BIOCHEMICAL AND MOLECULAR STUDIES OF CADMIUM RESISTANCE AND METAL BIOSORPTION IN BACTERIAL SPECIES ISOLATED FROM COCHIN ENVIRONMENT

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IN

BIOTECHNOLOGY

BY

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CERTIFICATE

This is to certify that the work presented in the thesis entitled **"Biochemical and molecular studies of cadmium resistance and metal biosorption in bacterial species isolated from Cochin environment"** is based on the original work done by Mr. Suresh Kumar M.K, under the supervision of Dr. G. S Selvam, Reader (on Lien), Department of Biotechnology, Cochin University of Science and Technology and myself, and no part there of has been presented for the award of any other degree.

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DECLARATION

I, hereby declare that the work presented in the thesis entitled **"Biochemical and molecular studies of cadmium resistance and metal biosorption in bacterial species isolated from Cochin environment"** is based on the original research carried out by me at the Department of Biotechnology, Cochin University of Science and Technology, under the guidance of Dr. G. S. Selvam, Reader (now at MKU, Madurai) and Dr. M. Chandrasekaran, Prof. & Head, Department of Biotechnology, Cochin University of Science and Technology, and no part there of has been presented for the award of any other degree.

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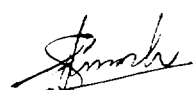


(M.K. Suresh Kumar)

CONTENTS

Dedicated to
my parents & Teachers

Chapter - I

General Introduction

1. GENERAL INTRODUCTION

1.1 PREFACE

Environmental pollution is one of the major problems being faced by society today due to the advent of urbanisation and industrialisation. Substances such as polycyclic aromatic hydrocarbons, pesticides, radioactive materials and trace metals released into the environment by industries are direct endangers to human life. Among them the trace metals and polycyclic aromatic hydrocarbons are common contaminants in the aquatic environment and affect aquatic life and fishery resources besides making their way into food chain and finally reach human sea food.

Heavy metals are released into the environment from various sources such as residential, industrial and stream water sources, though the major share is contributed by industries. Inland water systems act as a receiving body for effluents and rivers dislodge pollutants into the ocean. Further, heavy metals are a dangerous group of potentially hazardous pollutants particularly in estuaries and near shore water. Because of their intrinsically persistent nature, these metals are one of the major contributors of environmental pollution. The trace metals are not usually eliminated from the aquatic ecosystem by natural process and most metal pollutants are enriched in minerals and organic substances.

Among the different heavy metals, cadmium is soft and ductile, and related in its properties to mercury and zinc, because there is no cadmium ore, cadmium appears in the environment as a product of industrialisation and is found only near man's activities. Cadmium is suspected as a mutagen and a teratogen, as well as a carcinogen, with a

latency period of 20- to 50 years. Cadmium ions are particularly toxic and their deleterious effects on all forms of life are well known (Foster, 1983). In human body it causes serious damage to kidney and bone, and probably the best is known as *itai-itai* disease (Kloth *et al.*, 1995). Cadmium is found in zinc ores and is present in at least some trace quantity in all zinc products including galvanised steel and in a great variety of small objects, e.g. nails, screws, etc. Cadmium contamination in aquatic environment is mainly contributed by industries such as battery, electroplating, plastics, paints, fertilisers, refining and zinc mining operations.

Cadmium is included among the priority pollutants by most of the countries, and requires suitable treatment prior to its discharge into the environment. In India, the permissible concentration of cadmium in the industrial effluents discharged into inland surface water is 0.1mg l^{-1} (Puranik *et al.*, 1995).

Industrial wastewater treatment processes are broadly divided as physical, chemical and biological processes. Treatment involving the physical process is based on the physical properties of the contaminant and includes screening, sedimentation, floatation and filtration. The chemical method utilises the chemical properties of the effluent and the commonly used processes include chemical precipitation, chemical oxidation and reduction, ion exchange, filtration, reverse osmosis, electrochemical measurement and evaporative recovery. Whereas, the biological treatment processes include biosorption by which metals are sorbed and complexed to either living or dead biomass. The biological method for the removal of heavy metals from industrial-waste streams may provide an attractive alternative to physico-chemical processes.

Of the three methods of treatment, biological method is safe, efficient and could become an alternative to conventional physico-chemical processes. Biosorption, which naturally occurs in natural environment, is an eco-friendly process and once appropriate living cells, efficient in removing the trace levels of metals from natural environment, are recognised, it is easy to remove the contaminant pollutant from the environment efficiently.

However, this biological process is yet to draw the attention of environmental technologists for industrial application, owing to the dearth of knowledge, particularly on biochemistry and molecular mechanisms involved and employed by native bacteria, in the biosorption of metals from the environment.

Microorganisms in the environment are continuously exposed to metallic anions and cations and some of these ions are taken up as essential nutrients (i.e. magnesium, potassium, copper and zinc). Whereas, other metals such as mercury, lead, cadmium, arsenic and silver with no biological function, exert toxic effects on microbial cells (Martin and Pool, 1991).

Recently, much interest has been focussed on the effects of cations of metals and oxyanions of metalloids on both prokaryotic and eukaryotic cells. Metals are required for the growth, metabolism and differentiation of living cells and organisms (Gadd, 1992). Three mechanisms have been proposed for the toxic action of metals on biological systems: (i) the blocking of functional groups of important molecules such as enzymes and transport systems, (ii) displacement and/or substitution of essential ions,

and (iii) modification of the active conformation of biomolecules (Oshorn *et al.*, 1997). In response to this toxic assault, bacteria have developed an astonishing mechanism of resistance.

According to Ouseph (1992) cadmium enters in to the Cochin backwater environment mainly from the nearby industries as wastes. He has observed that cadmium concentration in the range 83-95% of the total cadmium in the environment released as pollutant, is available to the biota. Based on his study he has concluded that high content of Hg, Zn and Cd are reaching estuary through industrial wastes, in the range of 6-8.4 ppm at the discharge point. Evaluation of available data of Cochin estuary indicated that the estuary receives anthropogenic inputs of cadmium (Jayasree, 1993). Cadmium is observed to be adsorbed to native flora and fauna. Analysis of surficial sediments revealed accumulation arising out of anthropogenic inputs especially in the northern part of the estuary. Gradual decrease of dissolved cadmium was observed as the river water enters the estuarine regions. About 15-25 % dissolved cadmium in the water was associated with organic compounds. Cadmium was found to be the least abundant among the trace metals determined in these estuarine sediments (Jayasree 1993).

In the present study, humble efforts were made to understand the mechanism of resistance and biosorption of cadmium employed by bacteria isolated from effluents released by chemical industries located in Cochin, besides recognising the role of metal binding proteins and metalloenzymes in the process of biosorption of cadmium. Such an information would enable selection of suitable biotools and development of ideal

biotechnologies for the efficient management and bioremediation of heavy metal pollution in the aquatic environments and consequently conservation of environment and biodiversity.

1.2 REVIEW OF LITERATURE

1.2.1 Heavy Metals

Metals with a specific gravity greater than 5 g cm^{-3} have been termed heavy metals (Lapedes, 1974). The heavy metals, which are of great environmental concern, are listed in Table 1.1.

Table 1.1
Heavy metals which have an environmental effect

Cadmium	Nickel
Chromium	Silver
Copper	Tin
Cobalt	Zinc
Lead	Lanthanides
Mercury	Actinides

According to Jone and Foster, (1997), heavy metals are categorised into two groups (Table 1.2), based on the nature of their toxicity. Group one includes those heavy metals, which are so toxic and persistent and are called the black list elements. Group two includes those metals, which are environmentally harmful and are known as gray list elements.

Table 1.2
Grouping of heavy metals based on their toxicity

Black list	Gray list
Cadmium	Chromium
	Copper
	Lead
Mercury	Nickel
	Zinc

1.2.2 Sources of Heavy Metals in Environment

Metals usually occur, geologically, as ores. Mineral deposits are physically or chemically processed to yield pure forms of metals. The main sources of heavy metals in the environment are:

a) Mining operations

Mining and refining of ores is the main source of metal introduction into environment.

b) Domestic effluents and urban storm water run off

The increase in the domestic activities and urban water run off cause concomitant increase in the quantities of metals being released into the environment.

c) Industrial waste water

Owing to the rapid industrialisation, quantum of industrial wastewater containing various metal pollutants increases significantly. The metallic pollutants discharged from chemical industries are usually non-biodegradable and/or toxic to microorganisms so as to disturb the biological systems in the ecosystem. Numerous industrial processes produce aqueous effluent containing heavy metals as contaminants (Table 1.3).

Table 1.3

Heavy metals present in major industrial effluents (Babich *et al.*, 1986)

Industries	Al	Ag	As	Cd	Cr	Cu	Fe	Hg	Mn	Pb	Ni
Paper and pulp	-	-	-	-	+	+	-	+	+	+	+
Organic chemicals	+	-	+	+	+	-	+	+	+	+	-
Petrochemicals	-	-	+	+	+	-	+	+	-	+	-
Alkalies & inorganics	+	-	+	+	+	-	+	+	-	+	-
Fertilisers	+	+	+	+	+	-	+	+	+	+	+
Petroleum refining	+	-	+	+	+	+	+	+	-	+	+
Steel work foundries	-	-	+	+	+	-	+	+	-	+	-
Non-ferrous works	+	+	+	+	+	-	-	+	+	-	+
Vehicles, aircraft & paints	+	+	-	+	+	-	+	+	-	-	+

Heavy metals affect every level of organisation, from the society (behaviour) to the organism (reproductive) and sub-cellular level. Since the organism can not destroy the metals by metabolic degradation, it protects itself from heavy metal poisoning by decreasing the rate of uptake or by binding the metals to a ligand that will hinder the metal from disrupting normal physiological process or by increasing the rate of excretion of the metal (Roesijadi, 1994).

While some transition elements are essential to living systems, at very low concentrations, some are toxic at higher concentrations. The metal ions and their complexes have the potential to cause genetic damage. Relatively, small changes in the structure of metal complexes cause significant difference in their mutagenic activities

(Abbott, 1985). Toxic metals are well known as enzyme inhibitors and disrupt pathway of oxidative phosphorylation (Singerman, 1984).

A myriad of metal pollutants enters into fresh water from innumerable sources and their effects on aquatic life are exhibited at the cellular to ecosystem levels. Aquatic organisms are mostly affected by metal contamination, since most of the industrial effluents, either processed or raw water is discharged into the nearby inland water bodies. The accumulation of metals in estuarine environment was reviewed by Williom *et al.*, (1994). Numerous investigations were done on the water borne exposure of heavy metals on fish (Buckley *et al.*, 1982, Dixon and Hilton 1985, Cusimano, 1993, and Handy, 1994), and these studies have indicated the toxicity as well as sub lethal effects of metals on respiration and osmoregulation (Seller *et al.*, 1975, Mallatt 1985 and Spy *et al.*, 1991).

1.2.3 Treatment of Metal Contaminated Wastewater

For health reasons and environmental protection, municipal and industrial wastewater needs to be treated before proper discharge into the environment. Physico-chemical and biological treatment processes are used for the removal of metal contaminants from the wastewater.

1.2.3.1 Physico-Chemical Processes

The important physico-chemical processes generally employed for metal recovery from wastewater are as given below.

1.2.3.1.1 Chemical Precipitation

Precipitation as either hydroxide or sulphide has traditionally been the most commonly used technology for metal bearing wastewater treatment.

1.2.3.1.2 Oxidation/Reduction

Oxidation/reduction processes have been used in practice for the removal of dissolved metal ions in the wastewater, depending on the volume involved in the treatment, as either a batch or continuous process. pH control is particularly important as both oxidation and reduction reactions may have pH optima (Swaddle, 1990).

1.2.3.1.3 Electrolytic Techniques

Electrolysis is the most direct way of recovering metals from its ores, as long as these can be handled in a fluid state. Electrolysis of aqueous solution may be used to obtain less reactive metals such as Cu, Ni, Zn and Cr in high purity either from aqueous concentrations of the metal salts themselves or from anodes of crude metal prepared by other usually pyrometallurgical techniques (electrorefining). Since each metal ion/metal couples have a characteristic E^0 values, electrorefining can be highly selective in yielding very pure products (Swaddle, 1990).

1.2.3.1.4 Evaporation

Evaporation is used for wastewater treatment processes. Impurities are left behind when the water gets evaporated into the stream. Since particulate matter,

microorganisms, endotoxins, and organic and inorganic chemicals do not evaporate, they remain in the flow down (Swaddle, 1990).

1.2 3.1.5 Reverse Osmosis

Reverse osmosis, an extremely useful technique for separation and purification is employed in the treatment of industrial wastewater. This has also proved economical for large-scale treatment of wastewater (Gonazales, 1996).

1.2.3.1.6 Membrane Technologies

Membrane filtration (MF) is a physical separation process across a semipermeable membrane. Since the pore size is 1-20 μm, separation of high molecular weight species and particles, including metalloids, particulate matter, microorganisms, endotoxins, organic carbon and metal ions from waste stream is possible. The membrane filtration process depends on maintaining both a balanced flow between the product and concentrate stream, and a transmembrane differential pressure (Gonazales, 1996).

1.2.3.1.7 Ion Exchange

Sodium ion exchange on zeolite or on synthetic organic cation exchanger resins such as Dowex-50 is employed for wastewater treatment processes. One of the major problems with the use of ion exchanger is that any ion should be removed from the water before ion exchange softening. The ion present in the water is likely to be oxidised by the air to Fe^{3+}, which is very strongly absorbed by ion exchange resins or zeolites and cannot easily be removed. Other problems with ion exchangers include coating of the resin beads or zeolite particles with supported matter from turbid water or algal growth.

Ion exchange processes should not be used for treatment of boiler water for steam turbines (Swaddle, 1990).

1.2.3.1.8 Solvent Extraction

Industrial wastes often contain valuable constituents which can be recovered most effectively and economically, by means of extraction with an immiscible solvent such as petroleum ether, diethyl ether, benzene, chloroform or some other solvents. The solvent extraction procedure is used for measurement of surface-active agents and various heavy metals (Swaddle, 1990).

1.2.3.1.9 Electrodialysis

Electrodialysis utilises the fact that cations, but not anions, can readily pass through a cation exchange membrane, while the reversal is true of anion exchange membrane. Electrodialysis can help the recovery/removal of chemicals as well as metal ions from the wastewater (Swaddle, 1990).

