biology, pathogenecity and ecology of bacterial plasmids (Eds. S.B. Levy, R.C. Clowes, and E.L. konig). Plenus press, New York. pp: 179-789.

Silver, S. and Phung, L.T. 1996. Bacterial heavy metal resistance: new surprises. Ann. Rev. Microbiol. 50: 753-789.

Singh, M. 2001. Heavy metal pollution in freshly deposited sediments of the Yamuna river (the Ganges river tributary): A case study from Delhi and Agra urban centres, India. Environmental Geol. 6: 664-671.

Sinha, S. and Mukherjee, S.K. 2008. Cadmium-induced siderophore production by a highly Cd-resistant bacterial strain relieved Cd toxicity in plants through root colonization. Curr. Microbiol. 56:55–60.

Singh, B. and Sekhon, G. S. 1977. "Adsorption, desorption and solubility relationships of lead and cadmium in some alkaline soils." J. Soil Science. 28: 271-275.

Skei, J. M. 1978. Serious mercury contamination of sediments in a Norwegian semi-enclosed bay. Marine. Poll. Bullet. 9: 191-193.

Smith, B.J. and Orford, J.D. 1989. Scales of pollution in estuarine sediment around the North Irish Sea. In: Sweeney JC ed. The Irish Sea, a resource at risk. Special publication no.3. Geographical Society of Ireland. pp. 107–115.

Sobolev, D. and Begonia, M.F.T. 2008. Effect of heavy metal contamination upon soil microbes: Lead-induced changes in general and denitrifying microbial communities as evidenced by molecular markers. Int. J. Environ. Res. Public Health. 5: 450-456.

Soldatini, G.F., Riffaldi, R. and Levi-Minzi, R. 1976. "Lead adsorption by soils." Water, Air Soil Pollut. 6: 111-128.

Sun, G-X., Zhou, W-Q. and Zhong, J-J. 2006. Organotin Decomposition by Pyochelin, Secreted by *Pseudomonas aeruginosa* even in an iron-sufficient environment. Appl. Environ. Microbiol. 72: 6411–6413.

Sutherland, I.W. 2001. Biofilm exopolysaccharides: a strong and sticky framework. Microbiology. 147:3–9.

Sutherland, R.A. 2003 Lead in grain size fractions of road deposited sediment. Environ. Pollut. 121: 229–237.

Taghavi, S., Lesaulnier, C., Monchy, S., Wattiez, R., Mergeay, M. and van der Lelie, D. 2009. Lead (II) resistance in *Cupriavidus metallidurans* CH34: interplay between plasmid and chromosomally-located functions. Antonie van Leeuwenhoek. 96: 171–182.

Tauriainen, S., Karp, M., Chang, W. and Virta, M. 1998. Luminescent bacterial sensor for cadmium and lead. Biosens. Bioelectron. 13: 931–938.

Tay, J.H., Liu, Q.S. and Liu, Y. 2001. The role of cellular polysaccharides in the formation and stability of aerobic granules. Lett. Appl. Microbiol. 33: 222–226.

Teeling, H. and Cypionka, H.1997. Microbial degradation of tetraethyl lead in soil monitored by microcalorimetry. Appl Microbiol Biotechnol. 48: 275-279.

Templaton, A.S., Trainor, T.P., Spormann, A.M., Newville, M., Sutton, S.R., Dohnalkova, A., Gorby, Y. and Brown, G.E. 2003. Sorption versus biomineralization of Pb(II) with in *Burkholderia cepacia* biofilms. Environ. Sci. Technol. 37: 300-307.

Tong, S., von Schirnding, Y.E. and Prapamontol, T. 2000. Environmental lead exposure: a public health problem of global dimensions. Bull. World Health Org. 78: 1068-1077.

Trajanovska, S., Britz, M.L. and Bhave, M. 1997. Detection of heavy metal ion resistance genes in gram-positive and gran-negative bacteria isolated from a lead-contaminated site. Biodegradation. 8: 113-124.

Trevors, J.T. 1986. Plasmid curing in bacteria. FEMS Microbiol. Lett. 32: 149-157.

Trichopoulos, D. 1997. Epidemiology of cancer. In: De Vita, V.T. (Ed.), Cancer: Principles and practice of oncology. Lippincott Company, Philadelphia, pp: 231-258.

Turner, J.S., Glands, P.D., Samson, A.C.R. and Robinson, N.J., 1996. Zn^{+2}-sensing by the cyanobacterial metallothionein repressor SmtB: different motifs mediate metal-induced protein-DNA dissociation. Nucleic acids Res. 24: 3714-3721.

Turkdogan, M.K., Fevzi, K., Kazim, K., Ilyas, T. and Ismail, U. 2003. Heavy metals in soil, vegetables and fruits in the endemic upper gastrointestinal cancer region of Turkey. Environ. Toxic. Pharm. 13: 175–179.

Turner, A. 2010. Marine pollution from antifouling paint particles. Mar. Poll. Bullet. 60: 159–171.

van Hullebusch, E.D., Zandvoort, M.H. and Lens, P.N.L. 2003. Metal immobilisation by biofilms: Mechanisms and analytical tools. Re/Views Environ Sci Bio/Technol 2: 9–33.

Vantelon, D., Lanzirotti, A., Scheinost, A.C. and Kretzschmar, R. 2005. Spatial distribution and speciation of lead around corroding bullets in a shooting range soil studied by micro-X-ray fluorescence and absorption spectroscopy. Environ. Sci. Technol. 39: 4808-4815.

Velea, T., Gherghe, L., Predica, V. and Krebs, R. 2009. Heavy metal contamination in the vicinity of an industrial area near Bucharest. Environ. Sci. Pollut. Res. 16: 27-32.

Voleski, B. 1994. Advances in biosorbtion of metals: selection of biomass type. FEMS Microbiol. Rev. 14: 291-302.

Walker, D.J., Clemente, R., Roig, A. and Bernal, M.P. 2003. The effects of soil amendments on heavy metal bioavailability in two contaminated Mediterranean soils. Environ. Pollut. 122: 303–312.

Watt, G.C.M., Britton, A., Gilmour, H.G., Moore, M. R., Murray, G. D. and Robertson S. J. 2000. Public health implications of new guidelines for lead in drinking water: a case study in an area with historically high water lead levels. Food Chem. Toxicol.38: 73-79.

Wang, C.L., Ozuna, S.C., Clark, D.S. and Keasling, J.D. 2002. A deep-sea hydrothermal vent isolate, *Pseudomonas aeruginosa* CW961, requires thiosulfate for Cd^{+2} tolerance and precipitation. Biotechnol. Letts. 24: 637-641.

Wang, W., Shao, Z., Liu, Y. and Wang, G. 2009. Removal of multi-heavy metals using biogenic manganese oxides generated by a deep-sea sedimentary bacterium *Brachybacterium* sp. strain Mn32. Microbiol. 155: 1989–1996.

Webb, R.B. and Hass, B.S. 1984. Biological effects of dye on bacteria. VI. mutation induction by acridine orange and methylene blue in the dark with special reference to *Escherichia coli* WP6 (pol A1). Mutation Research/Genetic Toxicology. 137: 1-6.

Wingender, J., Neu, T.R. and Flemming, H.C. 1999. What are bacterial extracellular polymeric substances? In: Microbial extracellular polymeric substances (Wingender J, Neu TR, Flemming HC, eds). Springer, Berlin, pp 1–20.

Wuertz, S., Miller, C.E., Pfister, R.M. and Cooney, J.J. 1991. Tributyltin-resistant bacteria from estuarine and fresh water sediments. Appl. Environ. Microbiol. 57: 2783-2789.

Yamoka, Y., Inoue, H., Takimura, O. and Oota, S. 2002. Effect of iron on the degradation of triphenyltin by pyoverdines isolated from *Pseudomonas chlororaphis*. Organometal. chem. 16: 277-279.

Yildirim, V., Ozcan, S., Becher, D., Buttner, K., Hecker, M. and Ozcengiz, G. 2011. Characterization of proteome alterations in *Phanerochaete chrysosporium* in response to lead exposure. Proteome Science. 9:12.

Zimdahl, R.L. and Hassett, J. J. 1977. "Lead in Soil." In Lead in the environment. W. R. Boggess and B. G. Wixson (eds.), pp. 93-98. NSF/RA-770214. National Science Foundation, Washington, D.C.

Zanardini, E., Andreoni, V., Borin, S., Cappitelli, F., Daffonchio, D., Talotta,P., Sorlini,C., Ranalli,G., Bruni, S. and Cariati, F. 1998 Lead resistant microorganisms from red stains of marble of the Certosa of Pavia, Italy and use of nucleic acid based techniques for detection.Inter. Biodeter. Biodeg. 40: 171-182.

Zobell, C.E. 1941. Studies on marine bacteria. I. The cultural requirements of heterotrophic aerobes. J. Mar Res. 4: 42-75.

Zucconi, L., Ripa, C., Alianiello, F., Benedetti, A. and Onofri, S. 2003. Lead resistance, sorption and accumulation in a *Paecilomyces lilacinus* strain. Biol. Fertil. Soils. 37: 17-22.

CORRECTIONS IN THE THESIS

1. I have now included possible bioremediation strategies using lead resistant bacterial strains and also their use in monitoring lead contamination in the terrestrial and aquatic environment in the separate **conclusion** section of the thesis.

2. The main objective of this study was to isolate highly lead resistant bacteria from terrestrial and estuarine environments of Goa and explore their resistance mechanisms in order to employ them for bioremediation and biomonitoring of lead. Therefore, no attempt was made to study the community structure and dynamics of lead resistant bacteria during different seasons. During this study, I have done sampling three times to isolate highly lead resistant bacterial strains. There was no need to do sampling 4 times a year as my main objective was to study their resistance mechanisms rather than finding out presence of stable lead resistant microbial community.

3. Our aim was not to determine total organic carbon concentration in order to observe effect of TOC on lead resistant microbial community. We were also not interested in finding correlation between total organic carbon content of sample vis- a- vis total bacterial count along with total lead resistant bacterial count. Therefore, TOC of the sample from which lead resistant bacteria were isolated was not estimated. Aim of this study was to isolate highly lead resistant bacteria from terrestrial and estuarine econiches of Goa and explore their resistance mechanisms.

- Glucose (0.4%) was optimum concentration for the growth of lead resistant isolates whereas glucose above 0.4% caused precipitation of lead in the growth medium and results in decrease lead bioavailability (Fig. 2.24). Only bioavailable heavy metals exert toxic effect on bacterial cells and not sequestered (non bioavailable) metals.

Studies dealing with metal resistance in bacteria are done in commonly used minimal media instead of organic media rich in complex carbon sources, since complex media (Nutrient broth, Zobell marine broth) with complex carbon sources viz. oil and hydrocarbons tend to sequester metal ions thus make them unavailable to bacterial cells. Therefore simple carbon source like glucose and Tris- minimal medium with glucose was used in our study to study lead resistance.

4. It is ideal to do comparative study on environmental optimas for six selected isolates, but we have not presented any comparative data with reference to pH, temperature, NaCl

and carbon sources. In order to avoid confusion arising due to crowding of lines, if we combine 6graphs together as single graph, I have presented each graph separately.

I have very well included physiological characteristics of lead resistant bacterial isolates from lead battery waste in my Ph.D thesis.

5. I have used $Pb(NO_3)_2$, $CdCl_2$, $ZnCl_2$ and $HgCl_2$ since these are the most soluble salts of lead, cadmium, zinc and mercury respectively and most toxic. Most soluble metal salts give the real picture of metal resistance concentration, since soluble metals are readily bio-available for microorganisms.

6. It is evident from Fig 2.17 that *Providencia alcalifaciens* 2EA exposed to 1.4 mM lead nitrate showed less biomass and increased lag phase but total protein concentration was significantly high as compared to control because of high level expression of lead induced stress proteins (97 kDa and 147 kDa) which is clearly evident from SDS-PAGE analysis of lead exposed cells and control cells (Fig. 4.12).

It is evident from Fig 2.19 that *Pseudomonas aeruginosa* strain WI-1 exposed to 0.6 mM lead nitrate showed less biomass and increased lag but total protein concentration was significantly high as compared to control because of high level expression of lead induced stress proteins (11 kDa) which is clearly evident from SDS-PAGE analysis of lead exposed cells and control cells (Fig. 4.9). I have taken equal density of cells for protein estimation when exposed to 0.6 mM lead nitrate and in control conditions.

7. In all the SDS-PAGE experiments equal amount of protein was loaded in each well and electrophoresed. In all the SDS-PAGEs protein concentration in each well was **30 µg**.

8. I tried to isolate plasmid DNA from all the 11 lead resistant bacterial isolates out of which 10 isolates were found positive for plasmid (Fig. 4.2, 4.3, Fig. 4.7a, 4.7b, 4.7c, Fig. 4.14).

There are various heavy metal resistance mechanisms operational in bacteria which regulate the toxic level of biologically available metal ions in order to protect the cells from toxic effects of heavy metals on various vital metabolic processes. I have screened presence of *pbrA*, *smtA* and *BmtA* genes involved in lead resistant mechanism using plasmid/ genomic DNA of selected potential isolates (Figs. 4.2, 4.3, 4.4, 4.5, 4.8, 4.9, 4.10, 4.11, 4.12). Therefore I can conclude that there are multiple possible mechanisms

for lead resistance in natural isolates. But I do not have any idea weather all the above lead resistance regulating genes are present in a single lead resistant bacterial strains because I have not done sufficient experiments to prove it. We can't deny with the possibility of multiple resistance mechanism.

LIST OF PUBLICATIONS

1. **Naik, M.M.** and Dubey, S.K. 2011. Lead-enhanced siderophore production and alteration in cell morphology in a Pb-resistant *Pseudomonas aeruginosa* strain 4EA.**Current Microbiology**. 62:409–414.