1.2.3.1.10 Activated Carbon Adsorption

Adsorption technology has been examined for removing heavy metals. The adsorbents which has probably received more attention in granulated activated carbon (GAC), and the adsorptive characteristics of both commercial carbon and activated carbon for removing the waste material have been examined. Carbon adsorption process is an expensive treatment process for the recovery of metal ions (Urritia, 1997).

1.2.3.1.11 Coagulation-Flocculation

Coagulation and flocculation technology provides an effective removal of the polluting materials. Removal of metal up to 99% can be achieved. However, as increasingly more stringent standards are being required, the disposal of solid residues (the sludge) may pose problems. The most common coagulants are alum, ferric chloride and ferric sulphate (Gabriel, 1994).

1.2.3.2 Biological Processes

The biological process of metal bioremediation is a new inroad of environmental biotechnology. Several biological materials are used in practice for bioremediation.

1.2.3.2.1 Use of Plant Biomass

The use of plant biomass to remediate hazardous waste has great promises. Phytoremediation is the use of plant to make metal decontaminant and non-toxic. It is referred as bioremediation, botanical bioremediation or green remediation. The idea is to use rare plants that hyper accumulate metals to selectively remove and recycle metals contaminated in soil and wastewater excessively. Phytoremediation was introduced in 1983 and gained public exposure in 1994 and has increasingly been examined as a potential, practical and more cost-effective technology for metal removal (Rufus *et al.*, 1997).

Phytoremediation includes:

- Phytoextraction

  The use of plants to remove metal contaminants from soil

- Phytovolatization

  The use of plants to make volatile chemical species of soil elements

- Rhizofiltration

  The use of plant roots to remove contaminants from flowing water

- Phytostabilization

  The use of plants to transform soil metals to less toxic form but not removing the soil

1.2.3.2.2 Use of Microorganisms

Microorganisms can remove toxic metals and metalloids from contaminated waters and waste streams by converting them to forms that are precipitated or volatilised from solution. The adsorption of metals and metalloids onto microbial biomass can also prevent further migration of these contaminants (Lovely *et al.*, 1997).

1.2.4 Microbe-Metal Interaction

Microbe-metal interaction plays an important role in the remediation of hazardous metal contamination. Microorganisms remove a number of metals and metalloids from the environment or waste streams by reducing them to a lower redox state. Many of the organisms that catalyse such reactions use the metals or metalloids as terminal electron acceptor in anaerobic respiration (Lovely *et al.*, 1996). The microbial reduction of Cr^{6+} to Cr^{3+} has been one of the most widely studied forms of metal bioremediation (Wang and Shen., 1995).

Oremland, (1994) studied the microbial reduction of the highly soluble oxidised form of selenium, Se^{6+} to insoluble elemental selenium, Se^{0}. Microorganisms that conserve energy to support growth from Se^{6+} reduction are a natural mechanism of the

removal of selenium from contaminated surface and ground water (Anderson *et al.*, 1997).

Microorganisms reduce Hg^{2+} to volatile Hg^{0}, a mechanism for mercury resistance which naturally contribute to the volatilisation of mercury from contaminated environment and there is the possibility that the stimulation of this metabolism might enhance mercury remediation (Saouter *et al.*, 1995, Menson *et al.*, 1995).

Significant advances in the understanding of microbe-metal interactions have been made in recent years and it seems almost certain that novel microbe-metal interaction to be discovered. Many of the microbe-metal interactions have potential application for the remediation of metal contaminated environments and waste streams (Richard and Shuttleworth, 1997).

1.2.4.1 Precipitation

Microorganism promotes metal precipitation by producing ammonia, organic bases or hydrogen sulphides, which precipitate metals as hydroxides or sulphides. Sulphate reducing bacteria transform SO_4 to H_2S, which promotes the extra cellular precipitation of metals from solution. *Klebsiella aerogenes* is able to detoxify cadmium to a cadmium sulphide (CdS) form, which precipitate as electron-dense granules on the cell surface (Aiking *et al.*, 1982).

1.2.4.2 Intracellular Accumulation

The intracellular accumulation of many metals (usually Cd, Ag, Zn, Cu, Cr, Ni, U, Pb, Hg, Ti, As, Pt and Au) has been recorded occur in bacteria, fungi and algae. It

has been inferred, in several instances, that the accumulation of a metal results from the lack of specificity in a normal concentration. Metals may act as competitive substrates in transport system. Kelly *et al.*, (1984) reported that metals such as Ag, As, Hg, Zn, Pb and Cd are generally toxic and certain microorganisms show resistance to them. However, toxic metals may also be rendered innocuous by systems that lead to their intracellular deposition and accumulation.

1.2.4.3 Microbially Catalysed Metal Transformations

Bacteria catalyse chemical transformations of heavy metals. The transformation includes oxidation, reduction, methylation and demethylation (Silver and Misra, 1984). Elements such as mercury and arsenic are transformed by microbes from relatively non-toxic inorganic ions into toxic methylated forms. The same or other microbes degrade organo-metallic compounds. Oxidation and reduction by microbial enzymes also affect the bioavailability and toxicity of heavy metals (Silver, 1985).

1.2.4.4 Extracellular Metal Complexation

The extracellular accumulation of metal depends on the excretion/synthesis of extracellular polymers by the microorganism. The metal accumulation by extracellular polymer is generally considered as a positive phenomenon requiring no direct microbial activity and also the bacteria produce large amounts of extracellular organic material in the presence of toxic metal ions (Jones, 1967). Sag *et al.*,(1995) studied in Zoogloea the mechanism of metal removal by microbial polymers. There was physical entrapment of precipitated metals by the polymer matrix and the complexation of soluble metal species by changed constituents of the polymers. Many extracellular microbial polymers consist

of neutral polysaccharides, hexosamines, and organically bound phosphates that are capable of complexing metal ions. The complexation of metal ions by charged constituents may be likened to an ion-exchange type reaction and thus is affected by the chemical environment and the presence of other metal ions.

1.2.5 Metal Sorption on the Cell Surface

The mechanism by which microorganisms accumulate metals is important for the development of microbial process for the concentration, removal and recovery of metals from aqueous solution. Metal accumulation occurs at cell surface or within the cell wall matrices. The accumulation of metal at the cell surface is due to the result of complexation reaction between metal ions and the charged receptive constituent of the cell walls. The composition of cell wall of receptive constituent is highly species-dependant and differs considerably among gram negative and gram positive bacteria, yeast, filamentous fungi and algae (Mc Murrough and Ross, 1967).

The adsorption of metals and metalloids by the biological system is called biosorption. Biosorption process has been proposed (Brierley, 1990, Gadd, 1990 and Volesky, 1990) as an efficient and potentially cost-effective way of removal of toxic heavy metals from industrial effluents with metal concentration in the range 1-100 mgl^{-1}. Biosorption can be divided into two categories depending upon cell viability, viz., dead cell and live cell biosorption (Mittal *et al.*, 1997). The materials used for metal biosorption process are called biosorbents. The important biosorbents are bacteria, yeast, filamentous fungi and algae.

1.2.5.1 Bacteria

The use of bacterial biomass in metal sorption can be of great interest owing to its large diversity and few attempts have been made to exploit this in practice (Volesky *et al.*, 1998). The interaction of bacterial surface with soluble metals in the aqueous environments where microorganisms live is inevitable. Bacterial exchange of nutrients and waste with surrounding medium occur through diffusion, both internally and externally. Bacteria have adopted different shapes, which provide them with great surface area to volume ratio so as to optimise diffusion. The ability of bacterial cells to bind metals is associated with the characteristic and indisposable cell envelope (Thompson and Beveridge, 1993). The cadmium biosorption of bacterial species from aqueous solution reported in the literature is presented in Table 1.5.

Table 1.5

Biosorption of cadmium by bacterial biomass.

Bacterial sp	Accumulation of Metals (mg g^{-1})	Reference
Alcaligenes sp	10.0	Mc Entee *et al.*, (1983)
Bacillus sp	0.10	Cotoras *et al.*, (1992)
Citrobacter sp	0.20	Michel *et al.*, (1986)
Citrobacter sp	0.50	Macaskie *et al.*, (1987)
Klebsiella aerogenes	0.10	Tynaeka *et al.*, (1981)
Pseudomonas aeroginosa	0.40	Chopra *et al.*, (1971)
Pseudomonas flourescence	0.50	Falla *et al.*, (1993)
Pseudomonas putida	0.25	Denise *et al.*, (1985)
Rhizobium leguminosaram	0.50	Diane *et al.*, (1997)
Staphylococcus aureus	0.20	Kendo *et al.*, (1974)

1.2.5.2 Fungi and Yeast

Fungi and yeast can accumulate non-nutrient metals such as cadmium, mercury, lead, uranium and silver in substantial amounts. Both living and dead fungal cells possessed a remarkable ability for taking up toxic and precious metals. The uptake of metals by fungi and yeast have generated an interest in using them for removal of toxic metals from wastewater and recovery of precious metal from effluent or processed water (Shumate *et al.*, 1978).

Both living and dead cells of fungi are capable of metal uptake and thus could be good metal biosorbents. The use of dead biomass seems to be a preferred alternative for metal uptake studies. The wider acceptability of cells is due to the absence of toxic limitations, absence of requirements of growth media and nutrients in the feed solution, and the fact that biosorbent materials can be re-used and the metal uptake reactor can be easily modelled mathematically (Kapoor *et al.*, 1997).

The biosorption of metal by cell surface binding can take place in both living and dead cells and is of particular interest in the removal and recovery of metals. The cadmium biosorptive capacities of various fungi and yeast are presented the table 1.6.

Table 1. 6

Cadmium biosorption by fungal/yeast biomass

Biomass	Biosorptive Capacity (mg g^{-1})	Reference
Aspergillus niger	2.0	Mullen *et al.*, (1992)
Aspergillus oryzae	5.0	Kiff and Little (1986)
Aurobasidium pullulans	5.0	Gadd *et al.*, (1988)
Azomonas agilis	6.28	Kyung *et al.*, (1998)
Mucor rouxii	1.8	Mullen *et al.*, (1992)
Penicillium sp	3.0	Galun *et al.*, (1987)
Penicillium spinulosum	0.4	Townsley *et al.*, (1986)
Rhizopus nigricans	30.0	Tobin *et al.*, (1984)
Rhizopus orrhizus	12.0	Holan & Volesky (1995)
Saccharomyces cerevisiae	70.0	Volesky *et al.*, (1993)
Saccharomyces cerevisiae	12.2	Stoll *et al.*, (1994)
Streptomyces pimprina	500.0	Puranik *et al.*, (1995)

1.2.5.3 Algae

Algal biosorption process can be used for the removal of toxic metals and/or radionucleides from liquid effluents before their safe discharge in addition to their use as a recovery process for metals of value (Leusch *et al.*, 1996). For the metal biosorption photosynthetic microorganism and unicellular microalgae are used. The precipitation and crystallisation of metals may occur within and around the cell wall as well as the

production of algal polysaccharides and siderophores. The algal biomass is relatively an inexpensive biosorbent and is commonly used for biosorption process (Kuyucak, 1990). *Cladophora* sp, a filamentous alga has been shown to contribute significantly to the removal of heavy metals (Shumate and Strandberg, 1985). Some seaweed collected from ocean indicated impressive biosorbents for metal removal (Kuyucak and Volesky, 1988). Different algal-based biosorption systems reported in literature are summarised in Table 1.7.

Table 1.7
Cadmium biosorption by algal biomass

Algal biomass	Cadmium accumulation (% dry weight)	Reference
Anabaena cylindrica	0.25	Jakubawaki *et al.*, (1991)
Aphanocapsa sp.	0.37	Jakubawaki *et al.*, (1991)
Ascophyllum nodosum	0.55	Volesky (1994)
Asterionella formosa	0.30	Conway *et al.*, (1979
Chlorella salina	2.20	Knummongkol *et al.*, (1982)
Chlorella vulgaris	0.60	Wong *et al.*, (1979)
Chlorella hemosphaera	0.90	Da Costa *et al.*, (1992)
Cricosphaera elongata	0.01	Gnassia-Barelli (1982)
Fragilaria crotonensis	0.25	Conway *et al.*, (1979)
Halimeda opentia	0.37	Volesky (1994)
Nostoc UAM 208	0.09	Fernandez-Pinas (1991)
Oscillatoria woronichihli	1.12	Fisher (1984)
Sargassum notan	8.30	Volesky (1994)
Scenedesmus obliguus	10.0	Cain *et al.*, (1980)
Thalassiosira rotula	5.20	Dongmann *et al.*, (1983)

1.2.6 Factors Affecting Biosorption.

There are many factors that influence the biosorption of metals by microorganisms such as pH of the solution, temperature, contact time, physiology of culture used, initial metal concentration, and cation and anion concentration of external media.

1.2.6.1 pH of the Solution

Biosorption of metals, by microorganism, is dependent on the pH of the external medium. Biosorption of cationic metal species increase with increase in pH values. For the majority of biosorbents the optimum pH is slightly acidic to around neutral (4-7). Some metals which form anionic complexes adsorb most strongly at very acidic pH (1-2) (Kayucak *et al.*, 1988). Green and Darnall, (1990) classified metal ions into three classes based on their pH dependence of biosorption by microorganisms. The first group metals are tightly bound at pH>5 and can be desorbed at pH<2. The metals that fall into this group are Al^{3+}, Cu^{2+}, Pb^{2+}, Cr^{3+}, Cd^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+}, Fe^{2+}, Be^{2+}, and UO_2^{2+}. Second group of metals is biosorbed at high proton concentration (low pH). They are TcO^{4-}, $PtCl^{3-}_4$, CrO_4^{2-}, SeO^{2-}_4 and $Au(CN)_2^-$. The third class of metal species where biosorption is reported as being independent of pH are Ag^+, $Hg^{2+}$and $AuCl_4^-$ (Hosea *et al.*, 1986).

1.2.6.2 Temperature

Temperature has a significant effect on biosorption. There is relatively little information on the influence of temperature on the biosorption of metals by microbiota. In most metal biosorption studies with microorganisms, the temperature is usually kept closed. When the temperature varied between 0^0C and 30^0C a little effect was seen on

the biosorption of manganese and molybdenum by *Chlorella* cells (Nakajima and Sakaguchi, 1986).

1.2.6.3 Time

The rate of uptake is critical for the design and economy of any adsorption system, leaving aside the bioaccumulation phase of metal entry into living cells. DeRome and Gadd, (1987) found that time between ten minutes and two hours was sufficient for the biosorption process.

1.2.6.4 Physiological State of Culture Used

The physiological state of culture greatly affects the amount of metal sorbed by biomass commonly used in metal biosorption process. When the biomass is in dead state, the cells are permeable and allow metals to enter and bind to an internal component and surface of the cell as well as the external surface thus increasing the metal uptake/biosorption (Garnham, 1994). Martin and pool, (1991) explained that the changes in cell wall structure and/or polysaccharides and growth conditions are effecting biosorption of metal ions.

1.2.6.5 Initial Metal Concentration

The amount of metal concentration present in the external medium influences the biosorption capacities of biomass. When the concentration of the metal is high above tolerable levels, the initial response is an inhibition of microbial activity. As the concentration of metal increases, death ensures the microbial cells (Bibich *et al.*, 1980).

1.2.6.6 Cation and Anion Concentrations of External Medium

Concentrations of cations depress the biosorption of metals of interest or the other cations. Such effects can be explained in terms of competition between ions for the same metal binding sites on the microbial biomass. Green *et al.*, (1987) studied the selectivity of metal ions in microalgae *Chlorella vulgaris*. It tend to vary according to Al^{3+}, Ag^{+}> Ca^{2+}>Cd^{2+}>Pb^{2+}>Zn^{2+}=Co^{2+}>Cr^{3+}. In some situations cation concentrations increase the biosorption of anion metal species, as illustrated by the increased biosorption of pertechnetate by microalgae at increased external concentration of Na^{+}, K^{+}, Mg^{-} and Ca^{2+} (Garnham *et al.*, 1992). Anionic concentrations rarely affect the biosorption of metals.

1.2.6.7 Adsorption kinetics and Isotherms

The adsorption isotherms are plots of solute concentration in the adsorption state as a function of its concentration in the solution at constant temperature. Isotherm is plotted using the data at the equilibration of the experimental system (i.e., by plotting residual equilibration concentration of the solute versus concentration of the solute on the adsorbent). This gives valuable information, useful for the selection of an adsorbent. It also facilitates the evaluation of the feasibility of the adsorption process for a given application (Webber, 1985). Several equilibrium models have been developed to describe adsorption isotherm relationship. Two widely used adsorption models for biosorption of metals, viz. Langmuir and Freundlich models, are used for the evaluation of the adsorption data (Langmuir, 1918, Webber, 1985).

The Langmuir model:

$$Ceq/Q = 1/ (b \times Q\ max) + Ceq/Q\ max.$$

The Freundlich model:

$$In\ Q = In\ K = (1/n)\ In\ Ceq.$$

In practice it is difficult to design a treatment process on the basis of the equilibrium position of the reaction, because the reaction usually takes too long to reach completion. Therefore, adsorption data are plotted at various intervals of time and a with suitable rate expression of a treatment process. The problem next arises is obtaining a suitable rate expression governing the reaction. This requires certain assumptions to be made. The Langmuir rate equation can be applied with the assumption that the initial metal concentration changes significantly during the adsorption process. Frequently this assumption is well founded. However, the final metal concentration following the adsorption process is only a small fraction of the initial metal concentration of the initial metal concentration (Jancovicks, 1965). Another assumption is related to the desorption process. Earlier reports state that for low adsorbate concentration, the rate constants of desorption is much smaller than the rate constant for adsorption. Considering these facts, it is possible to neglect the desorption process or assume that the adsorption process is irreversible (Jancovicks, 1965).

1.2.7 Desorption

The metal adsorbed by the biomass can be desorbed. Desorption is usually achieved with an acid wash. The process of desorption itself is important in the eventual disposal of the used biomass adsorbent. Nakajima, (1982) showed that sodium carbonate could be used to desorb uranium from *Chlorella vulgaris*. Cotoras *et al.*, (1992)

demonstrated that a shift in pH is an effective desorption mechanism. Acids such as hydrochloric acid, sulphuric acid and nitric acid are usually used in desorption process. Some metals such as Ag, Au and Hg have no pH dependant adsorption and desorption is possible by the mixture of thiourea and formic ammonium sulphate. EDTA was the most efficient desorbent for the desorption of lead and zinc from *Streptoverticillium* biomass (Puranik *et al.*, 1997).

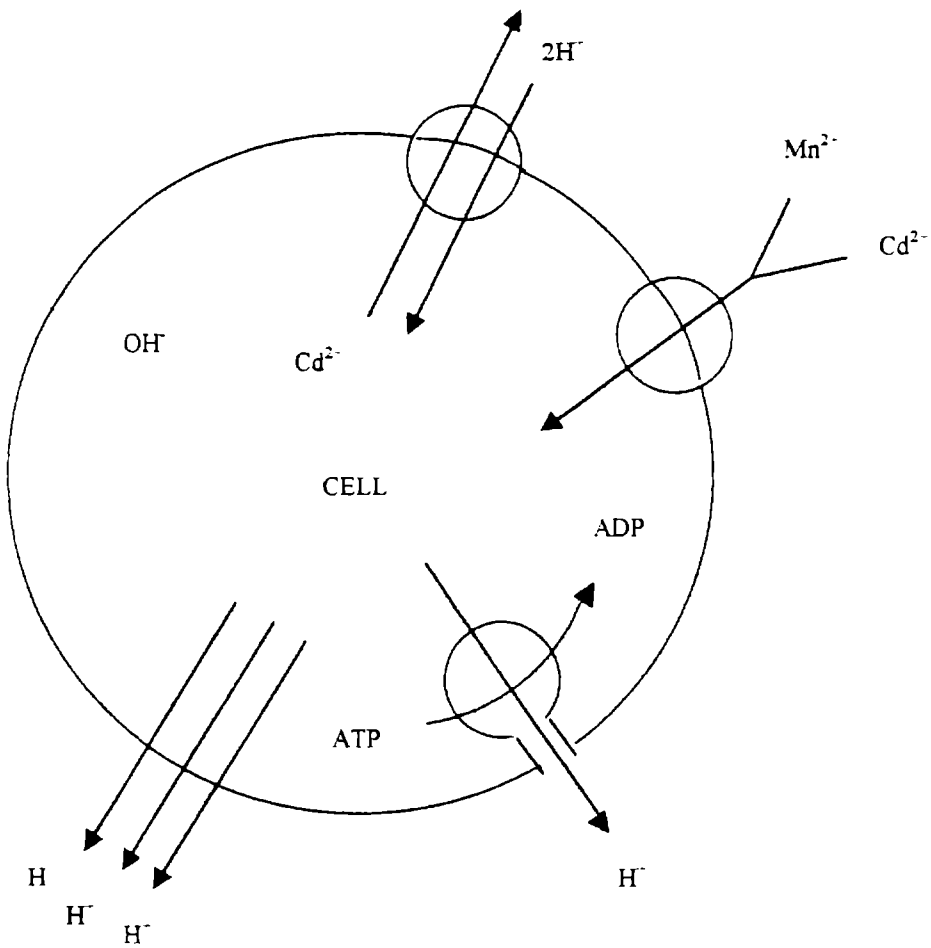
1.2.8 Metal Resistance in Bacteria

Bacteria have very efficient and different mechanisms for tolerating heavy metals. Often normal toxic levels of metals have no effect on growth of resistant strains. In bacteria, the genes controlling metal resistance are carried on plasmid, which provide the bacteria with a competitive advantage over other organisms when metals are present (Foster, 1983).

There are no currently acceptable concentrations of metal ions that can be used to designate metal sensitivity and resistance. The four basic mechanisms by which plasmids encode metal resistance are: (1) inactivation of the metals, (2) alteration of site inhibition, (3) impermeability of metals and (4) metal bypass mechanism (Trevors *et al.*, 1985) (Fig. 1.1). Cadmium ions are toxic to the bacteria but some of them have adopted various methods to survive in their habitat having high level of toxic contaminants. Bacteria have plasmid-encoded mechanism for resistance to antimicrobial substances including toxic heavy metals. The mechanism and molecular genetics of heavy metal

Figure 1.1

Model for the cadmium (II) uptake and efflux systems (Silver, 1985).



resistance in a wide range of bacteria have been studied extensively (Caguiat *et al.*, 1999). All bacterial cation efflux systems characterised todate are plasmid-encoded and inducible but differ in energy coupling and in the number and types of proteins involved in metal transport and regulation (Nies, 1992).

Bacterial plasmids encode resistance systems for toxic metal ions including Ag^{+}, 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+}. In addition to the understanding of the molecular genetics and environmental role of metal resistance, studies, during the last few years, have provided surprises and new biochemical mechanisms (Silver and Ji, 1994). Chromosomal encoded toxic metal resistance is known and their difference from plasmid mediated resistance is blurred. Some systems, such as copper transport ATPases and metallothionein cation-binding proteins are known to be from chromosomal origin. The largest group of metal resistance systems function by energy-dependent efflux of toxic ions (Silver and Phung, 1996).

1.2.9 Metal Binding Proteins

Live organisms either eukaryotic or prokaryotic have adopted several mechanisms to respond to the toxic effects of heavy metal ions especially cadmium, copper and zinc. One of the most common mechanisms is the expression of metallothionein protein in the cell. Metallothioneins (MTs) are ubiquitous low molecular weight proteins characterised by an unusually high cystein content and selective capacity to bind heavy metal ions, such as cadmium (Cd), copper (Cu) and zinc

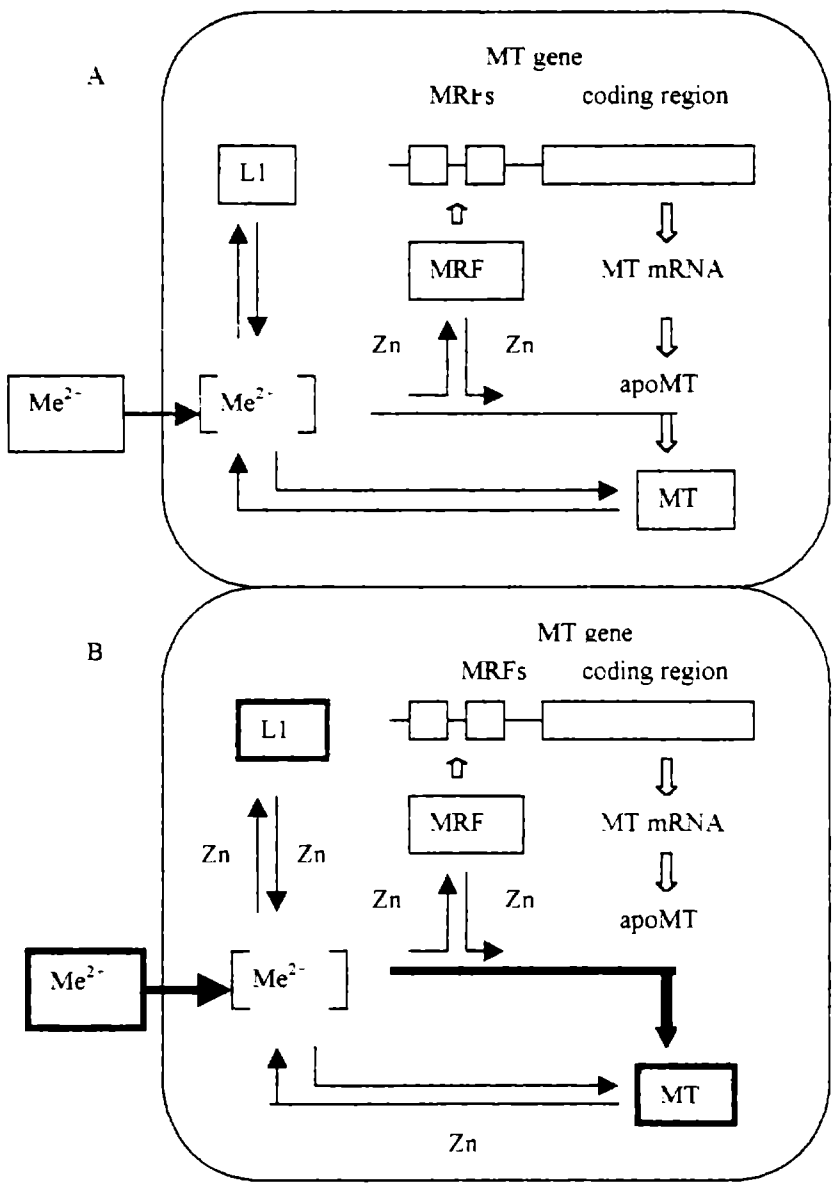
(Zn) (Tauseef *et al.*, 1987). Kotrba *et al.*, (1999) reported histidine rich and cystein rich metal binding peptide sequences genetically engineered, incorporated into *LamB* protein and expressed in *E.coli* cells. The isolated cell envelope of *E.coli* bearing newly added metal binding peptides showed up to 1.8 fold increase in Cd^{2+} binding capacity.

Accumulation of Cd and Zn salts in animals causes MTs induction in liver and kidney cells. It seems unlikely that protection against heavy metals would be the primary function of MTs. First, these ions are not present at high levels in most biotopes and probably do not exert a selection pressure significant enough to justify the expression of a special detoxification system. Second, if the role of MTs were purely protective, one would except to find these proteins only after exposure to toxic heavy metals; in fact, the basal level of expression of MT is relatively high (Karin, 1985).

According to a cellular model for MT induction proposed by Thiele, (1992), the induction of MT gene expression in eukaryotes can be assessed at both transcriptional and translational levels (Fig. 1.2). Information associated with an increase in the cellular content of metals is conveyed to the MT gene via metal activated transcription factors and initiate expression of specific proteins.

Figure. 1.2

Pathway for intracellular metal distribution and relationship to MT protein induction (Roesijadi, 1994)



1.3 SCOPE OF THE PRESENT STUDY

Earlier studies on cadmium are restricted to monitoring cadmium levels in the aquatic environment with a view to locate the presence of cadmium in aquatic environment as a pollutant and to trace the source of cadmium in the environment. In fact except for the few reports mentioned, under the preface section, which are basically review in nature, no detailed studies are available. The studies reported earlier very clearly indicate that Cochin backwater, a major water body which receives enormous load of effluents from a large number of chemical industries located in Cochin, commercial capital of Kerala, warrants urgent and immediate attention in respect of appropriate bioremediation process towards efficient management of metal pollution.