2. **Naik, M.M.,** Pandey, A. and Dubey, S.K. 2012. Bioremediation of metals mediated by marine bacteria. In: Bacteria in Environmental Management and Biotechnology (Eds. Satyanarayana, T., Prakash, A and Johri, B.N.), **Springer Verlag, NY, USA. doi 10.1007/978-94-007-2229-3_29.**

3. **Naik, M.M.,** Pandey, A. and Dubey, S.K. 2012 *Pseudomonas aeruginosa* strain WI-1 from Mandovi estuary possesses metallothionein to alleviate lead toxicity and promotes plant growth. **Ecotoxicology and Environmental Safety. Doi:10.1016/j.ecoenv.2011.12.015.**

4. **Naik, M.M.,** Pandey, A. and Dubey, S.K. 2012. Biological characterization of lead-enhanced exopolysaccharide from lead resistant *Enterobacter cloacae* strain P2B. **Biodegradation. DOI: 10.1007/s10532-012-9552-y.**

5. Pandey, A., **Naik, M.M.** and Dubey, S.K. 2010. Hemolysin, Protease, and EPS producing pathogenic *Aeromonas hydrophila* strain An4 shows antibacterial activity against marine bacterial fish pathogens. **Journal of Marine Biology. doi:10.1155/2010/563205.**

6. Pandey, A., **Naik, M.M.** and Dubey, S.K. 2010. Organic metabolites produced by *Vibrio parahaemolyticus* strain An3 isolated from Goan mullet inhibit bacterial fish pathogens. **African Journal of Biotecnology**. 9:7133-7140.

7. Pandey, A., **Naik, M.M.** and Dubey, S.K. 2011. Biological characterization of marine Fish pathogen, *Acinetobacter* sp. strain An2 producing antibacterial metabolites. **Journal of Scientific and Industrial Research**. 70:135-141.

8. **Naik, M.M.,** Pandey, A. and Dubey, S.K. 2012. P-type ATPase and MdrL efflux pump mediated lead and multi-drug resistance in estuarine bacterial isolates from Goa, India. **Antonie van Leeuwenhoek (communicated).**

9. Shamim, K., **Naik, M.M.,** Pandey, A. and Dubey, S.K. 2012. TBTC–induced significant alteration in cell morphology and adsorption of lead in *Aeromonas caviae* strain KS-1 from Mandovi estuary of Goa, India. **Environmental Monitoring and assessment (under review).**

10. **Naik, M.M.** and Dubey, S.K. 2012. Lead resistant *Providencia alcalifaciens* 2EA precipitate lead (Pb^{+2}) as insoluble lead phosphate. **International biodegradation and biodeterioration (under review).**

11. **Naik, M.M.** and Dubey, S.K. 2012. Metallothionein mediated lead resistance in bacteria from car battery waste. **Current Science (Under Review).**

Curr Microbiol (2011) 62:409–414
DOI 10.1007/s00284-010-9722-2

Lead-Enhanced Siderophore Production and Alteration in Cell Morphology in a Pb-Resistant *Pseudomonas aeruginosa* Strain 4EA

Milind Mohan Naik · Santosh Kumar Dubey

Received: 27 April 2010 / Accepted: 7 July 2010 / Published online: 27 July 2010

Abstract A lead-resistant bacterial strain 4EA from soil contaminated with car battery waste from Goa, India was isolated and identified as *Pseudomonas aeruginosa*. This lead-resistant bacterial isolate interestingly revealed lead-enhanced siderophore (pyochelin and pyoverdine) production up to 0.5 mM lead nitrate whereas cells exhibit a significant decline in siderophore production above 0.5 mM lead nitrate. The bacterial cells also revealed significant alteration in cell morphology as size reduction when exposed to 0.8 mM lead nitrate. Enhanced production of siderophore was evidently detected by chrome azurol S agar diffusion (CASAD) assay as increase in diameter of orange halo, and reduction in bacterial size along with significant biosorption of lead was recorded by scanning electron microscopy coupled with energy dispersive X-ray spectrometry (SEM-EDX). *Pseudomonas aeruginosa* strain 4EA also exhibits cross tolerance to other toxic metals viz. cadmium, mercury, and zinc besides resistance to multiple antibiotics such as ampicillin, erythromycin, amikacin, cephalexin, co-trimoxazole, mecillinam, lincomycin, ciphaloridine, oleondamycin, and nalidixic acid.

Electronic supplementary material The online version of this article (doi:10.1007/s00284-010-9722-2) contains supplementary material, which is available to authorized users.

M. M. Naik · S. K. Dubey (✉)
Department of Microbiology, Laboratory of Bacterial Genetics and Environmental Biotechnology, Goa University, Taleigao Plateau, Goa 403 206, India
e-mail: dubey_santosh1@rediffmail.com

Introduction

Toxic metals viz. cadmium, lead, and mercury without any known biological functions are one of the most severe environmental pollutants prevalent in industrial wastes and their release into natural water bodies and terrestrial ecosystems pose serious threat to humans as well as microorganisms. Heavy metals are toxic to cells in a variety of ways which include DNA damage, oxidative damage to proteins and lipids, and binding to essential proteins and enzymes [1, 10, 18]. Therefore, U.S. Environmental Protection Agency has included lead, mercury, and cadmium in their list of hazardous wastes [4]. Some natural microbial strains employing a variety of protective mechanisms can survive at very high concentrations of these toxic heavy metals without any impact on their growth and metabolism. This unique characteristic of heavy metal-resistant microbes including bacteria makes them an ideal tool for bioremediation of metal contaminated sites. Various strategies through which they resist high concentrations of heavy metals include efflux, reduction, oxidation, extracellular sequestration, and intracellular bioaccumulation [3, 18, 20, 21]. In order to counteract frequent exposure to toxic organic compounds and heavy metals bacteria also exhibit significant alteration in cell morphology [5, 17].

Microbes require iron for growth and proliferation but iron (Fe^{+3}) at neutral pH in aerobic environment forms insoluble ferric hydroxide unavailable to microorganisms since the solubility constant for ferric hydroxide is 4×10^{-38} and respond to iron limitation by producing iron chelating siderophores [9, 11]. *Pseudomonas aeruginosa* is known to secrete two chemically distinct iron chelators viz. pyoverdine and pyochelin to solubilise Fe^{+3} and transport into the bacterial cells via specific receptors [7, 16]. These

bacteria are very useful in agriculture since their siderophores exhibit good plant growth promoting activity under iron limiting conditions, control crop diseases, and rescue plants from heavy metal toxicity [19, 25]. Apart from Fe^{+3} these microbial siderophores also form stable complexes with metals such as Cd^{+2}, Pb^{+2}, and Zn^{+2} [9, 11, 16], and induced siderophore production in bacteria in response to Cd^{+2}, Zn^{+2}, and Cu^{+2} stress has also been reported [6, 21, 25]. Since microbial siderophores form stable metal–ligand complexes and influence the metal mobility in the environment and thus proved to be an important strategy to sequester toxic metals.

In the present investigation we have screened and identified a highly lead-resistant bacteria from soil sample contaminated with car battery waste besides its biological characterization with reference to lead-enhanced siderophore production, alteration in cell morphology, lead biosorption, growth behavior, cross tolerance to mercury, cadmium, and zinc along with antibiotic susceptibility.

Materials and Methods

Isolation of Lead-Resistant Bacterial Strain

Isolation of lead-resistant bacterial strain was done from soil sample contaminated with car battery waste from a battery manufacturing company of Goa, India. The soil sample was plated on PYT80 agar (10 mM 4-morpholineethanesulfonic acid (pH 6.8), 80 mg peptone, 80 mg yeast extract, and 80 mg tryptone in 1 l deionized double distilled water) plates amended with 100 μM lead nitrate by dilution plating technique [13]. Filter sterilized lead nitrate (1 M stock) was amended to the PYT80 agar medium after sterilization. The isolated bacterial colonies which appeared were further spot inoculated on fresh PYT80 agar plates with different concentrations of lead nitrate and the bacterial colony which appeared at highest concentration of lead nitrate was selected for further characterization.

Identification of Lead-Resistant Bacterial Isolate

Identification of the selected lead-resistant bacterial isolate was done based on morphological characterization, biochemical tests, FAME analysis (Sherlock version 6.0B), and 16S rDNA sequencing. 16S rDNA was PCR amplified using following eubacterial primers:

8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-ACGGCTACCTTGTTACGACTT-3′). PCR amplification was performed using PCR amplification kit (Bangalore Genei, India) and sequencing was done at Xcelris laboratories, Ahmedabad, Gujarat, India. 16S rDNA sequence was compared against GenBank database using NCBI BLAST search. Phylogenetic analysis of the bacterial isolate was also done constructing phylogenetic tree using MEGA4 software [8, 12, 22, 26].

Characterization of Lead-Resistant Isolate

Growth behavior and lead resistance limit of the selected lead-resistant isolate was studied in Tris-minimal medium (TMM) [15], supplemented with different concentrations of lead nitrate at 30°C, pH 7.2 with constant shaking at 150 rpm. Absorbance of the culture suspension was recorded at definite time intervals as OD_{600nm} using UV–Vis spectrophotometer (Shimadzu, UV-2450, Japan) and graph was plotted between Absorbance and time interval. Slight modification in Tris-minimal medium was done by using 0.05 mM β-glycerol phosphate instead of inorganic phosphate in order to avoid lead precipitation and 0.4% glucose as carbon source along with 0.1 μM $FeCl_3$ (Himedia, India). Cross tolerance of the isolate to $HgCl_2$, $CdCl_2$, and $ZnCl_2$ was also determined in TMM.

Characterization of Siderophore and Assay

Characterization of siderophore of lead-resistant strain was done using UV–Vis spectrophotometer and spectrofluorimeter (Shimadzu, RF-5301-PC, Japan). Siderophore production was monitored by chrome azurol S agar diffusion (CASAD) assay [24]. The selected strain was grown in TMM with different concentrations of lead nitrate; cells in stationary phase were harvested to collect yellow–green supernatant containing siderophore which was filter sterilized (0.22 μm). In CASAD assay, each well (5 mm) was filled with 40 μl of siderophore containing supernatant and incubated for 2 h at 30°C. After incubation the diameter of orange halo around each well was measured and compared. Desferal mesylate (Sigma, USA), was used as a positive control and sterile TMM as negative control.

Morphological Characterization

Scanning electron microscope coupled with energy dispersive X-ray spectrometer (SEM-EDX) (JEOL JSM-5800LV) was used to examine morphological changes and lead biosorption in terms of weight % as compared to major and minor elements present on the cell surface of lead-resistant strain exposed to toxic levels of lead nitrate during exponential growth phase. Cells grown in TMM without lead served as control for SEM-EDX analysis. Cells were also grown in TMM with different concentrations of lead nitrate and effect of lead on cell morphology was analyzed by fixing bacterial cells in 3% glutaraldehyde overnight with 50 mM potassium phosphate buffer at 4°C.

Cells were washed thrice with phosphate buffer and dehydrated in gradually increasing concentrations of ethanol, i.e., 10, 20, 50, 70, 80, 90, 95, and 100% for 15 min each [17], air dried and stored in vacuum chamber prior to SEM-EDX analysis.

Antibiotic Susceptibility Testing

Antibiotic susceptibility test of lead-resistant isolate was performed following Kirby–Bauer disc diffusion method [2], using Muller–Hinton agar and antibiotic discs (Himedia, India).

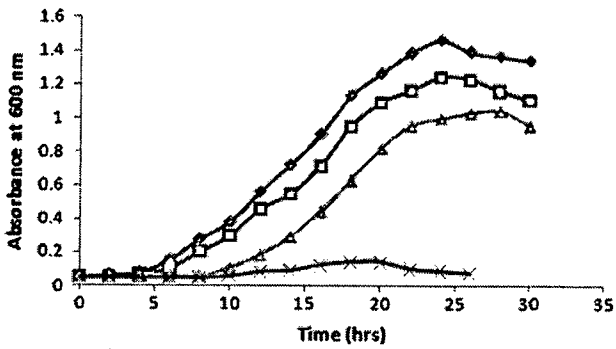


Fig. 2 Growth behavior of *Pseudomonas aeruginosa* strain 4EA in TMM amended with different concentrations of lead nitrate. *Filled diamond*: 0 mM (control), *filled square*: 0.5 mM, *filled triangle*: 0.8 mM, *times*: 0.9 mM

Results and Discussion

In the present investigation we have isolated a lead resistant bacterial strain from a terrestrial site contaminated with lead battery waste. This bacterial strain was found Gram negative, motile, rod shaped, and oxidative. It showed presence of enzymes such as oxidase, catalase, gelatinase, and appeared as yellow–green colony on cetrimide agar. Indole, methyl red and voges proskaeur's tests were found negative but bacterial isolate utilized citrate. Based on biochemical characteristics following Bergey's Manual of Systematic Bacteriology [14], Fatty acid methyl esters analysis and 16S rDNA sequencing followed by NCBI-BLAST search this lead resistant bacterial isolate was identified as *Pseudomonas aeruginosa* (Bankit no. 1341984) and designated as strain 4EA. Phylogenetic analyses clearly revealed its evolutionary relatedness with other *Pseudomonas aeruginosa* strains already reported (Fig. 1).

Pseudomonas aeruginosa strain 4EA could resist up to 0.8 mM lead nitrate in TMM (Fig. 2) and produced yellow-green, diffusible pigment. UV–Vis spectrophotometric and spectrofluorimetric analysis of this yellow-green pigment clearly revealed presence of two types of siderophores such as pyochelin and pyoverdine. Pyochelin showed

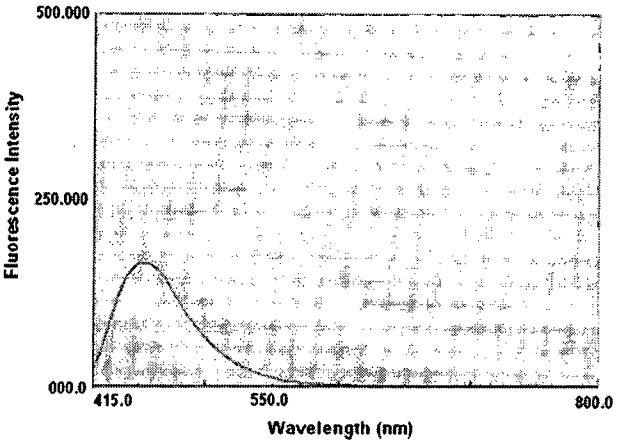


Fig. 3 Emission spectrum of pyoverdine of *Pseudomonas aeruginosa* strain 4EA when excited at 405 nm

absorbance maxima at 247 and 310 nm in UV range whereas pyoverdine showed strong absorbance at 370 nm. Pyoverdine exhibited emission at 460 nm when excited at 405 nm whereas pyochelin emitted at 448 nm when excited at 350 nm (Figs. 3, 4). The earlier UV–Vis spectrophotometric and spectrofluorimetric studies on microbial

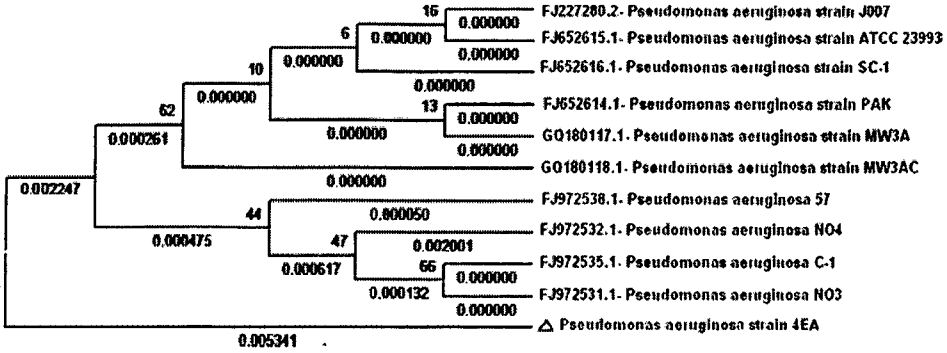


Fig. 1 Phylogenetic tree showing the evolutionary relationship of the test bacterium with other closely related bacteria using the Neighbor-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches

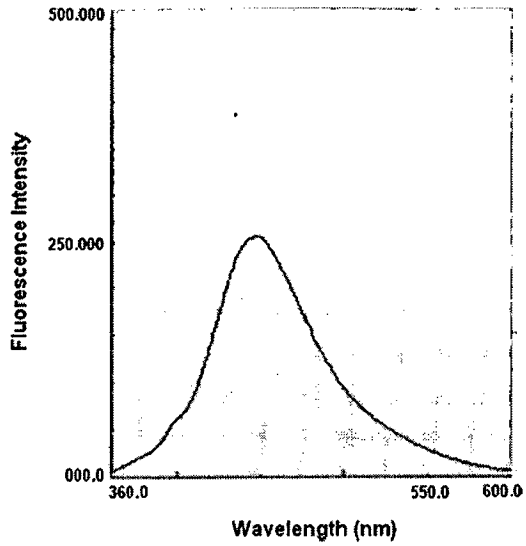


Fig. 4 Emission spectrum of pyochelin of *Pseudomonas aeruginosa* strain 4EA when excited at 350 nm

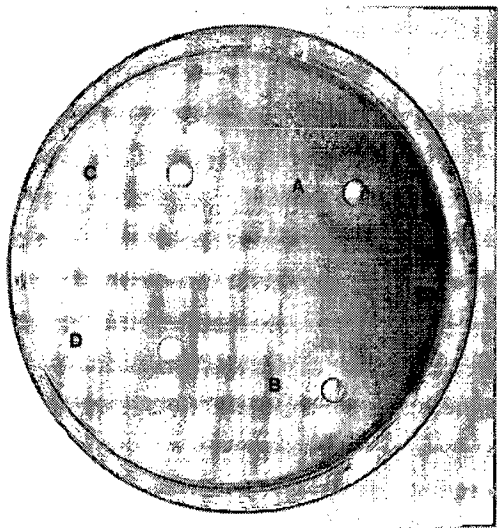


Fig. 5 CAS agar diffusion (CASAD) assay to demonstrate lead-enhanced siderophore production by *Pseudomonas aeruginosa* strain 4EA in terms of diameter of orange halo (cm). B, C and D indicate siderophore production in presence of 0.1, 0.3, and 0.5 mM lead nitrate, respectively, whereas A indicates basal level of siderophore production

siderophores (pyochelin and pyoverdine) have also shown similar results [7, 16, 19].

Lead enhanced gradual siderophore production was observed with increase in lead levels in the growth medium which was evidently revealed as increase in the diameter of orange halo on CAS agar plates using CASAD assay (Fig. 5). It is interesting to note that siderophore production was enhanced maximally up to 0.5 mM lead nitrate in TMM and was repressed above this level as evident from

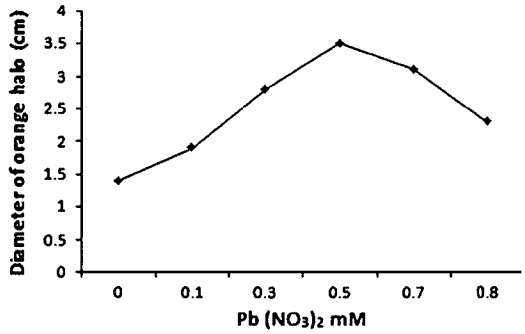


Fig. 6 Effect of different concentrations of lead nitrate on siderophore production by *Pseudomonas aeruginosa* strain 4EA in TMM in terms of diameter of *orange* halo (cm)

decrease in diameter of orange halo (Fig. 6). It is interesting to note that there are very few reports on metal induced siderophore production in microorganisms which is limited to Cd, Zn, and Cu only [6, 21, 25]. In the present study we have demonstrated lead-enhanced siderophore production in *Pseudomonas aeruginosa* for the first time. In addition to lead resistance this strain also showed cross tolerance to $ZnCl_2$, $CdCl_2$, and $HgCl_2$ as MIC for lead nitrate, zinc chloride, cadmium chloride, and mercuric chloride were 1, 0.7, 0.4, and 0.06 mM, respectively. The test bacterium also showed resistance to multiple antibiotics viz. oleondamycin (15 μg), nalidixic acid (30 μg), ampicillin (25 μg), erythromycin (15 μg), amikacin (10 μg), cephalexin (30 μg), co-trimoxazole (25 μg), mecillinam (33 μg), lincomycin (5 μg), and ciphaloridine (30 μg). The cell morphology of *Pseudomonas aeruginosa* strain 4EA was normal when grown without lead in the growth medium but significant change in cell morphology as reduction in cell size and shrinkage was evidently observed when cells were exposed to 0.8 mM lead nitrate. Interestingly this strain also showed 11% (as weight %) lead as compared to other elements present on the cell surface when grown in 0.8 mM lead nitrate which was clearly revealed by SEM-EDX analysis, whereas cells grown in TMM without lead nitrate demonstrated absence of any signal pertaining to lead (Fig. 7a, b).

In the present report, alteration in the cell morphology and enhancement of siderophore production in lead exposed *Pseudomonas aeruginosa* strain 4EA appear to be the response of bacterial cells against toxic lead. Lead is known to inhibit chlorophyll biosynthesis and hence suppresses the overall growth and bioproductivity of higher plants [23] but our studies have clearly demonstrated that lead resistant *Pseudomonas aeruginosa* strain 4EA has potential to biosorb very high levels of lead and also produce lead enhanced siderophores. Thus, this strain may serve as a bioinoculant for crop plants to supplement sufficient amount of iron (Fe III) by producing lead enhanced

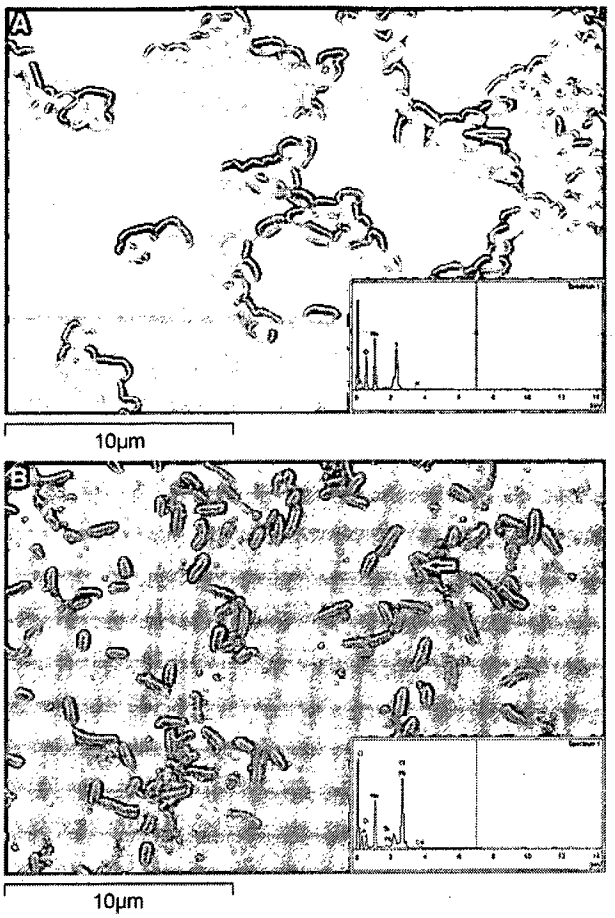


Fig. 7 Photomicrograph of *Pseudomonas aeruginosa* strain 4EA with and without exposure to lead nitrate using scanning electron microscopy-coupled with EDX (SEM—magnification, ×9000). **a** Control cells (no lead exposure) and EDX analysis (Arrow is pointing to the area considered for EDX analysis). **b** Cells exposed to lead nitrate (0.8 mM) and EDX analysis (*Arrow* is pointing to the area considered for EDX analysis)

siderophores along with biosorptive amelioration of lead present in contaminated fields.

Acknowledgments Milind Naik gratefully acknowledges U.G.C., Government of India for financial support as JRF. The authors are also thankful to Dr. M. S. Prasad and Mr. Vijay Khedekar from National Institute of Oceanography, Goa, India for scanning electron microscopy.

References

1. Asmub M, Mullenders LHF, Hartwig A (2000) Interference by toxic metal compounds with isolated zinc finger DNA repair proteins. Toxicol Lett 112–113:227–231
2. Bauer AW, Kirby WMM, Sherris JC, Turck M (1966) Antibiotic susceptibility testing by a standardized single disc method. Am J Clin Pathol 45:493–496
3. Borremans B, Hobman JL, Provoost A, Brown NL, van der Lelie D (2001) Cloning and functional analysis of the *pbr* lead resistance determinant of *Ralstonia metallidurans* CH34. J Bacteriol 183:5651–5658
4. Cameron RE (1992) Guide to site and soil description for hazardous waste characterization, vol I: Metals. Environmental Protection Agency EPA/600/4-91/029. p 250
5. Chakravarty R, Banerjee PC (2008) Morphological changes in acidophilic bacterium induced by heavy metals. Extremophiles 12:279–284
6. Clarke SE, Stuart J, Sanders-Loehr J (1987) Induction of siderophore activity in *Anabaena* sp. and its moderation of copper toxicity. Appl Environ Microbiol 53:917–922
7. Cox CD, Adams P (1985) Siderophore activity of pyoverdine for *Pseudomonas aeruginosa*. Infect Immun 48:130–138
8. Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791
9. Gilis A, Corbisier P, Baeyens W, Taghavi S, Mergeay M, van der Lelie D (1998) Effect of the siderophore alcaligin E on the bioavailability of Cd to *Alcaligenes eutrophus* CH34. J Ind Microbiol Biotechnol 20:61–68
10. Hartwig A, Asmuss M, Ehleben I, Herzer U, Kostelac D, Pelzer A, Schwerdtle T, Burkle A (2002) Interference by toxic metal ions with DNA repair processes and cell cycle control: molecular mechanisms. Environ Health Perspect 110:797–799
11. Hepinstall SE, Turner BF, Maurice PA (2005) Effect of siderophores on Pb and Cd adsorption to kaolinite. Clays Clay Miner 53:557–563
12. Kimura M (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120
13. Konopka A, Zakharova T, Bischoff M, Oliver L, Nakatsu C, Turco RF (1999) Microbial biomass and activity in lead contaminated soil. Appl Environ Microbiol 65:2256–2259
14. Krieg NR, Holt JG (1984) Bergey's manual of systematic bacteriology, vol 1. The Williams and Wilkins, Baltimore, USA, pp 140–309
15. Mergeay M, Nies DH, Schlegel HG, Gerits J, Charles P, Van Gijsegem F (1985) *Alcaligenes eutrophus* CH34 is a facultative chemolithotroph with plasmid-bound resistance to heavy metals. J Bacteriol 162:328–334
16. Namiranian S, Richardson DJ, Russell DA, Sodeau JR (1997) Excited state properties of siderophore pyochelin and its complex with zinc ions. Photochem Photobiol 65:777–782
17. Neumann G, Veeranagauda Y, Karegoudar TB, Sahin O, Mausezahl I, Kabelitz N, Kappelmeyer U, Heipieper HJ (2005) Cells of *Pseudomonas putida* and *Enterobacter* sp. adapt to toxic organic compounds by increasing their size. Extremophiles 9:163–168
18. Nies DH (1999) Microbial heavy-metal resistance. Appl Microbiol Biotechnol 51:730–750
19. Pandey P, Kang SC, Gupta CP, Maheshwari DK (2005) Rhizosphere competent *Pseudomonas aeruginosa* GRC1 produces characteristic siderophore and enhances growth of Indian mustard (*Brassica campestris*). Current Microbiol 51:303–309
20. Roane TM (1999) Lead resistance in two bacterial isolates from heavy metal-contaminated soils. Microb Ecol 37:218–224
21. Rossbach S, Wilson TL, Kukuk ML, Carty HA (2000) Elevated zinc induces siderophore biosynthesis genes and a *zntA*-like gene in *Pseudomonas fluorescens*. FEMS Microbiol Lett 191:61–70
22. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425
23. Sengar RS, Pandey M (1996) Inhibition of chlorophyll biosynthesis by lead in greening *Pisum sativum* leaf segment. Biol Plant 38:459–462

24. Shin SH, Lim Y, Lee SE, Yang NW, Rhee JH (2001) CAS agar diffusion assay for the measurement of siderophores in biological fluids. J Microbiol Method 44:89–95
25. Sinha S, Mukherjee SK (2008) Cadmium-induced siderophore production by a highly Cd-resistant bacterial strain relieved Cd toxicity in plants through root colonization. Current Microbiol 56:55–60
26. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599

Chapter 29
Bioremediation of Metals Mediated by Marine Bacteria

Milind M. Naik, Anju Pandey, and Santosh Kumar Dubey

Abstract Metals are an intrinsic part of the earth's crust. With rapid industrialization and urbanization, enormous amounts of industrial wastes including metal wastes are accumulating, which require special treatment. Wastes from mining and metal refining industries, sewage sludge, power plant wastes and waste incineration plants often contain substantial amounts of toxic heavy metals viz. Hg, Cd, Pb, As, Sb, Zn, Cu which pose serious treat to the environment and need to be removed from the source of pollution.