In this context, the present study was aimed at isolation of potential cadmium resistant bacteria from effluents, which are ideal source of such strains; characterise them for their efficiency to remove cadmium from effluents through the process of biosorption and to understand the role of plasmid metalloenzymes and metal binding proteins in metal sorption with a view to develop the strains as probable biotools for future use in metal biotechnology and bioremediation.

Objectives of the present study

Specific objectives of the present study include the following:

1. Isolation and characterisation of cadmium resistant bacteria from metal contaminated effluents released into environment by chemical industries located in Cochin.
2. Evaluation of the role of plasmid in cadmium resistance by bacteria.

3. Determination of the process of biosorption of cadmium by selected strains of bacteria isolated from industrial effluents.
4. Evaluate the role of metal binding proteins and metalloenzymes in cadmium resistance by selected strains of bacteria isolated from industrial effluents.

Chapter - II

Isolation and Characterisation of Cadmium Resistant Bacteria

2. ISOLATION AND CHARACTERISATION OF CADMIUM RESISTANT BACTERIA

2.1 INTRODUCTION

Toxic heavy metals are discharged into the environment through various industrial processes and the industrial waste contributes to environmental pollution. Heavy metals are normal constituents of living materials and are essential for the metabolic process at low concentration. However they are regarded as harmful when available in excess either in the environment or in the body of the organism (Suresh *et al.*, 1998). Heavy metals such as cadmium, chromium, lead, mercury, nickel, etc. in wastewaters, are hazardous to life in the environment.

The occurrence of metal resistant bacteria in anthropogenically polluted site is well documented by Diel *et al.*, (1990). Bacteria have been found to be useful, since they have the ability to survive in virtually all possible habitats and detoxify the toxic chemicals in the environment. Some microorganisms are responsible for metal transformation in environment and may serve as bioassay indicator organisms in polluted and non-polluted environments. Moreover, metal-resistant bacteria could have potential biotechnological application particularly in the bioremediation of toxic metals in waste water.

The estuarine and brackish water environments are increasingly being polluted, in the recent times, particularly due to industrial effluent discharged in to them. The conservation of our estuarine and brackish water environment thus is of paramount

importance, and their monitoring of pollution is highly essential. Cochin is an industrially important city of Kerala. There are many chemical and metallurgical industries operating and these industries discharge their processed and/or raw metal containing industrial and domestic effluents in to the surrounding inland water bodies, which seriously affect the aquatic life. Under normal conditions, the metals discharged into aquatic environments along with the effluents are attacked or modified by microorganisms in the aquatic environment. So, if the microflora capable of modifying or adsorbing the metals are known, it would enable development of an ideal bioremediation process.

In this context, it was desired to screen the effluents released by the chemical industries, in and around Cochin, for cadmium resistant bacteria towards recognition and utilisation of potential cadmium resistant bacteria for possible development of bioremediation technology for the future.

2.2 MATERIALS AND METHODS

2.2.1 Samples

Treated effluents from three different chemical industries (petrochemical, organic chemical and pesticide manufacturing industries) were used.

2.2.2 Sample Collection.

Effluent samples were collected from the discharge point of the three different chemical industries located in the industrial belt of Cochin. Samples for microbiological analysis were collected in sterile containers and transported to the laboratory in an icebox

immediately. The microbiological analysis was done within 3 hours of collection. The samples were maintained at 4^0C for minimising the changes in the physico-chemical properties, until used.

2.2.3 Analysis of Effluent Sample

Effluent samples were subjected to the following physico-chemical analysis.

2.2.3.1 Temperature

The temperature of the samples was recorded immediately after the collection, at the sampling point using a sensitive (1/10) thermometer (0-110^{0}C).

2.2.3.2 pH

Measurement of pH was carried out with a digital pH meter (Systronics India).

2.2.3.3 Determination of Heavy Metal Ions

The concentration of dissolved metal ions in the samples were determined using the Atomic Absorption Spectrophotometer (, Perkin Elmer, Model 2380 USA).

2.2.4 Isolation of Cadmium Resistant Bacteria

2.2.4.1 Medium

The nutrient agar (NA) medium (Hi-media) was used to enumerate the bacteria from the industrial effluent samples. Composition of the medium is given in Appendix-I.

2.2.4.2 Preparation of Serial Dilution Blank

One ml each of the effluent samples were made up to 10 ml with sterile double distilled water (10^{-1} dilution) serial dilution blanks and prepared up to 10^{-5} dilution.

2.2.4.3 Plating Procedure

Pour plate technique was employed. One ml each of serially diluted blanks was used as inoculum for inoculating nutrient agar media. After plating, the plates were incubated at 30^0C for 3-5 days and the total viable counts were observed. The bacterial colonies were observed for their colony morphology, shape, size and colour. Later, single celled colonies were picked and subcultured on nutrient agar slants and maintained at 4^0C.

2.2.5 Confirmation of Cadmium Resistance By Bacterial Isolates

2.2.5.1 Medium

Nutrient broth (NB) and nutrient agar media (Hi-media) were used for confirmation of cadmium resistance in bacterial isolates.

2.2.5.2 Inoculum And Inoculation Procedure

Pre-culture of the isolate was prepared, initially by inoculating 5 ml of nutrient broth taken in test tubes and incubating at 30^0C under agitated condition for a period of 12 hrs. The overnight grown cultures were serially diluted (10^{-5}) with 0.9% NaCl and 100 µl of the bacterial samples transferred on to nutrient agar supplemented with a range of $CdSO_4$ (0.001 - 0.020 mM) concentrations by spread plate technique.

The isolates with, comparatively, faster growth rate and ability to grow at maximum concentration of cadmium supplemented media were selected as a potential isolates for further studies.

2.2.6 Identification of Bacterial Isolates

The potential isolates were identified based on biochemical and morphological characteristics, out lined by Bergey's Manuel of Systematic Bacteriology Vol: 2 after purification by repeated streaking on nutrient agar plates.

The cultures were maintained on nutrient agar (Himedia) and subcultured periodically at regular intervals of 15 days. Stock cultures were maintained in the same medium. Purity of the cultures was checked once in a month by repeated streaking on nutrient agar plates.

2.2.7 Growth Studies

2.2.7.1 Media

Growth studies were carried out using nutrient broth (NB), and Tris Glucose Phosphate medium (TGP) medium (composition given in Appendix-I). The prepared medium was autoclaved at 121^0C, 15 lb pressure for 15 minutes and used.

2.2.7.2 Preparation of Inoculum

1. A loop full of 24 hrs old agar slope culture of the bacterial strain was first grown in 10 ml NB for 12 hrs at room temperature (28 ± 2^0C).

2. One ml of the culture broth was then aseptically transferred into 50 ml of nutrient broth and incubated, in a rotary shaker at 200 rpm, for 12 hrs at room temperature (28± 2^0C).
3. Cells were harvested by centrifugation (Kubota 6900 model, Japan) at 10,000 rpm for 10 min.
4. The harvested cells were washed repeatedly with sterile physiological saline (0.85% NaCl) and resuspended in 10 ml of the same saline.
5. The prepared cell suspension (0.5 OD at 600 nm) was used as inoculum, and stock was kept at 4^0C,until used.

2.2.7.3 Measurement of Growth

The growth of bacteria in the medium was determined in terms of turbidity in the culture broth, by measuring absorbance at 600 nm in UV visible spectrophotometer (Milton Roy ,Genesys spectronic 5, USA). Growth was expressed as optical density (OD).

2.2.8 Optimisation of Growth Parameters

Various environmental parameters that influence the growth of *Alcaligenes* sp, *Pseudomonas* sp and *Staphylococcus* sp, isolated from the effluent, were studied to optimise the growth. The different parameters optimised for growth include incubation temperature, pH, carbon sources, nitrogen sources and medium. The growth optimization studies were carried out in both nutrient broth and mineral base medium.

2.2.8.1 Temperature

Optimum temperature for maximum growth was determined by growing the bacteria at various incubation temperatures (25, 30, 35, 40 and 50^0C) for a period of 24 hrs on a rotary shaker. Growth was determined as mentioned under section 2.2.7.3.

2.2.8.2 pH

Optimum pH required for maximum growth was determined by subjecting the bacteria to various pH (5-10) conditions, adjusted in the media using 1.0N HCl/NaOH. After inoculation and incubation for 24 hrs at room temperature (28 ± 2^0C) on a rotary shaker, growth was determined as per the procedure described under section 2.2.7.3.

2.2.8.3 Carbon Sources

The effect of carbon sources on growth was tested by the addition of casein, glucose, galactose, sodium gluconate, starch and sucrose in the medium at 0.5% (w/v) level. After 24 hrs of incubation at room temperature (28 ± 2^0C) on a rotary shaker, growth was determined as per the procedure described under section 2.2.7.3.

2.2.8.4 Nitrogen Sources

The effect of additional nitrogen sources that support maximum growth was estimated using organic nitrogen sources such as beef extract, peptone, yeast extract and tryptone, and inorganic nitrogen source such as NH_2SO_4, $NaNO_3$, KNO_3 0.5% (w/v) level. After 24 hrs of incubation, at room temperature (28 ± 2^0C), on a rotary shaker, growth was determined as per the procedure described under section 2.2.7.3.

2.2.8.5 NaCl Concentration

Optimum concentration of NaCl for maximum growth was determined by subjecting the bacteria to various level of NaCl concentration in the media (1-5 %). After incubation for 24 hrs on a rotary shaker, growth was determined as per the procedure described under section 2.2.7.3.

2.2.8.6 Media

Suitable medium for maximum growth of bacteria was selected by growing the strains in different media such as TY medium, CY medium, MG medium, LB medium and NB medium for a period of 24 hrs (Composition of media given in Appendix-I), and growth was determined as mentioned under section 2.2.7.3.

2.2.9 Heavy Metal Resistance Profile

Heavy Metal Resistance Profile of bacteria for the heavy metals, viz. Cd, Cu, Co, Hg, Pb and Zn (1.0 mM), was determined by growing the strains in agar media supplemented with the desired heavy metals.

2.2.10 Antibiotic Resistance Profile

Selected strains were tested for resistance to antibiotics, viz. ampicillin, chloramphenicol, gentamycine, kanamycine, streptomycin and tetracycline incorporated in LB medium at a conc. of 50 µg/ml (Appendix-I).

2.3 RESULTS

Effluent samples from the three stations recorded different temperatures (18^0C, 19^0C and 24^0C respectively for station I, II and III). Effluent samples collected from station I were highly alkaline compared to station II and III, which were acidic in nature. From the data presented in the Table 2.1, it is seen that the effluent samples, collected from the three different stations contained cadmium, copper, cobalt, lead and nickel (Table 2.1).

In station I, lead was detected in high concentration followed by cobalt, copper, cadmium and nickel. In station II cobalt was high followed by lead, nickel, cadmium and copper. Whereas, in station III copper was high followed by lead, cobalt, cadmium and nickel.

In general, the concentration of the metal varied for the stations. Cadmium was detected in the range of 15.08 - 18.76 ppm. Copper was recorded in the range of 13.36-31.31 ppm. Cobalt varied from 26.40 to 76.27 ppm. Lead varied from 31.70 to 274.16. Nickel was detected in the range of 12.81-19.58 ppm.

Twelve strains were obtained from the industrial effluents. All the 12 strains were tested for their resistance to different conc. of cadmium. Data presented in Table 2.2 indicate that BTS CRL1, BTS HOC6 and BTS HIL11 were resistant of all concentrations to cadmium tested. While BTS CRL5 was sensitive to all concentration

tested. Strain BTS CRL3, BTS CRL4 and BTS HOC7 were sensitive to concentrations above 0.001 mM.

The three strains, BTS CRL1, BTS HOC6 and BTS HIL11 were identified based on their morphological and biochemical characteristics, as *Pseudomonas* sp, *Alcaligenes* sp and *Staphylococcus* sp.

The growth curve obtained for *Pseudomonas* sp, *Alcaligenes* sp and *Staphylococcus* sp are presented in (Fig 2.1a). From the results it is clearly evident that *Pseudomonas* sp enter into the logarithmic phase after 4 hrs of inoculation and continued till 12 hrs in nutrient broth medium. A very long log phase was observed in TGP medium, which continued up to 34 hrs, and a long stationary phase was noticed. In *Alcaligenes* sp the log phase started after 4 hrs of inoculation and continued up to 7 hrs in both NB and TGP medium. A very long stationary phase was observed in TGP medium till 22 hrs and in NB medium, the stationary phase was extended up to 16 hrs (Fig.2.1b). In *Staphylococcus* sp, the log phase was observed during 3-12 hrs after inoculation in nutrient broth, while in TGP medium it was between 3-18 hrs. A short stationary phase was noticed in NB in contrast to a long stationary phase, was observed in TGP medium (38 hrs) (Fig 2.1c). The growth rate of these strains in the presence of cadmium was consistently slower compared to their respective controls (Fig 2.2 a, b & c).

Effect of temperature on growth was tested in nutrient broth at different temperatures viz. 25^0C to 50^0C. The results presented in Fig 2.3 a, b & c. suggests that all the isolates preferred an optimum temperature of 30^0C for maximum growth,

although significant level of growth was recorded at 35^0C. Higher temperatures 50^0C, did not favour the growth of all cultures.

Effect of pH on growth of the three potential isolates was determined by subjecting them to various levels of pH (5-10) in the nutrient broth. From the results presented in Fig 2:4 a, b, c. it is inferred that all the three isolates could grow well over a wide range of pH 6 - 9, with an optimum at pH 7. They did not grow well at extreme pH conditions (pH 5 and pH 10).

Different carbon sources were incorporated in the mineral base medium (1% w/v) to test the effect of carbon sources on growth. Data presented on Fig 2:5 indicated that glucose, galactose and sodium gluconate favoured maximum growth followed by casein and maltose. Starch supported least growth rate in *Pseudomonas* sp. In *Staphylococcus* sp, maximum growth was observed with glucose followed by maltose, sodium gluconate, and galactose. Starch and casein gave least growth rate. In the case of *Alcaligenes* sp all the carbon sources, except starch, supported considerable level of growth rate.