Microorganisms from metal polluted habitats possess a variety of inherent mechanisms to tolerate high levels of toxic metals which include precipitation of metals as phosphate, sulphide, carbonate; volatilization via methylation/ethylation; physical exclusion in membranes and extracellular polymeric substances (EPS); energy driven metal efflux system and intracellular sequestration mediated by metallothionein like proteins. For the last several decades, metal resistant microorganisms including marine bacteria have been considered a potential alternative for heavy metal recovery and bioremediation resulting in the development and refinement of many bioremediation technologies for removal of toxic metals form contaminated soils and aqueous mining and industrial wastes/effluents. Interestingly, these bioremediation technologies are economically viable, environmental friendly and value added processes.

Keywords Bioremediation • Metallothioneins • Heavy metals • Marine environment • Biosurfactants • Biosorption

M.M. Naik (✉) • A. Pandey • S.K. Dubey
Department of Microbiology, Goa University, Taleigao Plateau, Goa 403206, India
e-mail: dubey_santosh1@rediffmail.com

T. Satyanarayana et al. (eds.), *Microorganisms in Environmental Management: Microbes and Environment*, DOI 10.1007/978-94-007-2229-3_29,

29.1 Introduction

Heavy metals are intrinsic part of the earth crust. With rapid industrialization and urbanization enormous amount of industrial waste containing toxic heavy metals have been generated which need special treatment before release into the natural environment viz. terrestrial, aquatic and atmospheric environment. These toxic metals viz. Hg, Cd, Pb, Zn, Cr, Te, As, Sb and Cu released directly into the marine environment pose serious threat to the health and productivity of the marine biota including marine flora and fauna (Skei 1978; De et al. 2007, 2008; Jayaraju et al. 2010). Wastes from industries, sewage sludge, power plants and incineration plant often contain substantial amount of toxic heavy metals, which are of serious environmental concern and need to be removed from the source of pollution itself. It is interesting to mention that even Antarctic Ocean water, which is considered relatively more pristine than any other ocean water, is also contaminated with heavy metals due to anthropogenic activities (Bonner 1984). For instance, high level of several heavy metals in krills have been already reported (Yamamoto et al. 1987). Similarly level of Cd in caridean decapods, *Chorismus antarcticus* and *Notocrangon antarcticus* was highest among marine crustaceans (i.e.13 mg/kg dry weight). Some bacterial isolates from Antarctic Ocean have also shown high resistance to common antibiotics and heavy metals which clearly indicates that Antarctic Ocean is contaminated with heavy metals (De Souza et al. 2006).

For last several decades metal resistant microorganisms including bacteria have been considered a potential alternative for clean up and bioremediation of heavy metal contaminated environmental sites. Many bioremediation technologies have been developed for detoxification and removal of toxic heavy metals from metal contaminated aquatic sites and industrial wastes employing various indigenous metal resistant bacteria from heavy metal contaminated marine sites (Francis and Tebo 1999; Rathgeber et al. 2002; Iyer et al. 2005; De et al. 2007, 2008; Wang et al. 2009).

Microorganisms including bacteria possess a variety of mechanisms to tolerate and bioremediate high levels of toxic metals which include precipitation of metals as phosphates, carbonates and sulphides; volatilization via methylation/ethylation/reduction; intracellular bioaccumulation mediated by low molecular weight metallothionein proteins; ATP mediated efflux system; biosorption of metals at cell surface and sequestration in extracellular polymeric substances viz. EPS (Nakanura 1989; Blindauer et al. 1992; Nies 1999; Roane 1999; Rossbach et al. 2000; Borremans et al. 2001; Edgcomb et al. 2004; Mire et al. 2004; Naik and Dubey 2010). It is important to note that bioremediation technologies based on microbes are economically viable, cost effective, environment friendly. [AU1]

29.2 Heavy Metal Pollutants in Marine Environment

Although essential metals viz. Zn, Cu, Mn, Mo, Ni, Co, Mg and Fe are required in trace amounts for various metabolic processes of organisms, they are toxic at higher concentrations (Gadd 1992; Choudhuri and Srivastava 2001).Toxic metals viz. Cd,

Pb, Hg, Cr and metalloids such as As and Te which are non-essential, non-biodegradable persist indefinitely in marine ecosystem as a result of natural and anthropogenic activities posing serious environmental and health problems to the marine biota. Consumption of seafood contaminated with mercury and cadmium has been reported to cause serious diseases such as Minamata and itai-itai in coastal population of Japan respectively (Lanford and Ferner 1999; Matsuda et al. 2003). Therefore, these heavy metals have been included as the most hazardous substances in the US-EPA list of priority (Cameron 1992).

29.3 Level of Heavy Metal Pollutants in Marine Environment

All heavy metals exist in surface water in colloidal, particulate and dissolved phases, although dissolved concentrations are generally low (Kennish 1992). The colloidal and particulate metals may be found as (i) hydroxides, oxides, silicates, sulphides; or (ii) adsorbed to clay, silica or organic matter. The soluble forms are generally ions or un-ionized organometallic chelators or complexes. Non-essential heavy metals and metalloids of particular concern to surface water systems are Cd, Cr, Hg, Pb, As and Sb (Kennish 1992).The level of heavy metals in trough sediments , Kara sea, arctic ocean have been determined which revealed presence of cadmium < 0.5 ppm; As 2 ppm; Co 23 ppm; Cr 112 ppm; Cu 30 ppm; Hg 310 ppb; Ni 51 ppm; Pb 17 ppm; Sb 1.8 ppm and Zn 96 ppm respectively (Siegel et al. 2001). Whereas in Minamata bay very high level of mercury was reported which caused a serious neurological disorder in humans referred as "Minamata disease". The level of total mercury in sea water of Minamata bay ranged from 56 to 285 ng/L and 2.1–506 ng/L (Kumagai and Nishimura 1978). Interestingly, surface sediment sample from semi-enclosed bay, "Gunnekleivfjorden" from Southwest, Norway contained mercury ranging from 90 to 350 ppm (Skei 1978).

The behaviour of metals in natural waters is a function of substrate sediment composition, the suspended sediment composition, and the water chemistry. Sediment composed of fine sand and silt will generally have higher levels of adsorbed metals than quartz and detrital carbonate rich sediments. Metals also have a high affinity for humic acid, organo clays and oxides coated with organic matter (Connell and miller 1984). The water chemistry of the system controls the rate of adsorption and desorption of metals to and from sediment. Adsorption removes the metal from the water column and stores the metal in the substrate. Desorption returns the metal to the water column where recirculation and bioassimilation may take place. Metals may be desorbed from the sediment if the water experiences increase in salinity, decrease in redox potential or decrease in pH.

The contamination levels and distribution characteristics of heavy metals in coastal waters and sediments from Tianjin Bohai Bay, China revealed that Pb and Zn were the main heavy metal pollutants in the coastal waters of the bay. High levels of Pb and Zn appeared especially near the estuary, indicating that river discharge was the main pollution source. Analysis of data for the period 1987–2004 indicated that Pb pollution in coastal waters of Tianjin Bohai Bay originated primarily from

river discharge before 2001. Pb levels did not decrease after 2001 when annual runoff levels declined; indicating that Pb pollution by atmospheric deposition had increased due to the use of leaded petrol in motor cars. Pb, Zn, and Cd were the dominant polluting elements in superficial sediments from Tianjin Bohai Bay, with levels in excess of the corresponding upper limits of environmental background values. High concentrations of polluting elements were found in tidal sediments near water bodies such as Qihe and Dagu estuaries (Meng et al. 2008).

29.4 Heavy Metal Resistant Marine Bacteria

29.4.1 Heavy Metal Resistant Marine Bacteria from Coastal Waters

Since presence of metal resistant bacterial isolates in the environment directly suggests metal contamination, unusual rise in the number of mercury resistant bacteria along the Indian coast indicates significant mercury contamination. Several heavy metal resistant marine bacteria from coastal waters of India were isolated and evaluated for their ability to transform/efflux heavy metals viz. mercury, cadmium and lead. Interestingly, these marine bacteria were highly resistant to mercury and were capable of growing at 25 ppm or higher levels of mercury indicating their potential for detoxification not only of Hg, but also Cd and Pb along with polychlorinated biphenyls (Ramaiah and De 2003; De et al. 2006, 2007, 2008).These metal tolerant bacteria removed mercury by means of volatilization and were successful to detoxify mercury amended waters. Mercury resistant marine, *Pseudomonas aeruginosa* strain CH07 resists lead by EPS – entrapment mechanism which was clearly revealed by SEM-EDX analysis (De et al. 2007). Antarctic water samples were also examined for the presence of metal and antibiotic resistant bacteria (De Souza et al. 2006).The bacterial isolates from these waters showed varying degrees of resistance to antibiotics viz. chloramphenicol, ampicillin, streptomycin, tetracycline and kanamycin along with metals such as Cr, Cd, Zn and Hg which proved that even pristine environments are not devoid of metal pollutants and metal resistant bacteria. The metal resistant marine bacteria include *Enterobacter cloacae*, *Alcaligenes faecalis*, *Bacillus pumillus, Bacillus* sp., *Pseudomonas aeruginoasa, Alteromonas* sp., *Vibrio harveyi, Aeromonas* sp., *Corynebacterium* sp., *Brevibacterium iodinium, Streptomyces* sp., *Salinobacter* sp., *Micromonospora* sp., *Saccharomonospora* sp., *Flavobacterium* sp., *Moraxella* sp., *Micrococcus* sp. *and Aeromonas* sp. (Mire et al. 2004; Iyer et al. 2005; De Souza et al. 2006; De et al. 2008; Selvin et al. 2009; Matyar et al. 2010).

29.4.2 *Heavy Metal Resistant Marine Bacteria from Marine Sediments*

As a consequence of anthropogenic activities marine environment around the world is also contaminated by heavy metals. These toxic metal contaminants usually accumulate in the sediments and interestingly concentration of heavy metals is by several orders of magnitude higher in the sediment than their levels in surface water. It has long been recognised that aquatic microorganisms have a strong affinity for surfaces and that the majority of benthic bacteria are not suspended in waters but are attached to sediment particles. Adherent bacteria from Belgian continental plate showing metal contaminated marine sediment belongs to γ and δ proteobacteria (Gillan and Pernet 2007). Water and sediments of the Seine estuary are also contaminated by chemicals, especially Cadmium, which interestingly favours survival, growth and selective enrichment of Cadmium resistant bacteria. Among them 11 distinct isolates were found to carry *cadA* gene e.g. *Staphylococcus* sp., *Micrococcus* sp. and *Halobacillus* sp. (Oger et al. 2003).

Arsenite resistant bacteria were detected in deep sea sediments on the SouthWest Indian Ridge. Phylogenetic analysis based on 16S rRNA revealed that they mainly belonged to proteobacteria and actinobacteria. Denaturing gradient gel electrophoresis revealed that *Microbacterium esteraromaticum* was the dominant member in the arsenite enriched communities and this was reconfirmed by 16S rRNA gene library analyses. There is a significant diversity in arsenite resistant bacteria inhabiting the deep sea sediment which may play a role in the biogeocycling of arsenic in the marine environments (Chen and Shao 2009).

A deep sea sedimentary manganese-oxidizing bacterium, *Brachybacterium* sp. strain Mn 32 showed high Mn(II) resistance (MIC 55 mM) and Mn (II) – oxidizing/removing abilities. Strain Mn 32 removed Mn(II) employing two pathways : (i) oxidizing soluble Mn (II) to insoluble biogenic Mn oxides – birnessite (δ-MnO_2 group) and manganite (γ-MnOOH); (ii) the biogenic Mn oxides further adsorb more Mn(II) from the culture medium (Wang et al. 2009).

Few studies have focused on the mechanisms of adaptation to mercury contamination in marine sediment microbial communities. High frequency of Gram-negative bacterial isolates that are resistant to mercury were isolated from the aerobic culturable marine microbial community of marine sediment of Brunswick, GA, and Skidaway island near Savannah, GA, USA (Reyes et al. 1999).

29.4.3 *Heavy Metal Resistant Marine Bacteria from Hydrothermal Vent, Sponges and Coral Reefs*

Hydrothermal vents are highly rich sources of heavy metals which are considered to be toxic to living organisms. Since deep-sea hydrothermal vent fluids are enriched with toxic metals, it was hypothesized that (i) the biota in the vicinity of a vent is

adapted in the presence of toxic metals and (ii) metal toxicity is modulated by the steep physico-chemical gradients that occur when anoxic, hot fluids are mixed with cold oxygenated seawater. These metal enriched waters provide a constant selection pressure making the deep-sea hydrothermal vent environment a likely place to find heavy metal resistant microorganisms. For instance, heavy metal resistant heterotrophic bacteria were also isolated from the deep sea hydrothermal vent polychaete, *Alvinella pompejana* (Jeanthon and Prieur 1990). Mercury adaptation among bacteria from a deep-sea hydrothermal vent has also been reported (Vetriani et al. 2005). Deep-sea hydrothermal vents lie deep below the ocean and spew waters enriched with metals such as cadmium, zinc, lead, iron, and mercury. *Pseudomonas aeruginosa* CW961, an isolate from the vicinity of a deep-sea hydrothermal vent, grew in the presence of 5 mM Cd^{2+} and removed Cd^{2+} from solution. Sulphate was sufficient for growth when Cd^{2+} was not present in the culture medium; however, thiosulfate was necessary for Cd^{2+} precipitation and cell survival in the presence of Cd^{2+} (Wang et al. 2002). Three hyperthermophilic vent archaea, the sulfur-reducing heterotrophs, *Thermococcus fumicolans* and *Pyrococcus* strain GB-D and the chemolithoautotrophic methanogen *Methanocaldococcus jannaschii* were tested for tolerance to heavy metals viz. Zn, Co, and Cu. The sulphide addition consistently ameliorated the high toxicity of free metal cations by the formation of dissolved metal-sulphide complexes as well as solid precipitates (Edgcomb et al. 2004).

Coral reef flora and fauna are at risk of exposure to high concentrations of heavy metals from contaminant inputs such as processing plant and mine effluent discharged into rivers and marine ecosystems (Gladstone 1996). Coral reef sponges often harbour communities of symbiotic microorganisms that fulfil necessary functions for the well-being of their hosts. Sponges invariably filter a large volume of seawater and potentially accumulate heavy metals and other contaminants from the environment. Sponges, being sessile marine invertebrates and modular in body organization, can live many years in the same location and therefore have the capability to accumulate anthropogenic pollutants such as toxic heavy metals over a long time period. Sponges and corals are often host to abundant and diverse communities of symbiotic bacteria and algae that contribute to their nutrition and health. Microbial communities associated with the sponge, *Rhopaloeides odorabile* were used as bioindicators for sublethal copper stress (Webster and Webb 2001). Not only do sponges have potential for monitoring elevated concentrations of heavy metals but also examining changes in their microbial symbionts which is a novel and sensitive bioindicator for the assessment of pollution on important microbial communities. The sponge associated bacteria including *Streptomyces* sp. MSI01, *Salinobacter* sp. MSI06, *Roseobacter* sp. MSI09, *Pseudomonas* sp. MSI016, *Vibrio* sp. MSI23, *Micromonospora* sp. MSI28, *Saccharomonospora* sp. MSI36 and *Alteromonas* sp. MSI42 showed resistance against Cd, Hg and Pb (Selvin et al. 2009).

29.5 Biochemical and Genetic Mechanisms of Heavy Metal Bioremediation by Marine Bacteria

29.5.1 Intracellular Sequestration by Metallothioneins and Other Metal Binding Proteins

Metallothioneins are small cysteine rich, low molecular weight proteins ranging from 3,500 to 14,000 Da and ubiquitous in eukaryotes but also present in bacteria and cyanobacteria (Hamer 1986).These proteins have potential to sequester multiple heavy metal ions viz. Hg^{+2}, Cd^{+2}, Pb^{+2}, Zn^{+2}, Ag^{+1} and Cu^{+2} through thiol groups of cysteine and also participate in metal homeostasis. Cysteine constitutes nearly 30% of the total amino acids present in the protein. Metallothioneins and other metal binding proteins present in diverse cyanobacterial and bacterial species regulate the intracellular toxic metal concentration through highly specific metal binding. Metallothioneins limit metal availability and is thereby believed to confer protection against deleterious effects of high concentration of toxic heavy metals in *Synecococcus* PCC 7942, *Anabaena* PCC 7120, *Pseudomonas putida* and *Pseudomonas aeruginosa* (Blindauer et al. 2002). Typically, the metals for which metallothioneins have the highest affinity are Cd, Pb, Hg, Cu and Zn but also bind to Ni though with lower affinity. The heterotrophic marine bacterium, *Vibrio alginolyticus* interestingly showed production of extracellular copper-binding compounds when exposed to copper in a sea water medium. Fractionation and analysis of copper and methionine incorporation in culture supernatant fractions showed that the copper-binding compound co-eluted with material which was radiolabeled with ^{35}S methionine. This suggested that the copper-binding compound is a protein (Schreiber et al. 1990).The data suggest a potential role for macromolecules excreted by heterotrophic bacteria in control of copper ion activity in seawater. Two copper-inducible supernatant proteins having molecular masses of 21 and 19 kDa (CuBPl and CuBP2) were identified in marine bacterium, *Vibrio alginolyticus* and these proteins were 25 and 46 times amplified in supernatants of copper-challenged bacterial cultures as compared with controls without copper (Harwood-Sears and Gordon 1990).

29.5.2 Exopolymers, Biosurfactants and Other Biomolecules

Prokaryotic microorganisms in nature produce diverse and chemically complex exopolymeric substances (EPS) comprising of a variety of high molecular weight organic molecules viz. polysaccharides, proteins, nucleic acids, phospholipids along with other non-polymeric constituents of low molecular weight. EPS localized on outer bacterial cell surface mediate exchange of nutrients and essential metal ions with surrounding environments and also play important role in cell adhesion, formation

of biofilms, flocks and protects cells from hostile environments (Vu et al. 2009). Electrostatic interaction of metal cations with negatively charged functional groups involved in metal immobilisation by EPS are COO^-, HPO_4^-, OH^- and SO_4^{-2} and possess both adsorptive and adhesive properties (Pal and Paul 2008). EPS is believed to play a substantial role in sequestration of toxic heavy metals with varying degree of specificity and affinity thus prevent their entry inside bacterial cells and protect bacterial cells from toxic heavy metals such as Pb, Cd, Hg and Zn. Enzymatic activities in bacterial EPS are also involved in detoxification of heavy metals by transformation and subsequent precipitation resulting in entrapment of metals in these polymeric substances (Loaec et al. 1998; Bhaskar and Bhosle 2006; Poli et al. 2010). Therefore use of microbial sorbents like EPS can be used to bioremediate heavy metals from marine waters since marine pollution control is a major challenge to the environmental biotechnologists. The use of isolated microbial biopolymer in metal bioremediation process is more economically viable and ecofriendly alternative to environmentally risky chemical methods such as precipitation, coagulation, ion exchange and electrochemical processes.

Bacterial extracellular polymeric substances work as a carrier of heavy metals in marine food chain since metal binding properties of bacterial EPS and its possible role in bioaccumulation of Cu and Pb in marine food chain was investigated using a partially purified and chemically characterized microbial EPS isolated from *Marinobacter* sp. (Bhaskar and Bhosle 2006). Marine isolates of sulphate reducing bacteria (SRBs) have been reported to release EPS in the liquid medium during growth which forms complex with Ni, Cr and Mo and may be employed for bioremediation of marine ecosystem (Beech and Cheung 1995). It has been demonstrated that heavy metal resistant, deep sea hydrothermal vent bacterial species also produce EPS with capacity to bind metals and other toxic substances (Loaec et al. 1998; Wuertz, et al. 2000). EPS produced by Antarctic bacterial isolate contained uronic acid and sulphate which may act as carrier of heavy metals in southern ocean environments (Mancuso, et al. 2005). In marine *Pseudomonas aeruginosa* CH07 Lead was entrapped in EPS indicating it as a possible resistance mechanism (De et al. 2007) (Fig. 29.1). Accumulation of hexavalent chromium by an exopolysaccharide producing marine *Enterobacter cloacae* which is not only resistant to chromium but also showed enhanced growth and exopolysaccharide production in the presence of 25, 50 and 100 ppm Cr (VI) (Iyer et al. 2004).

Surfactants and emulsifiers comprise a unique class of molecules that are distinguished by their capacity to interface between water-soluble and oil phases. This amphipathic quality is conferred by the presence of both polar and non-polar moieties, endowing these molecules with a hydrophilic-hydrophobic nature. Marine microorganisms produce low and high molecular weight biosurfactants. The low molecular weight biosurfactants include glycolipids viz. trehalose tetraesters, dicorynomycolates, fructose lipids, sophorolipids and rhamnolipids and lipopeptides which include surfactin and viscosin. Preferred use of biosurfactants for bioremediation of heavy metals over chemical surfactants and emulsifiers is due to their lower toxicity, better environmental compatibility and economic viability. Among the various bioactive compounds, biosurfactants (BS)/bioemulsifiers (BE) are

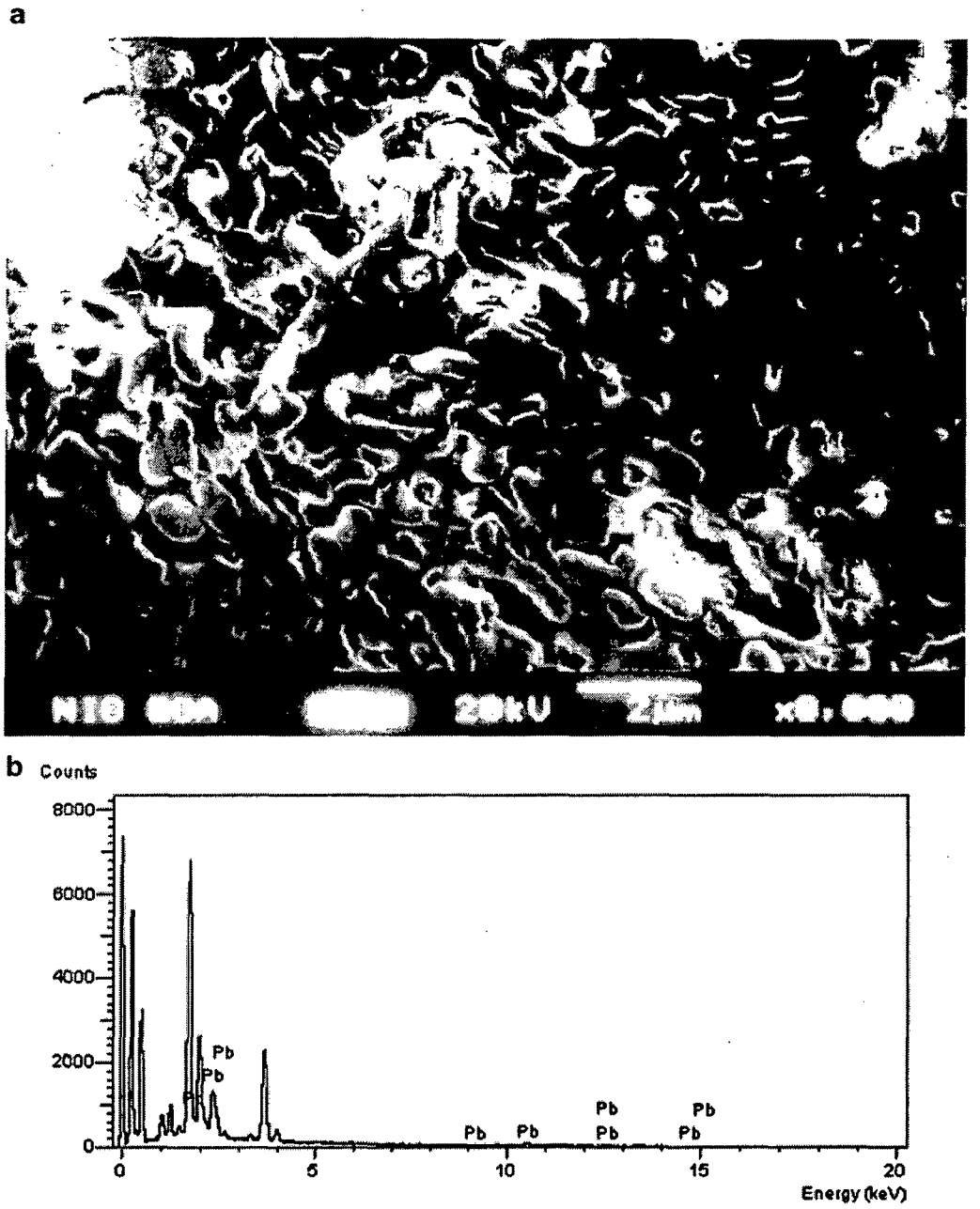


Fig. 29.1 Removal of Pb from Sea water nutrient broth (SWNB) amended with 50 mg/l Pb. **a)** SEM picture of the EPS-entrapped Pb (black arrow). **b)** The signal reflected from Pb as revealed by EDS. Results shown here are for the mercury-resistant marine Pseudomonas strain CH07. (Courtesy of De Jaysankar, in Microbes and Environment 22(4):336–345, 2007)

attracting major interest and attention due to their structural and functional diversity. Marine microorganisms such as *Acinetobacter* sp., *Arthrobacter* sp., *Pseudomonas* sp., *Halomonas* sp., *Myroides* sp., *Corynebacteria* sp., *Bacillus* sp., *Alteromonas* sp. have been studied for production of BS/BE and exopolysaccharides (Satpute et al. 2010). Hexavalent chromium reduction and trivalent chromium tolerance of

312 marine *Bacillus* sp. MTCC 5514 is known to be mediated by its extracellular
313 enzyme, chromate reductase and its biosurfactant (Gnanamani et al. 2010). An
314 excellent alternative for enhanced metal bioremediation is the use of microbial
315 biomolecules such as microbial surfactants and extracellular polymers which would
316 increase the efficiency of metal reduction/sequestration by microorganisms to
317 achieve field bioremediation of metal contaminated sites (Singh and Cameotra
318 2004). It is interesting to note that these biomolecules exhibit dual advantage in
319 bioremediation of sites contaminated with metals and organic compounds since
320 microorganisms have capability to utilize organic compounds such as hydrocarbons
321 as carbon source and simultaneous sequestration of heavy metals.

322 *29.5.3 ATPase Mediated Efflux*

323 In order to maintain heavy metal homeostasis, intracellular concentration of toxic
324 heavy metal ions has to be tightly controlled. Soft-metal-transporting P-type
325 ATPases are group of proteins involved in transport of heavy metals across the bio-
326 logical membranes and thus responsible for bacterial heavy metal resistance. ATPase
327 mediated efflux pump prevents over accumulation of metals inside the bacterial
328 cells by effluxing excessive heavy metals outside the cells. The best examples of
329 Zn(II)/Cd(II)/Pb(II)-translocating ATPases are encoded by gene, *zntA* from *E. coli*
330 and *cadA* gene from *S. aureus* plasmid pI258 (Silver 1999; Rensing et al. 1999).
331 Complete operon *pbrUTRABCD* confering efflux mediated lead resistance has been
332 well studied in *Ralstonia metallidurans* CH34 (Borremans et al. 2001; Taghavi et al.
333 2009).The mechanism of Zn resistance in multiple metal-resistant *Pseudomonas*
334 *putida* strain S4 is based on inducible efflux. An ATPase in this strain mediates
335 active extrusion of Zn^{+2}, which occurred during the exponential phase of growth
336 (Choudhuri and Srivastava 2001).