The effect of nitrogen sources on growth of *Pseudomonas* sp, *Staphylococcus* sp, *Alcaligenes* sp was determined by incorporating different nitrogen sources into the mineral base medium. The result shown in Fig 2.6 suggest that among the nitrogen sources tested, tryptone supported maximum growth of *Pseudomonas* sp and *Alcaligenes* sp while yeast extract gave maximum growth rate for *Staphylococcus* sp. Other organic nitrogen sources also could support significant level of growth. In the case of inorganic

nitrogen sources tested, maximum growth was observed with NH_2SO_4 supplemented medium for all the isolates.

Data presented in Fig 2.7 evidence that maximum level of growth occurred in the absence of NaCl in the medium for *Pseudomonas* sp. Further, the growth declined progressively along with increase in NaCl concentration. In *Alcaligenes* sp no significant change was observed in the absence or presence of NaCl in the medium up to 3%. But at the higher concentration of NaCl the level of growth rate declined. In the case of *Staphylococcus* sp, the maximum growth was observed at 2% of NaCl concentration, and higher concentration of NaCl retarded the growth rate of the organism.

The results obtained for the optimization of growth media for *Pseudomonas* sp, *Alcaligenes* sp and *Staphylococcus* sp suggest that TY medium supported maximum growth. Nevertheless other media supported considerable level of growth for all the strains (Fig 2.8 a, b & c).

It was observed that all the three isolates could exhibit heavy metal resistant profile, and showed resistance to Cu, Pb, Zn and sensitive to Hg (Table 2.3).

All the isolates, *Pseudomonas* sp, *Alcaligenes* sp and *Staphylococcus* sp showed resistance to ampicillin and chloramphenicol, while sensitive to other antibiotics.

Table 2.1

Analysis of effluent samples collected from industries.

Station	Metal conc. ± SD (ppm)			Temperature (° C)	pH
Station-I	Cadmium	(Cd)	15.09 ± 0.07	24.0	11 ± 0.5
	Copper	(Cu)	16.89 ± 1.13		
	Cobalt	(Co)	51.71 ± 0.99		
	Lead	(Pb)	1.35 ± 0.05		
	Nickel	(Ni)	12.82 ± 0.05		
Station-II	Cadmium	(Cd)	16.54 ± 0.72	18.0	3 ± 0.5
	Copper	(Cu)	13.36 ± 0.42		
	Cobalt	(Co)	76.27 ± 1.38		
	Lead	(Pb)	31.70 ± 0.98		
	Nickel	(Ni)	19.58 ± 0.46		
Station-III	Cadmium	(Cd)	18.77 ± 0.57	19.0	5 ± 0.5
	Copper	(Cu)	31.31 ± 0.70		
	Cobalt	(Co)	26.15 ± 0.57		
	Lead	(Pb)	274.16 ± 9.28		
	Nickel	(Ni)	18.29 ± 0.61		

Table 2.2

Resistance to different conc. of cadmium by bacterial strains isolated from chemical industrial effluents

	Cd^{2+} Solution (mM)							
Strains	0.001	0.002	0.003	0.004	0.005	0.01	0.02	0.03
BTS CRL-1	R	R	R	R	R	R	R	R
BTS CRL-2	R	R	R	R	R	S	S	S
BTS CRL-3	R	S	S	S	S	S	S	S
BTS CRL-4	R	S	S	S	S	S	S	S
BTS CRL-5	S	S	S	S	S	S	S	S
BTS HOC-6	R	R	R	R	R	R	R	R
BTS HOC-7	R	S	S	S	S	S	S	S
BTS HOC-8	R	R	R	R	R	S	S	S
BTS HOC-9	R	R	R	R	S	S	S	S
BTS HIL-10	R	R	R	R	S	S	S	S
BTS HIL-11	R	R	R	R	R	R	R	R
BTS HIL-12	R	R	S	S	S	S	S	S

R- resistant, S- sensitive

Table 2.3

Heavy metal resistance profile of selected bacterial species isolated from Industrial effluents (Expressed as Colony Forming Unit-cfu)

Metal ions (1.0 mM)	*Pseudomonas* sp (cfu)	*Alcaligenes* sp (cfu)	*Staphylococcus* sp (cfu)
$Co(NO_3)_2$	18×10^7	32×10^7	21×10^7
$CuSO_4$	32×10^7	46×10^7	44×10^7
$Pb(NO_3)_2$	25×10^7	24×10^7	19×10^7
$ZnSO_4$	39×10^7	45×10^7	52×10^7
$HgCl_2$	-	-	-
$CdSO_4$	-	-	-
Control	41×10^8	36×10^8	33×10^8

Figure 2.1.a

Growth curve of *Pseudomonas* sp in Nutrient Broth and Tris Glucose Phosphate medium

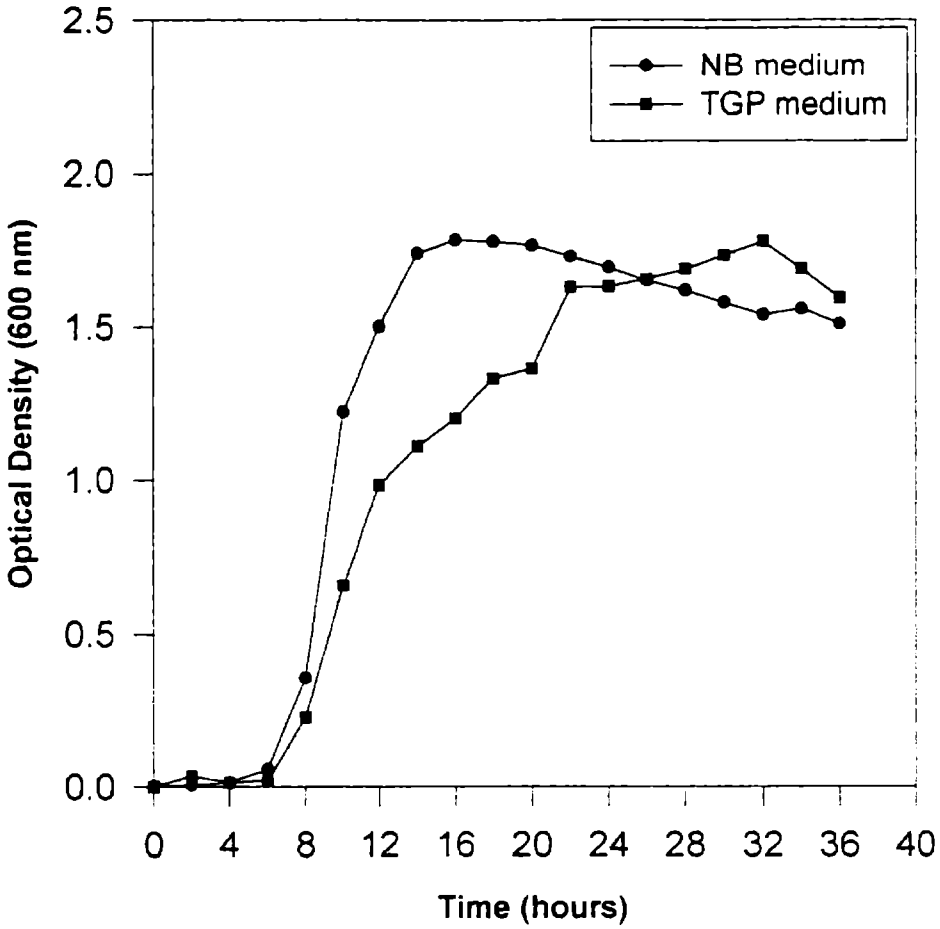


Figure 2.1.b

Growth curve of *Alcaligenes* sp in NB and TGP medium

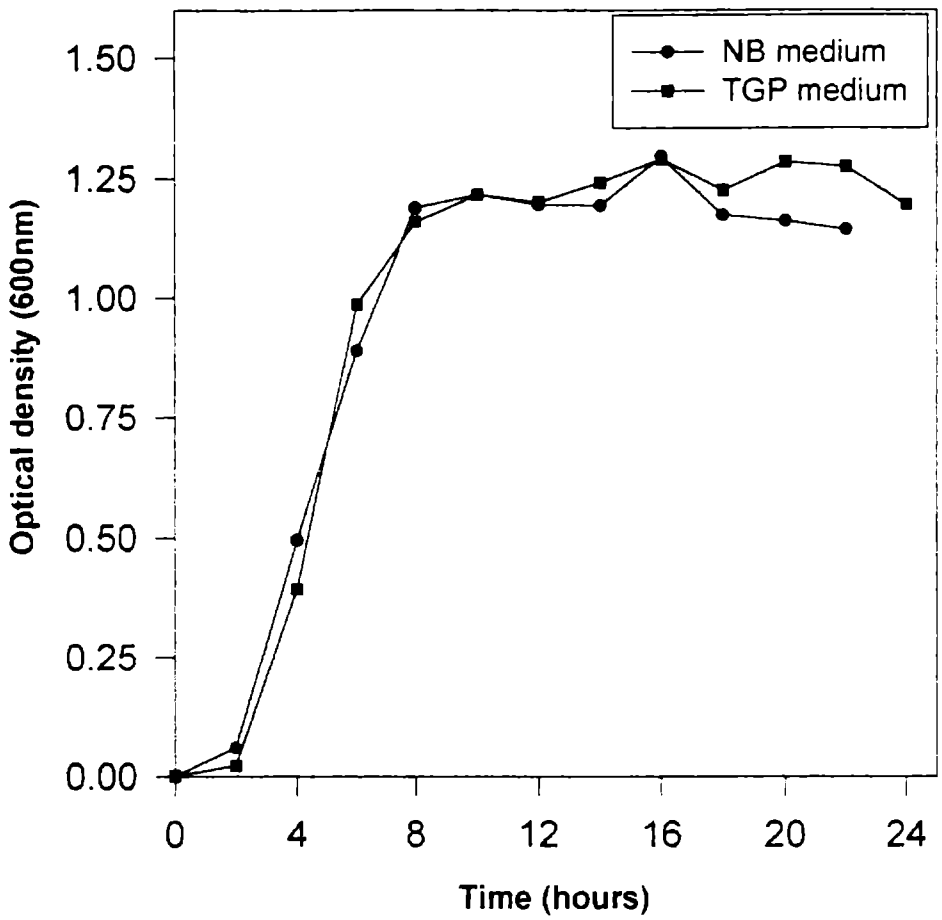


Figure 2.1.c

Growth curve of *Staphylococcus* sp in Nutrient Broth and Tris Glucose Phosphate medium