337 *29.5.4 Biosorption*

338 Biosorption of metals is mediated by several mechanisms such as ion exchange,
339 chelation, adsorption and diffusion through cell walls and membranes. Biosorption
340 encompasses those physico-chemical mechanisms by which metal ions are removed
341 from an aqueous solution. This phenomenon is often attributed to the binding of
342 metals on to the bacterial cell surface and EPS (Fig. 29.2) (Van Hullebusch et al.
343 2003). For example bacterial biomass was produced by culturing polysaccharide-
344 producing marine *Bacillus circulans* in liquid medium containing glucose as carbon
345 source and biomass was used to remove copper and cadmium ions from aqueous
346 solutions through biosorption. A biomass of 1.48–1.52 g dry weight/l was found to [AU2]
347 remove 80% of copper and 44% of cadmium from solutions containing 495 ppm
348 copper and 492 ppm cadmium respectively (Sahoo et al. 1992). Heavy metal chelation
349 property of exopolysaccharide produced by *Enterobacter cloacae*, a marine

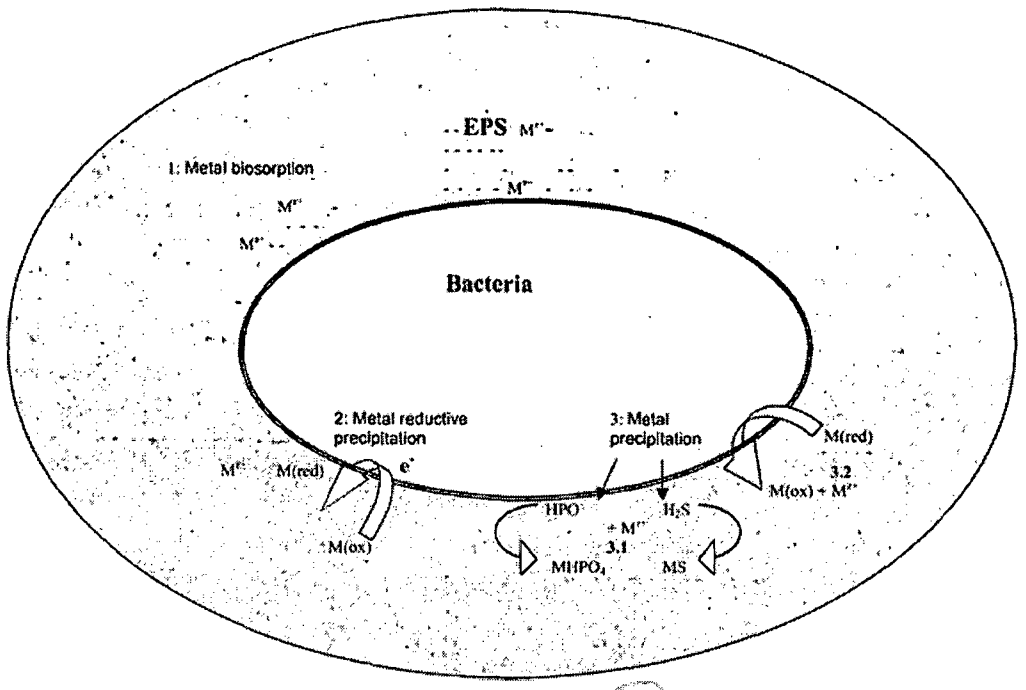


Fig. 29.2 Schematic representation of the interaction between metals and biofilm. 1: Metal biosorption by EPS; 2: Reductive precipitation of metal by enzymatic transformation; 3: 3-1: Metal precipitation as sulfide and phosphate; 3-2 Metal precipitation as biogenic oxide (iron and manganese) and metal adsorption on biogenic oxides. (Courtesy of Lens et al. 2003 ; Environ. Sci. Bio/Technol. 2: 9–33)

bacterium, isolated from the West Coast of India has already been reported. The exopolysaccharide demonstrated excellent chelating properties with respect to cadmium (i.e. 65%) followed by copper (20%) and cobalt (8%) at 100 mg/l level of respective heavy metal (Iyer et al. 2005). Mn(II)-oxidizing spores of marine *Bacillus* sp. strain SG-1 may be used successfully for bioremediation of Mn due to their inherent physically tough nature, unique metal binding capacity and oxidative precipitation (Francis and Tebo 1999). Photosynthetic bacterium, *Rhodobacter sphaeroides* S and another marine photosynthetic bacterium *Rhodovulum* sp. PS88 in a batch culture were capable of significant cadmium removal in the presence of 30 g/l Sodium chloride and divalent cations Mg^{2+} and Ca^{2+} (Watanabe et al. 2003).

29.5.5 Bioprecipitation as Phosphates and Sulphides

Marine bacteria immobilise toxic metals through bioprecipitation as phosphates and sulphides and protect bacterial cells from toxic effects of metals. Three pleiotropic, quorum sensing-defective *Vibrio harveyi* mutants were observed to precipitate soluble Pb^{+2} as an insoluble compound. The compound was purified and subjected to X-ray diffraction and elemental analyses. These assays identified the precipitated compound as $Pb_9(PO4)_6$, an unusual and complex lead phosphate salt that is produced synthetically

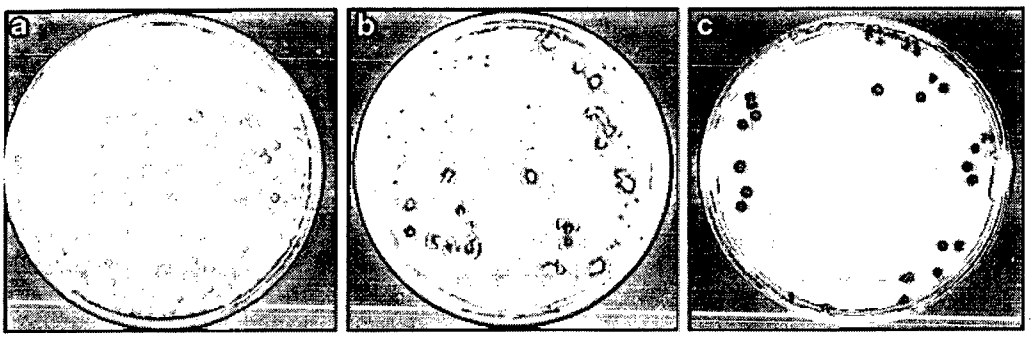


Fig. 29.3 Plates used for the enumeration of metalloid-resistant or - reducing bacteria in bacterial-film-like formations taken from the Melarie Summit site, Main Endeavor Field. (**A**) Metalloid-free control plate; (**B** and **C**) Plates containing 100 μg Na2SeO3 per ml and 100 μg K2TeO3 per ml, respectively. The red-orange color of colonies (**B**) is due to the accumulation of elemental Se, and the black color (**C**) is due to the accumulation of elemental Te. (Courtesy of Vladimir Yurkov; Appl. Environ. Microbiol. 68(9):4612–4622, 2002)

at 200°C (Mire et al. 2004). Sulphate reducing bacteria (SRBs) are anaerobic heterotrophic bacteria in marine environment which also precipitate heavy metals as insoluble metal sulphides e.g. ZnS, PbS, CdS and CuS (Chamberlain et al. 1988). A fluorescent *Pseudomonas* strain CW-96-1 isolated from a deep-sea vent sample under aerobic conditions tolerated cadmium up to 5 mM and Cadmium was removed by precipitation on the cell wall as cadmium sulphide (Wang et al. 1997, 2002).

29.5.6 Redox Reactions and Volatilization

Certain heavy metals viz. Cr, Mo, Se, U, Tc and Au can be immobilised by bacteria following enzymatic reduction of these metals into an insoluble lower redox state. Under oxidizing conditions, they occur as highly soluble and mobile ions, however, under reducing conditions they usually form insoluble phases (Van Hullebusch et al. 2003). Various marine mercury resistant bacterial isolates detoxify toxic mercury through reductive volatalization of mercury. Hg^{+2} gets reduced to elemental mercury (Hg^0) through catalytic activity of mercuric reductase enzyme encoded by *merA* gene (De et al. 2007, 2008). Various species of bacteria reduce selenite to elemental selenium which is insoluble and nontoxic thus eliminate its toxic characteristics. Tellurite- and selenite-reducing bacterial strains were isolated in high numbers from ocean water near hydrothermal vents, bacterial films, and sulfide-rich rocks. Growth of these isolates in media containing K_2TeO_3 or Na_2SeO_3 resulted in the accumulation of metallic tellurium or selenium (Figs. 29.3 and 29.4) (Rathgeber et al. 2002). Copper and nickel tolerant marine sulphate reducing bacteria (SRBs) were isolated from 90/10 copper-nickel alloy and were shown to attach and grow on this alloy. Using energy dispersive X-ray analysis, X-ray photoelectron spectroscopy and electrochemical polarization analyses it was confirmed that intracellularly immobilized metal sulphides produced resemble with synthetic sulphides

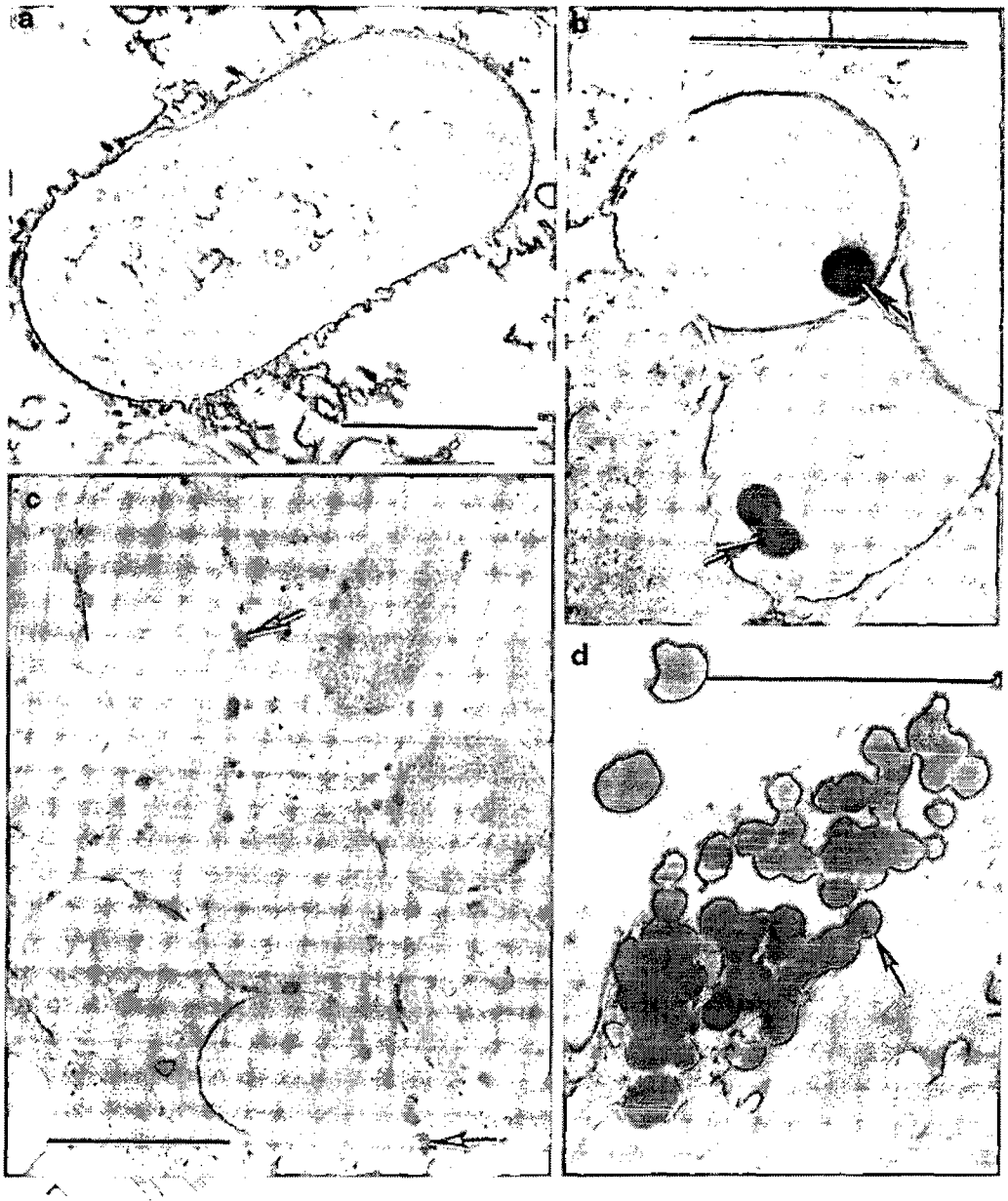


Fig. 29.4 Electron microscopy of ultrathin sections (**A**) Strain Se-1-2-red grown in metalloid-free medium; (**B** and **C**) intracellular localizations of Se (**B**) and Te (**C**), as reduction products of selenite and tellurite, in strains Se-1-2-red (48-h-old culture) and Te-1-1 (72-h-old culture), respectively; (**D**) granules of Se released from cells of Se-1-2-red (48-h-old culture). Bars: 0.5 μm. (Courtesy of Vladimir Yurkov; Appl. Environ. Microbiol. 68(9):4612–4622, 2002)

(Chamberlain et al. 1988). A deep sea sedimentary manganese-oxidizing bacterium, *Brachybacterium* sp. strain Mn 32, showed high Mn(II) resistance (MIC 55 mM) and Mn(II)-oxidizing/removing abilities. This bacterial strain removed Mn (II) employing a simple pathway involving oxidation of soluble Mn (II) to insoluble biogenic Mn oxides (Fig. 29.5) (Wang et al. 2009).