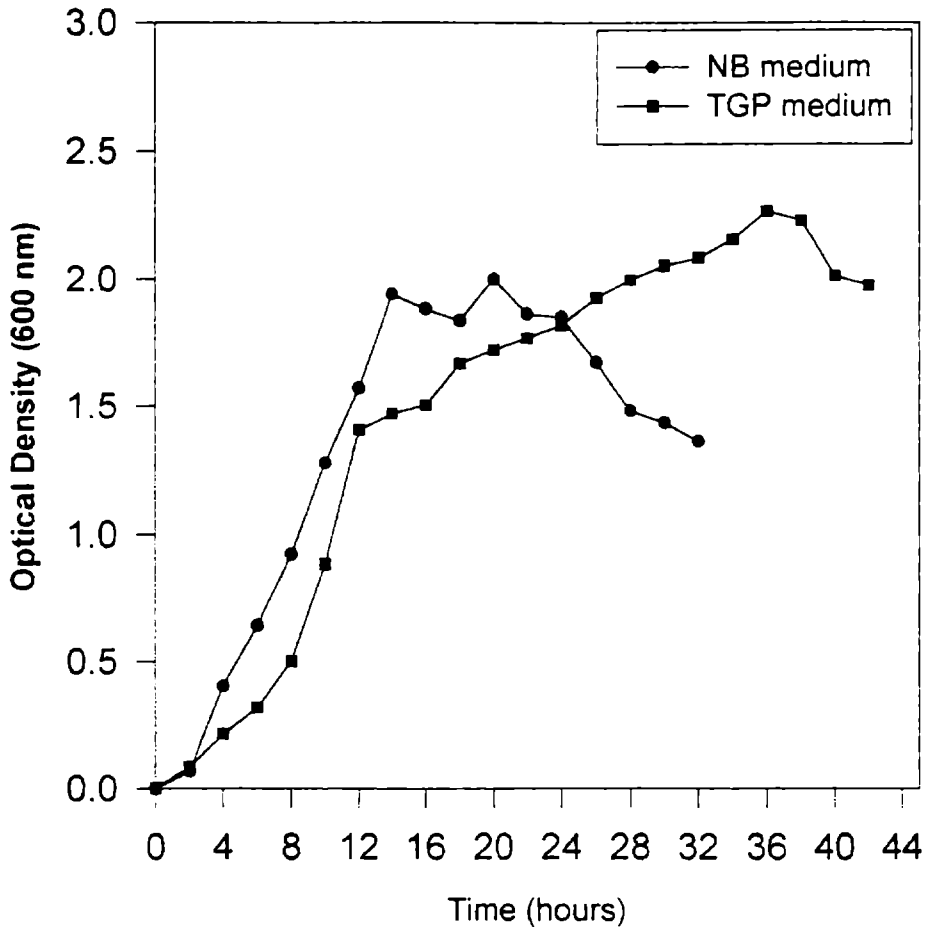


Figure 2.2.a

Growth of *Pseudomonas* sp in presence of cadmium ions

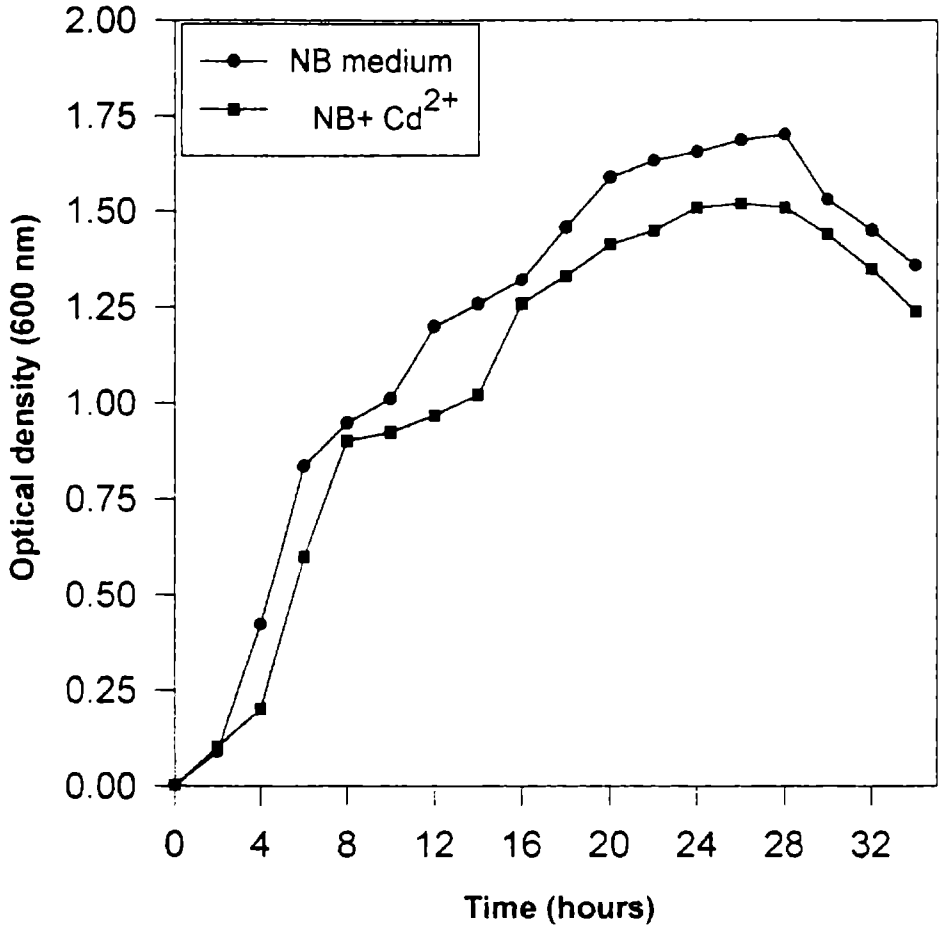


Figure 2.2.b

Growth of *Alcaligenes* sp in presence of cadmium ions

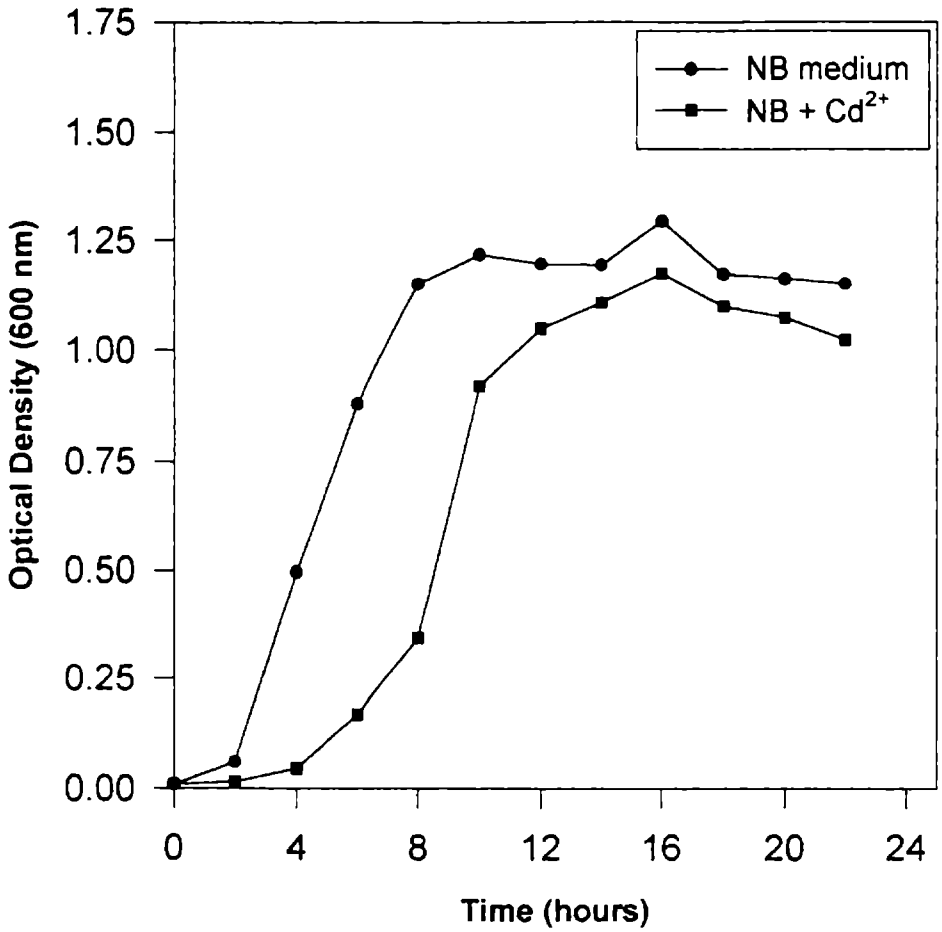


Figure 2.2.c

Growth curve of *Staphylococcus* sp in presence of cadmium ions

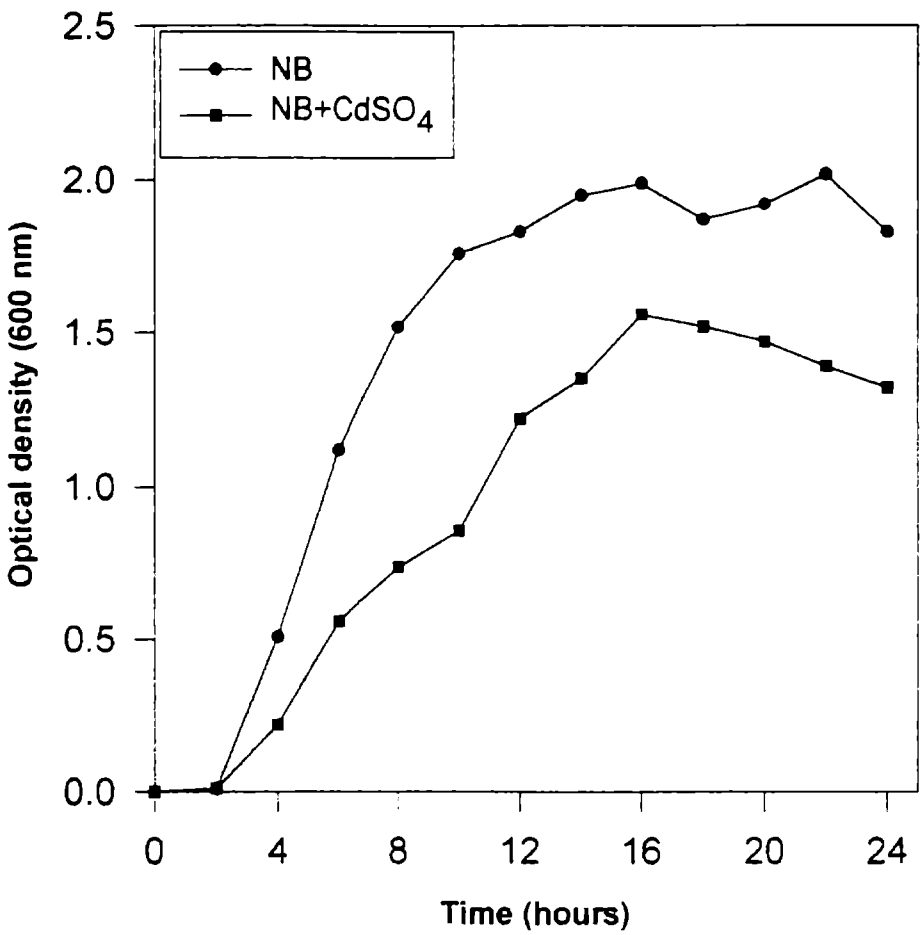


Figure 2.3.a

Optimisation of temperature
***Pseudomonas* sp**

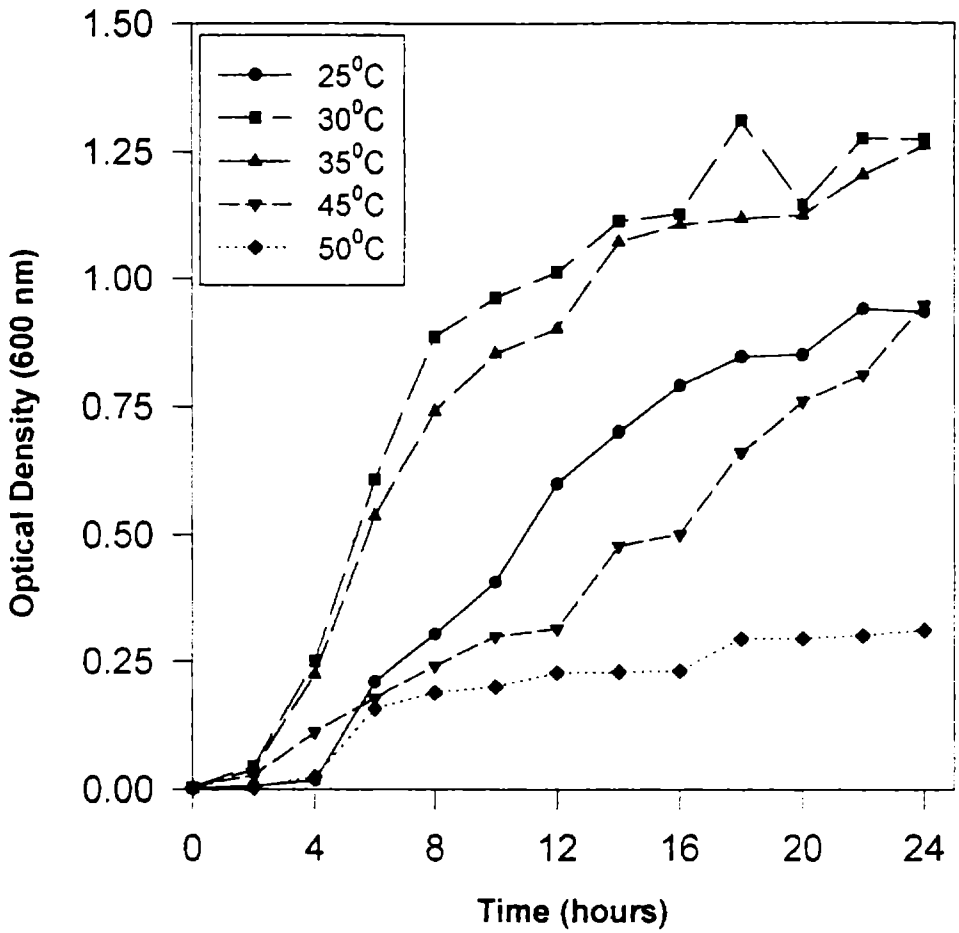


Figure 2.3.b

Optimisation of temperature
***Alcaligenes* sp**

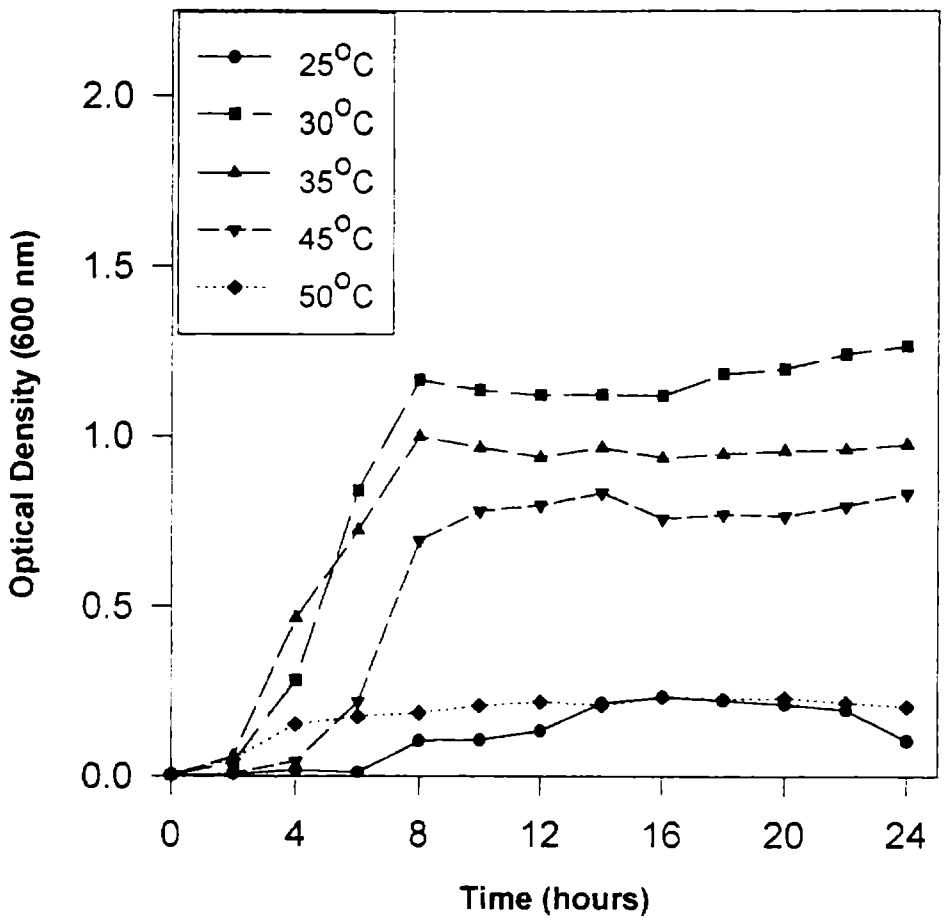


Figure 2.3.c

Optimisation of temperature
***Staphylococcus* sp**

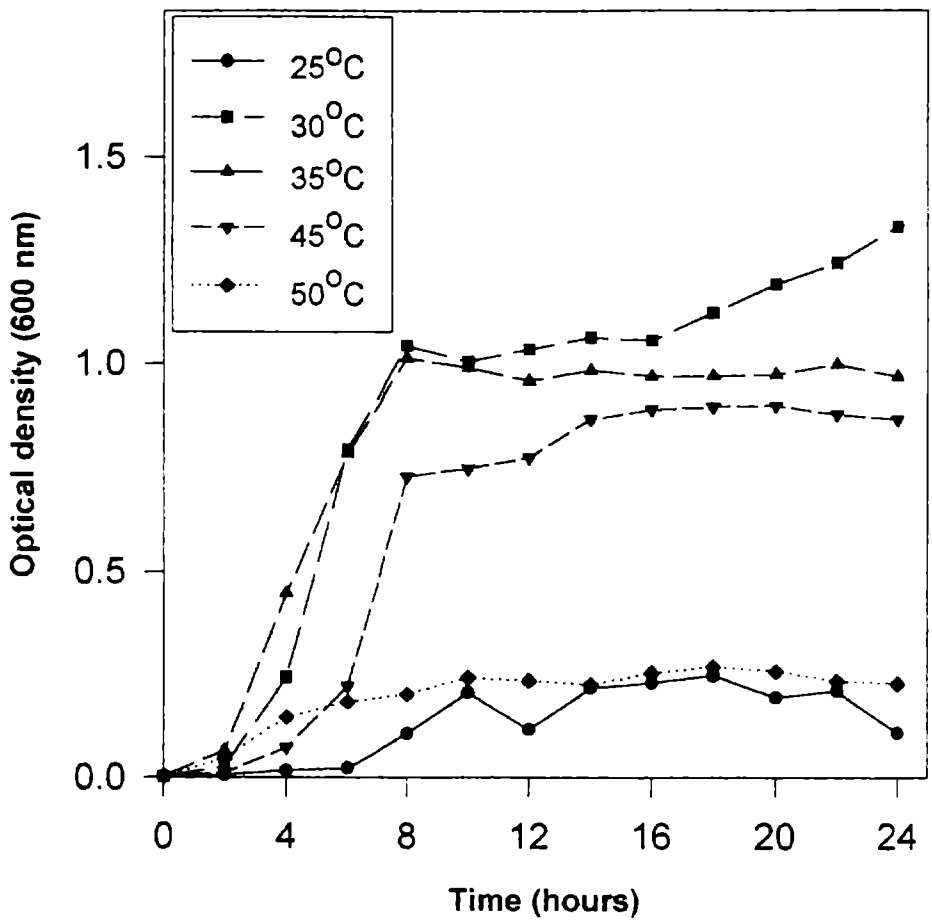


Figure 2.4.a

Optimisation of pH in *Pseudomonas* sp

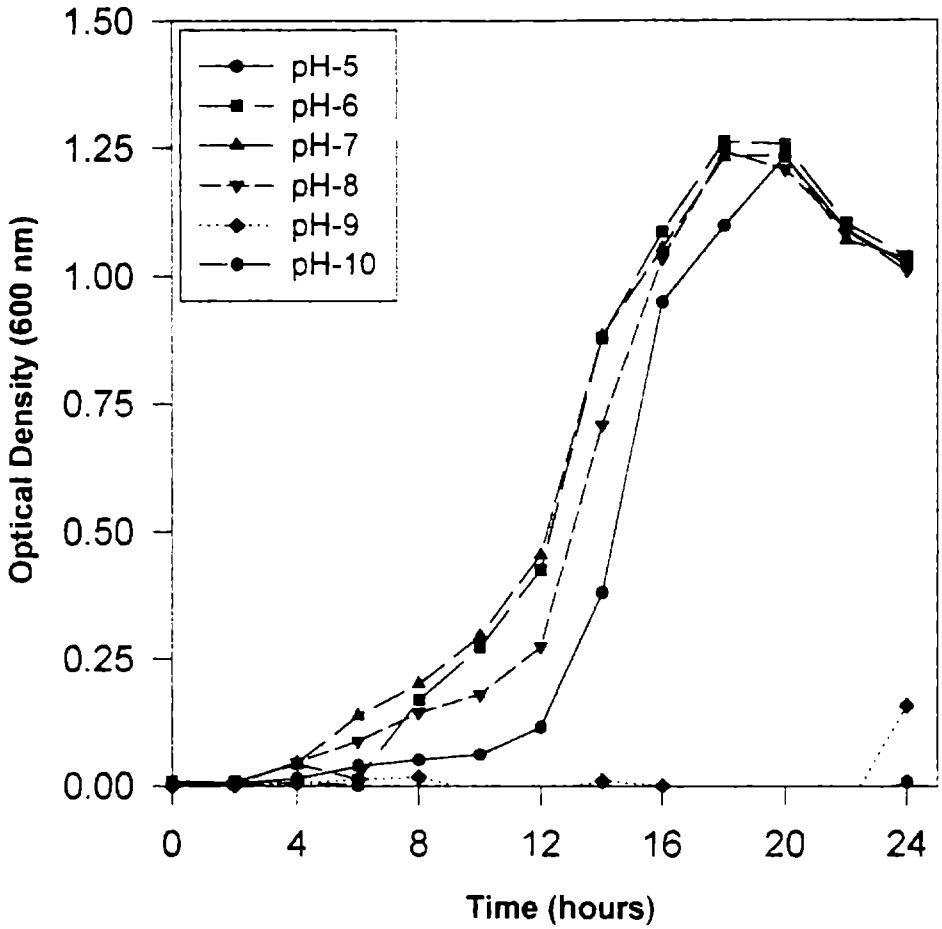


Figure 2.4.b