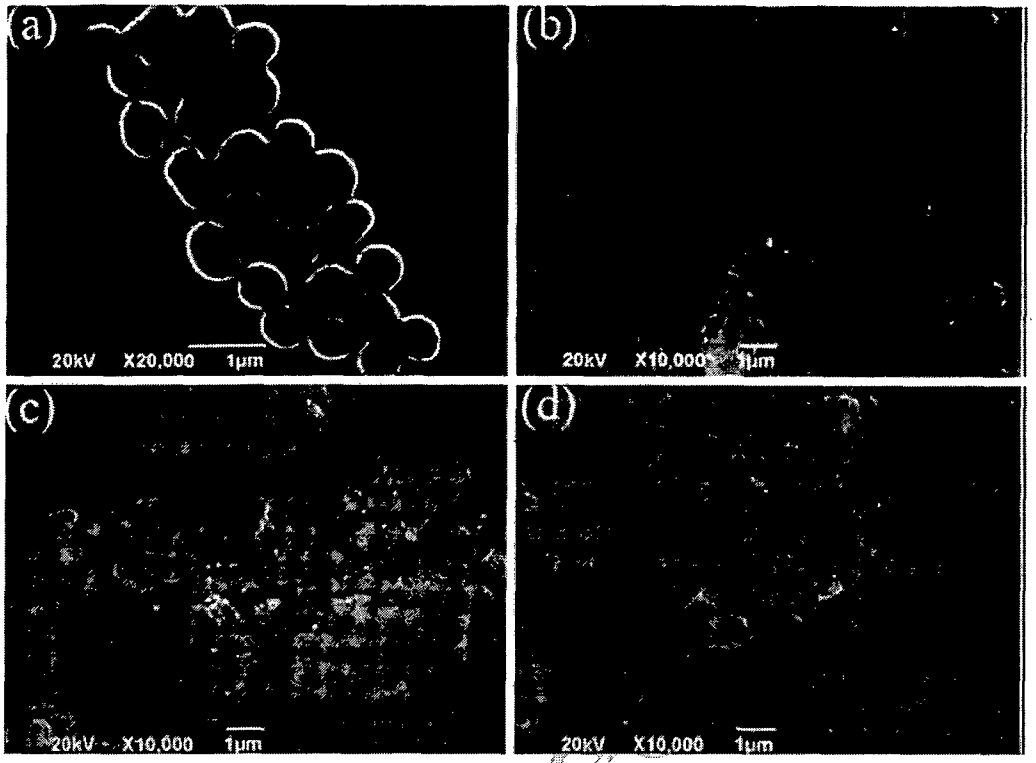


Fig. 29.5 SEM images of (**a**) cells of Brachybacterium sp. strain Mn32 (after 9 days of cultivation in liquid A medium without MnCl2); (**b**) biogenic Mn oxides produced on the cell surface of strain Mn32 (9 days of cultivation with 0.2 M MnCl2 in liquid A medium); (**c**) commercial MnO_2; (**d**) fresh synthetic MnO2 (Courtesy of Wang et al.: Microbiology 155:1989–1996, 2009)

29.5.7 Metal Resistance Conferring Bacterial Genes and Their Significance in Metal Bioremediation

Bacterial metallothionein genes *bmtA* from *Anabaena* PCC 7120, *Pseudomonas aeruginosa* and *Pseudomonas putida* encoding metallothionein that bind multiple zinc and copper ions with high affinity have already been cloned. Thiol modification demonstrates that cystein coordinates zinc in all these proteins and participates primarily in metal homeostasis. Bacterial isolates with this specific protein accumulate very high intracellular levels of metals without any deleterious effects on cell physiology and metabolism. This unique property of hyper-metal accumulating bacterial isolates can be employed in heavy metal bioremediation (Blindauer et al. 2002). Two copper-inducible supernatant proteins having molecular masses of 21 and 19 kDa (CuBPl and CuBP2) were identified in marine bacterium, *Vibrio alginolyticus* and these proteins were 25–46 times amplified in supernatants of copper-challenged cultures as compared with controls, which help in metal accumulation and therefore homeostasis of metals (Harwood-Sears and Gordon 1990).

Volatilisation of mercury by reducing Hg^{+2} to highly volatile elemental mercury, Hg^0 is mediated by the enzyme mercuric reductase encoded by the gene *merA* which

is a Hg detoxification mechanism employed by marine bacteria viz. *Alcaligenes faecalis*, *Bacillus pumilus*, *Pseudomonas aeruginosa* and *Bacillus iodinium* (De et al. 2008). Similarly *mer B* gene in marine bacteria encodes organomercurial lyase which is responsible for break down of organomercurial compounds causing cancer. Marine bacteria possessing this gene can be used to detoxify marine waters contaminated with organomercurials by using bioremediation technology.

Bacterial plasmids possess genetic determinants encoding resistance to several toxic metal ions viz. Ag^{+2}, AsO_2^-, AsO_4^{-3}, Cd^{+2}, Co^{+2}, CrO_4^{-2}, Cu^{+2}, Hg^{+2}, Ni^{+2}, Pb^{+2}, Sb^{+3}, TeO_3^{-2}, Tl^+ and Zn^{+2} (Silver 1999). The function of most resistance systems is based on the energy dependent efflux of toxic ions. Efflux systems are ATPases and others are chemiosmotic cation/proton pumps e.g. *czc* system conferring resistance to Cd^{+2}, Zn^{+2} and Co^{+2}. Some arsenate resistant bacterial isolates possess *ars* operon including *arsC* gene encoding arsenate reductase enzyme which reduce arsenate (As^{+5}) to arsenite (As^{+3}) and *arsA* gene encoding ATPase enzyme which efflux toxic arsenite to the cell exterior in order to maintain metal homeostasis (Silver 1999). ATPase based efflux pumps prevent over accumulation of toxic metals inside the bacterial cells by effluxing excessive heavy metals outside the cells. For instance Zn(II)/Cd(II)/Pb(II)-translocating ATPases are encoded by *zntA* in *E. coli* and *cadA* of *S. aureus* plasmid pI258 confers cadmium efflux mediated by cadmium ATPase (Silver 1999; Rensing et al. 1999).

29.5.8 Genetic Engineering of Marine Bacteria for Bioremediation of Metals

Significant developments in the field of molecular biology, microbiology, microbial biochemistry, biotechnology and genetics resulted in successful development of genetically engineered microbes for bioremediation of toxic metals. A number of genetic engineering approaches have been developed recently which proved useful in introducing the desired traits in bioremediating marine bacteria for designing microbes with novel remedial properties (Chen et al. 1999; Pieper and Reneke 2000).

Insertion of metallothionein encoding *bmtA* gene into a suitable expression vector and its transformation in a marine bacteria is already accomplished. These genetically modified marine bacteria expressing extremely high amount of metallothionein can be employed to bioremediate marine sites highly contaminated with heavy metals. These hyper-metal accumulating bacteria maintain metal homeostasis by reducing metal bioavailability. Expression of metallothioneins on the cell surface by fusion with cell surface proteins improves the bioaccumulation capacity of bacteria (Chen et al. 1999).

Marine bacterial isolates with natural plasmids have been well characterized which possess *merA* gene responsible for reduction of Hg^{+2} to elemental Hg^0 which is volatile and non toxic. This plasmid can also be transformed in marine bacteria or *merA* gene can be cloned into marine bacteria using standard genetic engineering techniques. These genetically engineered bacteria can be employed to

detoxify mercury contaminated marine sites. Introducing genes responsible for resistance to multiple heavy metals into a marine bacterium makes it resistant to multiple heavy metals and may be used to bioremediate sites contaminated with multiple heavy metals.

Marine bacteria can be genetically engineered to express high amount of metal binding groups viz. carboxyl, hydroxyl, sulphate, phosphoric, amine for biosorption of high amount of toxic metals either on cell surface or microbial products such as EPS and biosurfactants. Modification of bacterial isolates which can over produce EPS and biosurfactants may be a good strategy for bioremediation of significant amount of heavy metals. Microbial enzymes can be modified to increase their kinetics to reductively precipitate heavy metal ions or increase efficiency of enzyme to effectively detoxify metals such as mercury.

29.6 Metal Bioremediation Technologies Based on Marine Bacteria and Future Prospects

Natural marine bacterial isolates have got surprisingly tremendous potential to tolerate, sequester and remove toxic metal pollutants from ambient environment. Therefore, it is imperative and highly desirable to characterize such marine bacterial isolates with reference to their biochemical and genetic mechanism of resistance against heavy metals. Highlighting the fundamental mechanism of resistance in marine bacteria would prove useful in designing bioremediation sites polluted with toxic metals. The combination of genetic engineering of bacterial catalysts with judicious eco-engineering of polluted sites will be of paramount importance in future bioremediation strategies. Valuable properties already present in certain strains can be combined or improved through state-of-the-art genetic engineering tools. In future, expression of metallothioneins (MTs) on the surface of environmentally robust organisms such as marine *Pseudomonas aeruginosa* could prove to be a very promising strategy. Application of genetically engineered microorganisms (GEMs) in bioremediation has received a great deal of attention but has largely been confined to laboratory environment. Their practical impact and delivery under field conditions need to be studied. GEMs can also be employed to develop bioreporter sensors for biomonitoring sudden changes in the level of marine metal pollutants released due to several anthropogenic activities and natural geochemical cycling. However, ecological and environmental concerns and regulatory constrains are major obstacles for testing GEMs in the field. Legislations and biosafety norms should be strictly adhered to in this regard before employing these bacteria in metal bioremediation under field conditions. Since release of GEMs is banned worldwide their release to the environment will not be possible. Therefore, only alternative for metal remediation remains to be under controlled laboratory conditions.

An excellent option for enhanced bioremediation of metal contaminated sites is use of microbial products viz. surfactants, emulsifiers and extracellular polymers which would increase the efficiency of metal reducing/sequestering organisms for

field bioremediation. Sustainable and ecofriendly biotechnologies should be used in future for bioremediation and environmental impact assessment (EIA) should be performed prior to introducing metal resistant, marine GEMs in fields for bioremediation of toxic metals.

Acknowledgements The valuable help provided by Mr. Kashif Shamim, Ms Brenda D' Costa and Ms Dnyanada Khanolkar in preparation of manuscript is highly appreciated.

References

I.B. Beech, C.W.S. Cheung, Int. Biodeter. Biodegrad. **35**, 59–72 (1995)

P.V. Bhaskar, N.B. Bhosle, Mar. Poll. Ecotoxicol. **32**(2), 191–198 (2006)

C.A. Blindauer, M.D. Harrison, A.K. Robinson, J.A. Parkinson, P.W. Bowness, P.J. Sadler, N.J. Robinson, Mol. Microbiol. **45**(5), 1421–1432 (2002)

W.N. Bonner, Conservation and the Antarctic, in *Antarctic Ecology*, ed. by R.M. Laws, vol. II (Academic, London, 1984), pp. 821–850

B. Borremans, J.L. Hobman, A. Provoost, N.L. Brown, D. van der Lelie, J. Bacteriol. **183**, 5651–5658 (2001)

[AU3] R.E. Cameron, *Guide to Site and Soil Description for Hazardous Waste Characterization*, vol I. Metals (Environmental Protection Agency, Washington, DC, 1992), EPA/600/4-91/029

A.H.L. Chamberlain, S.E. Simmonds, B.J. Garn, Marine 'copper-tolerant' sulphate reducing bacteria and their effects on 90/10 copper-nickel (CA 706). Int. Biodeter. **24**(4–5), 213–219 (1988)

S. Chen, Z. Shao, Extremophiles **13**, 39–48 (2009)

W. Chen, F. Bruhlmann, R.D. Richins, A. Mulchandani, Curr. Opin. Biotechnol. **10**, 137–141 (1999)

R. Choudhuri, S. Srivastava, World J. Microbiol. Biotechnol. **17**, 149–153 (2001)

D.J. Connell, G.J. Miller, *Chemistry and Ecotoxicology of Pollution* (Wiley, New York, 1984)

M.-J. De Souza, S. Nair, P.A. Loka Bharati, D. Chandramohan, Ecotoxicology **15**, 379–384 (2006)

J. De, N. Ramaiah, Curr. Sci. **91**(3), 68–71 (2006)

J. De, N. Ramaiah, N.B. Bhosle, A. Garg, L. Vardanyan, V.L. Nagle, K. Fukami, Microbes Environ. **22**(4), 336–345 (2007)

J. De, N. Ramaiah, L. Vardanyan, Mar. Biotechnol. **10**, 471–477 (2008)

V.P. Edgcomb, S.J. Molyneaux, M.A. Saito, K.B.S. Lloyd, C.O. Wirsen, M.S. Atkin, A. Teske, Appl. Environ. Microbiol. **70**(4), 2551–2555 (2004)

C.A. Francis, B.M. Tebo, J. Mol. Microbiol. Biotechnol. **1**(1), 71–78 (1999)

G.M. Gadd, FEMS. Microbiol. Lett. **100**, 197–204 (1992)

D.C. Gillan, P. Pernet, Biofouling **23**(1), 1–13 (2007)

W. Gladstone, Trace metals in sediments, indicator organisms and traditional sea foods of the Torres Strait. Report Series 5a. Townsville, Australia. Great Barrier Reef Marine Park Authority, 1996

A. Gnanamani, V. Kavitha, N. Radhakrishnan, G.S. Rajkumar, G. Sekaran, A.B. Mandal, Colloids Surf. B Biointerfaces **79**(2), 334–339 (2010)

D.H. Hamer, Ann. Rev. Biochem. **55**, 913–951 (1986)

V. Harwood-Sears, A.S. Gordon, Appl. Environ. Microbiol. **56**(5), 1327–1332 (1990)

A. Iyer, K. Mody, B. Jha, Mar. Poll. Bull. **49**(11–12), 974–977 (2005)

N. Jayaraju, B.C.S. Reddy, K.R. Reddy, Anthropogenic impact on Andaman coast monitoring with [AU4] benthic foraminifera, Andaman sea, India. Environ. Earth. Sci. **62**(4), 821–829 (2010). doi:10.1007/s12665-010-0569-4

543 C. Jeanthon, D. Prieur, Progr. Oceanogr. **24**(1–4), 81–88 (1990)
544 M.J. Kennish, *Ecology of Estuarine: Anthropogenic Effect* (CRC Press, Boca Raton, 1992),
545 p. 494
546 M. Kumagai, H. Nishimura, J. Oceanogr. Soc. Jpn. **34**, 50–56 (1978)
547 N.J. Lanford, R.E. Ferner, J. Human Hypertens. **13**, 651–656 (1999)
548 M. Loaec, R. Olier, J. Guezennec, Carbohydr. Polym. **35**, 65–7018 (1998)
549 N.C.A. Mancuso, J. Guezennec, J.P. Bowman, Mar. Biotechnol. **7**, 253–271 (2005)
550 K. Matsuda, E. Kobayashi, Y. Okubo, Y. Suwazono, T. Kido, M. Nishijo, H. Nakagawa,
551 K. Nogawa, Arch. Environ. Health **58**, 218–222 (2003)
552 F. Matyar, T. Akkan, Y. Ucak, B. Eraslan, Environ. Monit. Assess. **167**, 309–320 (2010)
553 W. Meng, Y. Qin, B. Zheng, L. Zhang, J. Environ. Sci. **20**(7), 814–819 (2008)
554 C.E. Mire, J.A. Tourjee, W.F. O' Brien, K.V. Ramanujachary, Appl. Environ. Microbiol. **70**(2), [AU5]
555 855–8864 (2004)
556 M.N. Naik, S.K. Dubey, Curr. Microbiol. **62**, 409–414 (2010)
557 K. Nakanura, Bull. Environ. Cont. Toxicol. **42**, 785–790 (1989)
558 D.H. Nies, Microbial heavy-metal resistance. Appl. Microbiol. Biotechnol. **51**, 730–750 (1999)
559 C. Oger, J. Mahillon, F. Petit, FEMS Microbiol. Ecol. **43**, 173–183 (2003)
560 A. Pal, A.K. Paul, Indian. J. Microbiol. **48**, 49–64 (2008)
561 D.K. Pieper, W. Rencke, Curr. Opin. Biotechnol. **11**, 262–270 (2000)
562 A. Poli, G. Anzelmo, B. Nicolaus, Mar. Drugs **8**, 1779–1802 (2010)
563 N. Ramaiah, J. De, Microb. Ecol. **45**, 444–454 (2003)
564 C. Rathgeber, N. Yurkova, E. Stackbrandt, J.M. Beatty, V. Yurkov, Appl. Environ. Microbiol.
565 **68**(9), 4613–4622 (2002)
566 C. Rensing, M. Ghosh, P. Rosen, J. Bacteriol. **181**(19), 5891–5897 (1999)
567 N.S. Reyes, M.E. Frischer, P. Sobesca, FEMS Microbiol. Ecol. **30**, 273–284 (1999)
568 T.M. Roane, Microb. Ecol. **37**, 218–224 (1999)
569 S. Rossbach, T.L. Wilson, M.L. Kukuk, H.A. Carty, FEMS Microbiol. Lett. **191**, 61–70 (2000)
570 D.K. Sahoo, R.N. Kar, R.P. Das, Biores. Technol. **41**(2), 177–179 (1992)
571 S.K. Satpute, I.M. Banat, P.K. Dhakephalkar, A.G. Banupurkar, B.A. Chopade, Biotechnol. Adv.
572 **28**(4), 436–450 (2010)
573 D.R. Schreiber, F.J. Millero, A.S. Gordon, Mar. Chem. **28**(4), 275–284 (1990)
574 J. Selvin, P. Shanmugha, K.G. Seghal, T. Thangavelu, N. Sapna Bai, Microbiol. Res. **164**(3),
575 352–363 (2009)
576 F.R. Siegel, J.H. Kravitz, J.J. Galasso, Environ. Geol. **40**, 528–542 (2001)
577 S. Silver, Gene **197**, 9–19 (1999)
578 P. Singh, S.S. Cameotra, Biochem. Biophys. Res. Commun. **319**(2), 291–297 (2004)
579 J.M. Skei, Marine. Poll. Bullet **9**(7), 191–193 (1978)
580 S. Taghavi, C. Lesaulnier, S. Monchy, R. Wattiez, M. Mergy, D. van der Lelie, Antonie Van
581 Leeuwenhoek **96**, 171–182 (2009)
582 E.D. Van Hullebusch, M.H. Zandvoot, P.N.L. Lens, Environ. Sci. BioTechnol. **2**, 9–33 (2003)
583 C. Vetriani, Y.S. Chew, S.M. Miller, J. Yagi, J. Coomb, R.A. Lutz, T. Barkey, Appl. Environ.
584 Microbiol. **71**(1), 220–226 (2005)
585 B. Vu, M. Chen, R.J. Crawford, E.P. Ivanova, Molecules **14**, 2535–2554 (2009)
586 C.L. Wang, P.C. Michels, S.C. Dawson, S. Kitisakkul, J.A. Baross, J.D. Keasling, D.S. Clark,
587 Applied. Environ. Microbiol. **63**(10), 4075–4078 (1997)
588 C.L. Wang, S.C. Ozuna, D.S. Clark, J.D. Keasling, Biotechnol. Lett. **24**, 637–641 (2002)
589 W. Wang, Z. Shao, Y. Liu, G. Wang, Microbiology **155**, 1989–1996 (2009)
590 M. Watanabe, K. Kawahara, K. Sasaki, N. Noparatnaraporn, J. Biosci. Bioeng. **95**(4), 374–378
591 (2003)
592 N.S. Webster, R.I. Webb, Environ. Microbiol. **3**(1), 19–31 (2001)
593 S. Wuertz, E. Muller, R. Spaeth, P. Pfleiderer, H.-C. Flemming, J. Ind. Microbiol. Biotechnol. **24**,
594 116–123 (2000)
595 Y. Yamamoto, K. Honda, R. Tatsukawa, Proc. Nipr. Symp. Polar Biol. Natl. Inst. Polar. Res. Tokyo.
596 Jpn. **1**, 198–204 (1987)

Hindawi Publishing Corporation
Journal of Marine Biology
Volume 2010, Article ID 563205, 9 pages
doi:10.1155/2010/563205

Research Article

Hemolysin, Protease, and EPS Producing Pathogenic *Aeromonas hydrophila* Strain An4 Shows Antibacterial Activity against Marine Bacterial Fish Pathogens

Anju Pandey, Milind Naik, and Santosh Kumar Dubey

Laboratory of Bacterial Genetics and Environmental Biotechnology, Department of Microbiology, Goa University, Taleigao Plateau, Goa, 403206, India

Correspondence should be addressed to Santosh Kumar Dubey, santoshdubey.gu@gmail.com

Received 9 June 2010; Revised 22 August 2010; Accepted 1 October 2010

Academic Editor: Pei-Yuan Qian

A pathogenic *Aeromonas hydrophila* strain An4 was isolated from marine catfish and characterized with reference to its proteolytic and hemolytic activity along with SDS-PAGE profile (sodium dodecyl sulphate-Polyacrylamide gel electrophoresis) of ECPs (extracellular proteins) showing hemolysin (approximately 50 kDa). Agar well diffusion assay using crude cell extract of the bacterial isolate clearly demonstrated antibacterial activity against indicator pathogenic bacteria, *Staphylococcus arlettae* strain An1, *Acinetobacter* sp. strain An2, *Vibrio parahaemolyticus* strain An3, and *Alteromonas aurentia* SE3 showing inhibitory zone >10 mm well comparable to common antibiotics. Further GC-MS analysis of crude cell extract revealed several metabolites, namely, phenolics, pyrrolo-pyrazines, pyrrolo-pyridine, and butylated hydroxytoluene (well-known antimicrobials). Characterization of EPS using FTIR indicated presence of several protein-related amine and amide groups along with peaks corresponding to carboxylic and phenyl rings which may be attributed to its virulent and antibacterial properties, respectively. Besides hemolysin, EPS, and protease, *Aeromonas hydrophila* strain An4 also produced several antibacterial metabolites.

1. Introduction

Aeromonas infections are one of the most common bacterial diseases diagnosed in marine and cultured freshwater fish. *Aeromonas hydrophila* is found in diverse habitats, including soil, water, and is pathogenic to warm and cold-blooded animals [1]. Aquatic environment along with seafood is thus important potential source for the transmission of *Aeromonas hydrophila* resulting in human infections. *Aeromonas* spp. have been involved in wound infections, sepsis, outbreaks of water, and food-borne gastroenteritis [2]. Virulence in *Aeromonas hydrophila* is multifactorial which consists of aerolysins, hemolysins, enterotoxins, and proteolytic enzymes which play significant role in pathogenesis.

EPS, (exopolysaccharides) also play very important role in the interaction between bacteria and their environment as they are organic molecules formed by polymerization of organic fractions, carbohydrates, proteins, and humic substances [3]. In recent years there has been a growing interest in the isolation and characterization of microbial EPS owing to their importance in adhesion, nutrient sequestration, chelation of heavy metals, detoxification of toxic compounds and protection against osmotic shock [4]. Despite their importance, very few studies have been done on chemical characterization of EPS produced by *Aeromonas hydrophila*.

Over 120 of modern drugs have been isolated from terrestrial microorganisms, which includes important antibiotics and other drugs [5]. Although the ocean covers more than 70% of the earth's surface, microbial bioactive compounds of marine origin have been largely unexplored [6]. Marine environment is a special niche for many unique microorganisms, which produce bioactive compounds to adapt to particular environmental conditions [7]. There is an increasing demand of therapeutic drugs from diverse natural resources. Earlier studies focused on terrestrial plants and microbes proved extremely fruitful, yielding many organic

African Journal of Biotechnology Vol. 9(42), pp. 7134-7140, 18 October, 2010
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

Organic metabolites produced by *Vibrio parahaemolyticus* strain An3 isolated from Goan mullet inhibit bacterial fish pathogens

Anju Pandey, Milind M. Naik and S. K. Dubey*

Department of Microbiology, Laboratory of Bacterial Genetics and Environmental Biotechnology, Goa University, Goa, India.

Accepted 17 September, 2010

Identification and action of several antibacterial metabolites produced by a fish pathogen *Vibrio parahaemolyticus* strain An3 from marine ecosystem of Goa has been demonstrated. Antibacterial activity of the crude cell extract of the test bacterium has been evaluated against indicator pathogenic bacterial strains such as *Acinetobacter* sp. An2, *Aeromonas hydrophila* strain An4, *Staphylococcus arlettae* strain An1 and *Alteromonas aurentia* strain SE3 by agar well diffusion method which clearly demonstrated comparatively more significant inhibitory effect on indicator bacteria as compared to several commonly used antibiotics. Gas chromatography mass spectrometry (GC-MS) analysis of crude cell extract of the test organism interestingly revealed presence of indole, phenyl acetic acid, n-(3-methyl-1, 2, 4-oxadiazol-5-yl) - 1- pyrrolidine carboximidamide, pyrrolopyrazines, tetramethyl pyrazine and other important phenolic compounds which may be responsible for antibacterial activity against indicator microorganisms tested. It has been clearly demonstrated that *V. parahaemolyticus* strain An3 produced several medically important organic metabolites during cultivation suggesting it as a potential candidate for production of several antibacterial metabolites to control pathogenic bacterial strains causing serious fish and human diseases.

Key words: Antibacterial, gas chromatography mass spectrometry, metabolites, pathogenic bacteria, well diffusion.

INTRODUCTION

There is an increasing demand of therapeutic drugs from diverse natural resources. After many years of extensive research, the importance of terrestrial bacteria as source of valuable bioactive compounds has been very well established and exploited. As a result, the ocean and metabolites of marine organisms including associated microorganisms have now become the main focus of drug discovery research (Finical, 1993).These studies are concerned with bacteria and fungi isolated from sea water, sediments, invertebrates and fish (Kelecom, 2002). Bacteria occurring in aquatic ecosystems may have the ability to inhibit the growth of other microorganisms by producing antimicrobial substances such as antibiotics and bacteriocins. Their inhibitory mechanisms include: (i) Production of antibiotics, bacteriocins, siderophores, lysozymes, and proteases and (ii) alteration of pH through production of organic acids (Jorquera et al., 1999).

Vibrio spp. are common inhabitants of aquatic environment and are found free living as well as associated with various marine organisms such as squids, shrimps, corals, fish, molluscs, seagrasses and sponges. Some species are found as symbionts in specialized luminous organs of marine fish and invertebrates, whereas a number of other species are well-known pathogens of humans or

*Corresponding author. E-mail: santoshdubey.gu@gmail.com. Tel: 091-832-6519359, 091-832-2217770, 091-9923568466. Fax: 091-832-2225201.

Abbreviations: **GC-MS,** Gas chromatography mass spectrometry; **PCR** , polymerase chain reaction; **SYEP,** sea water based yeast extract peptone agar; **TCBS,** thiosulfate citrate bile salts sucrose; **TSI,** triple sugar iron; **ONPG,** o-nitrophenyl-β-d-galactopyranoside; **VP,** Voges-Proskauer; **BLAST,** basic local alignment search tool.

Journal of Scientific & Industrial Research
Vol. 70, February 2011, pp. 135-141

Biological characterization of marine fish pathogen, *Acinetobacter* sp. strain An 2 producing antibacterial metabolites

Anju Pandey, Milind Mohan Naik and Santosh Kumar Dubey*

Laboratory of Bacterial Genetics and Environmental Biotechnology, Department of Microbiology, Goa University, Goa 403 206, India

Received 23 July 2010; revised 31 December 2010; accepted 07 January 2011

This study presents antibacterial activity of several organic metabolites produced by a fish pathogen, *Acinetobacter* sp. strain An 2, from marine ecosystem of Goa. Cell extract demonstrated stronger inhibitory effects on Gram negative bacteria as compared to common antibiotics. GC-MS analysis of crude cell extract revealed presence of potential antimicrobials (butylated hydroxytoluene, phenol, pyrrolo-phenol, benzo-quinone, pyrrolo-pyrazine, phthalic acid butyl octyl ester and penta-fluoro-propionic acid hepta-decyl ester). FTIR analysis of its exopolysaccharide (EPS) revealed presence of amine, amide, carboxylic and phenyl groups. Therefore, this strain can be exploited as a potential candidate for several antibacterial drugs to combat bacterial pathogens causing serious fish and human diseases.

Keywords: *Acinetobacter* sp., Antibacterial activity, Crude cell extract, EPS, Indicator

Introduction

Although ocean covers more than 70% of earth's surface, microbial bioactive compounds of marine origin have been largely unexplored[1]. Marine microorganisms produce biologically active compounds to adapt to particular environmental condition[2-4]. Earlier studies reported[5-7] many organic biologically useful compounds including anticancer drugs and potential contribution of marine organisms including bacteria to the discovery of novel bioactive molecules. Several bacteria present in aquatic ecosystems inhibit growth of other microorganisms by producing antimicrobial substances (antibiotics and bacteriocins). Other inhibitors include siderophores, lysozymes, proteases, and organic acids[8]. Several biologically active substances, isolated from marine bacteria (*Pseudomonas, Yersinia, Aeromonas, Brevibacterium, Bacillus,* and *Alteromonas*), have demonstrated antibacterial activity[6,9-13]. Endophytic (*Acinetobacter baumannii*) secrete an antifungal compound, which inhibit growth of some fungal plant pathogens[14]. Microbial exopolysaccharide (EPS) plays an important role in interaction between bacteria and their environment[15] and is reported useful in adhesion, nutrient aquisition, heavy metal sequestration, detoxification of toxic compounds and protection against osmotic shock[16].

This study presents isolation of a fish pathogenic strain of *Acinetobacter,* which inhibits growth of three Gram negative pathogenic indicator bacterial isolates (*Vibrio parahaemolyticus* strain An 3, *Aeromonas hydrophila* strain An 4 and *Alteromonas aurentia* strain SE 3). This study also demonstrated its antibacterial activity by agar well diffusion assay and detected antibacterial organic metabolites in crude cell extract (CCE) by GC-MS and in EPS by FTIR.

Experimental Section

Bacterial Strain

Isolation and Screening of Marine Bacteria

Marine fishes with visible hemorrhagic symptoms and lesions on their body were screened from Goa, and infected body parts (mouth, fins and gills) were washed with sterile deionized double distilled water and swabbed with sterile cotton wool. A suspension of swab was prepared in saline and used for isolation of pathogenic bacteria on nutrient agar plates by serial dilutions. Discrete colonies were picked up for further characterization.

*Author for correspondence
Tel: 91-832-6519359; Fax: 91-832-2225201
E-mail: dubey_santosh1@rediffmail.com;
santoshdubey.gu@gmail.com