Optimisation of pH in *Alcaligenes* sp

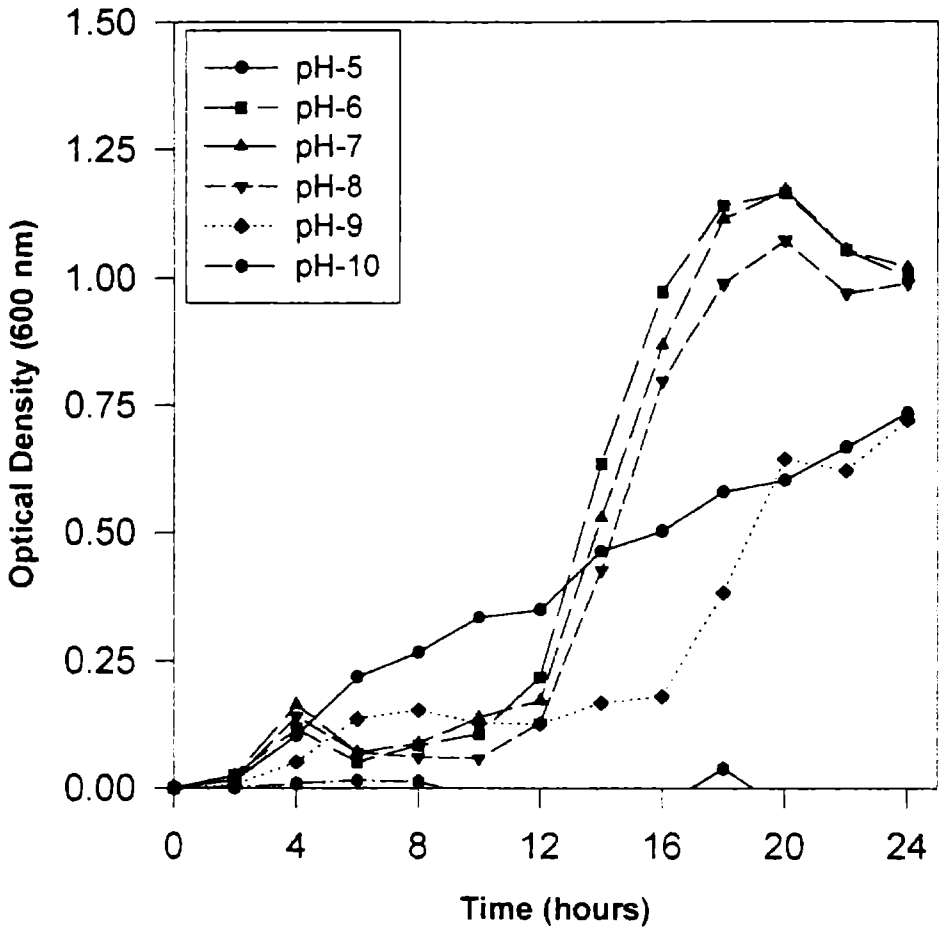


Figure 2.4.c

Optimisation of pH
in *Staphylococcus* sp

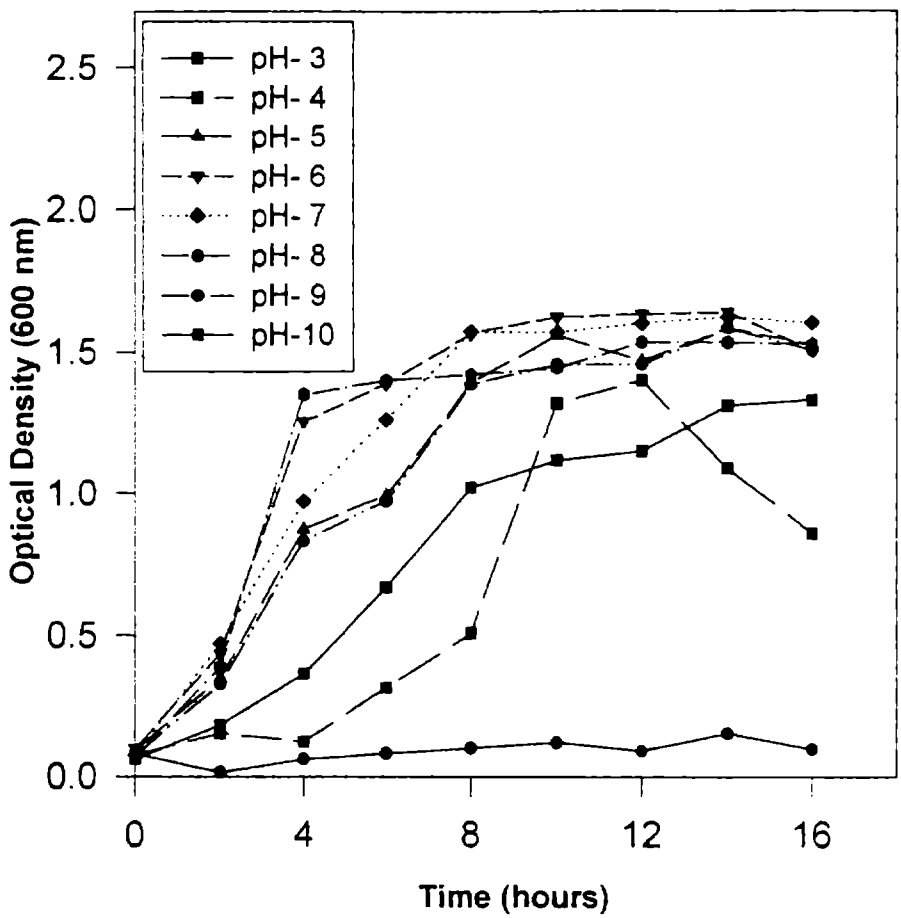


Figure 2.5

Optimisation of carbon sources in bacterial species

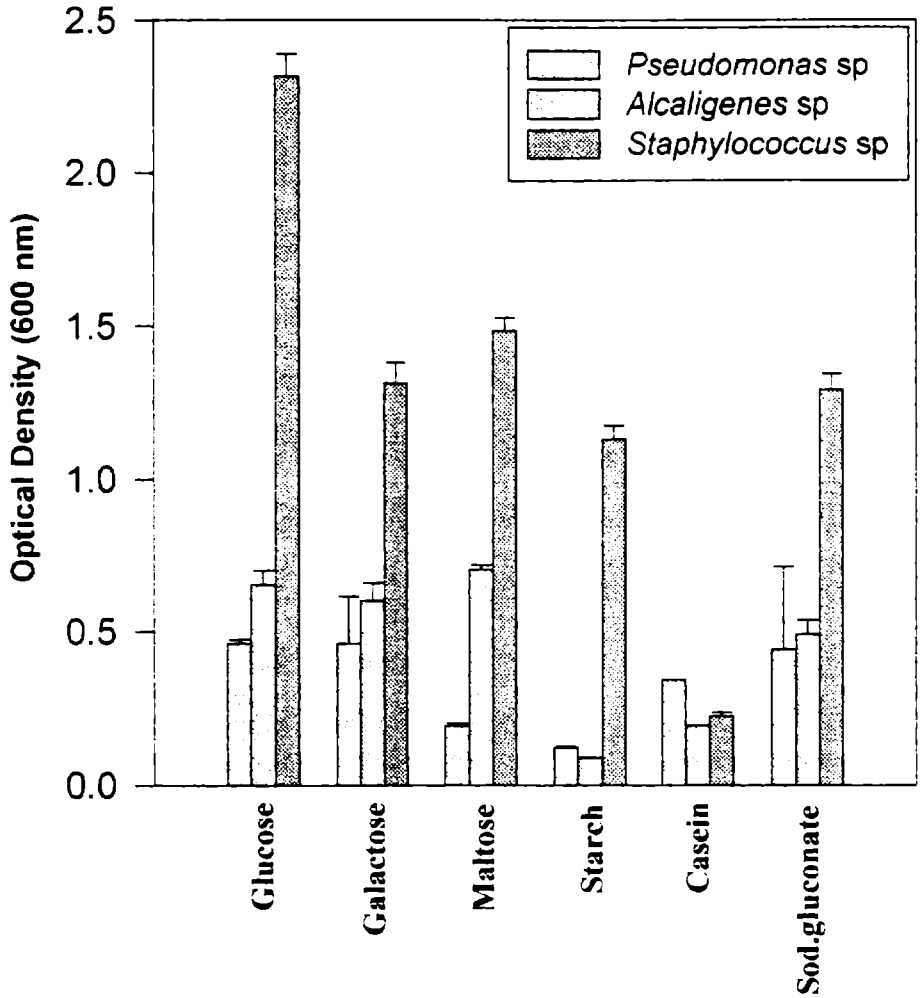


Figure 2.6

Optimisation of nitrogen sources in bacterial species

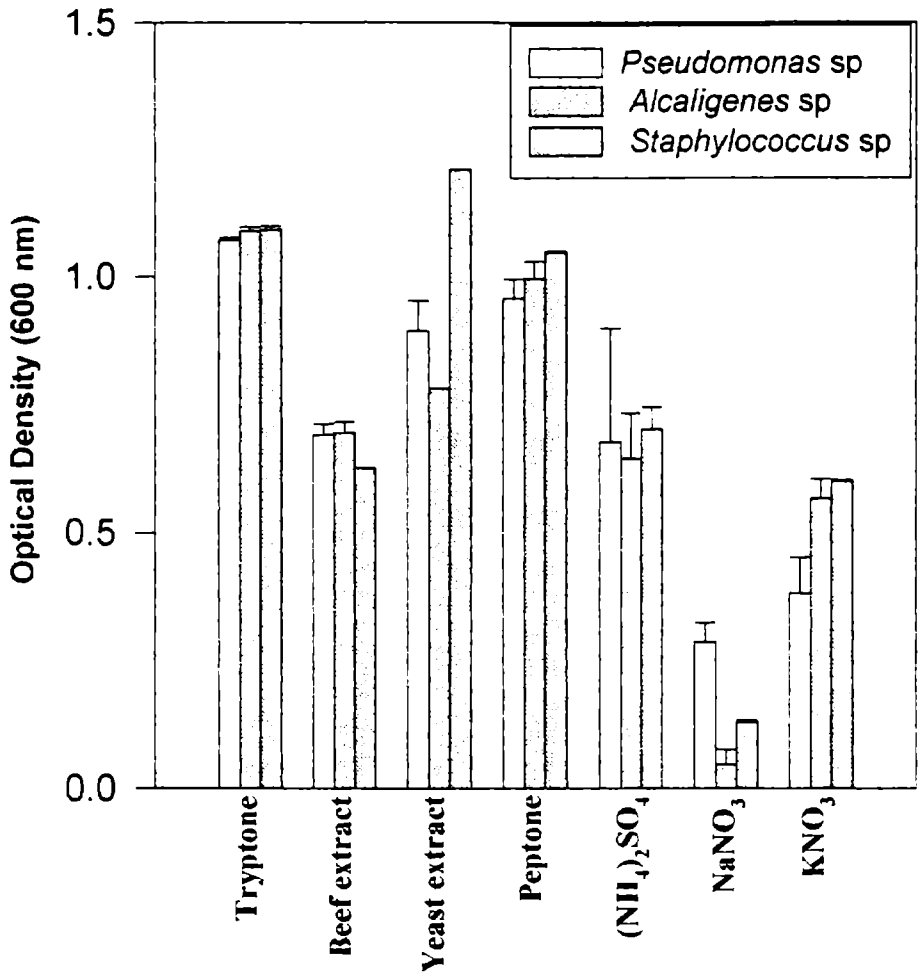


Figure 2.7

Optimisation of NaCl concentration of bacterial species

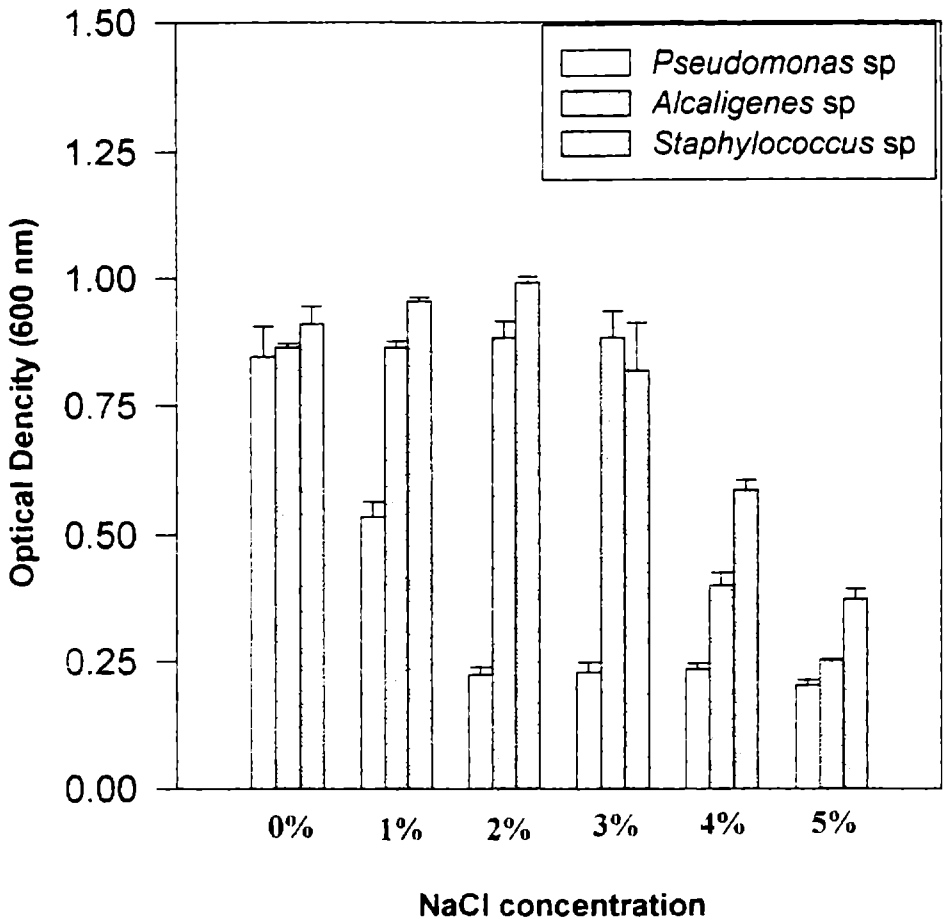


Figure 2.8.a

Optimisation of growth media
***Pseudomonas* sp.**

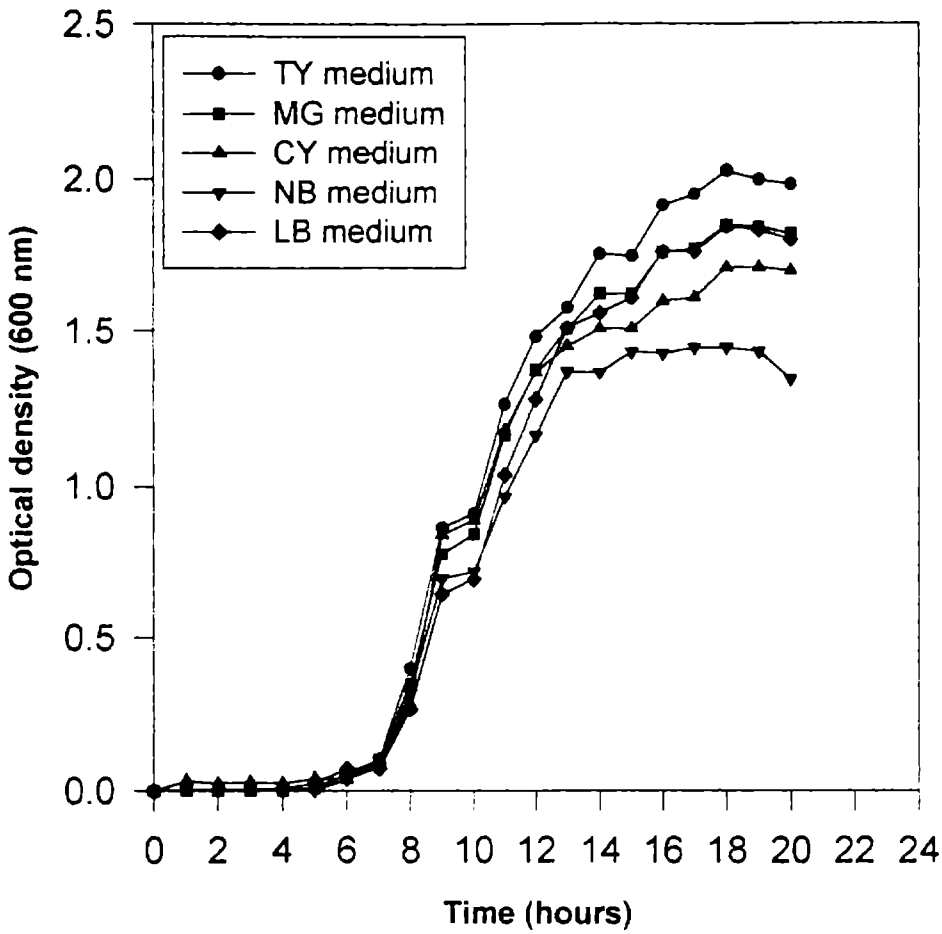


Figure 2.8.b

Optimisation of growth media
***Alcaligenes* sp**

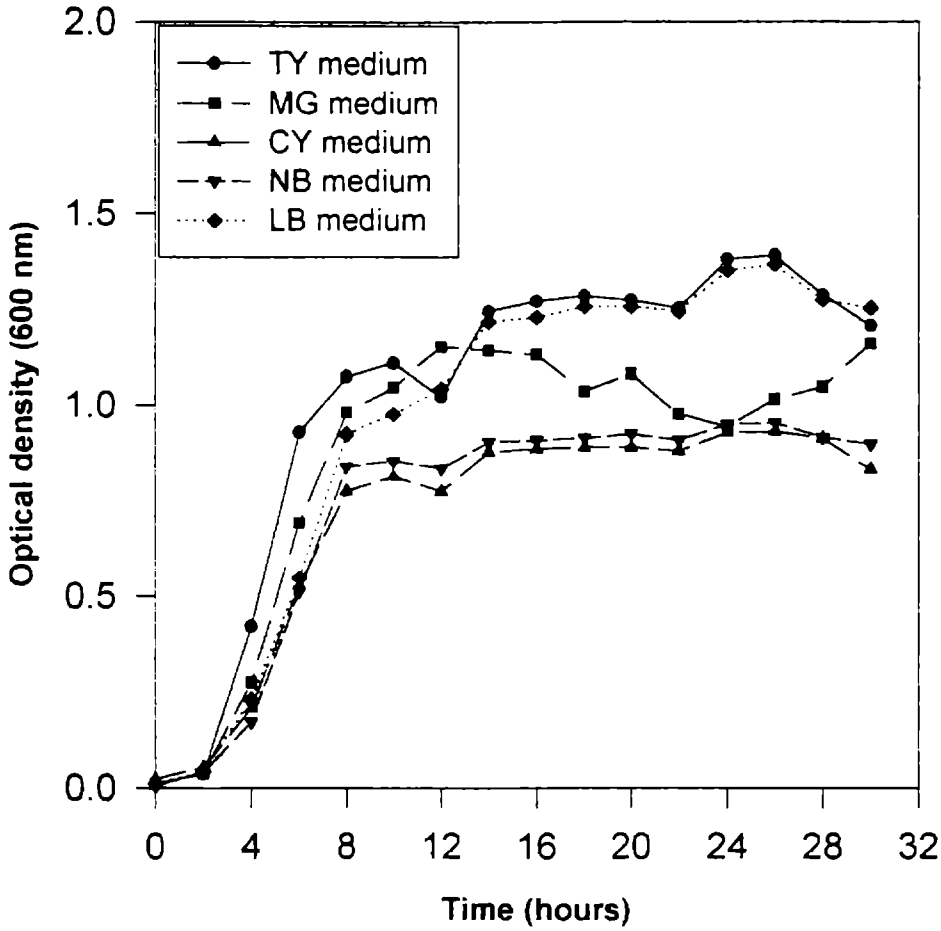
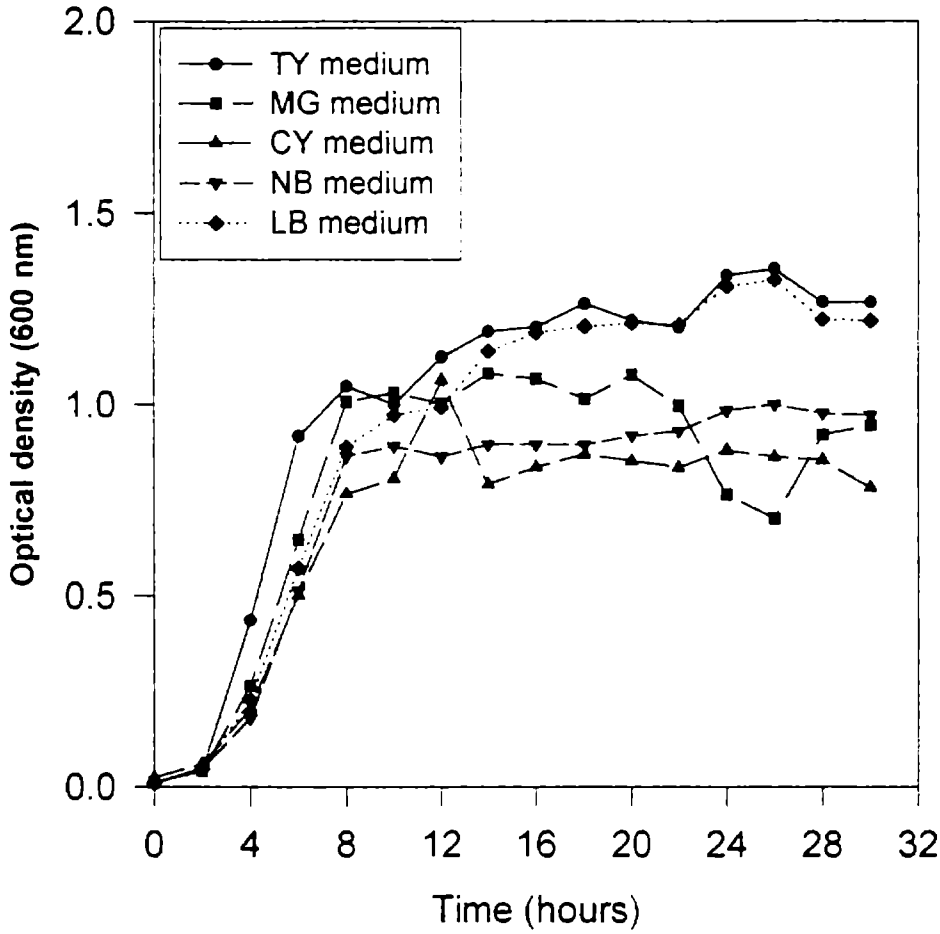


Figure 2.8.c

Optimisation of growth media
***Staphylococcus* sp**



2.4 DISCUSSION

Toxic metals are increasingly being discharged into the environment through industrial waste water. The waste water from chemical industries often contains excessive amounts of cadmium, cobalt, copper, chromium, lead etc. The microorganism in natural environment can not destroy the metal by metabolic degradation. The only way by which an organism can protect itself from the heavy metal poisoning is by binding the metal to a ligand that will prevent the metal from interfering in normal physiological processes of the cell or by increasing the rate of excretion. Metal resistant strains, in general, have superior ability to tolerate high concentration of toxic ions and hence are more desirable. In many applications it is not practical to use live organism which has necessitated the use of microbial biosorbent (Omer *et al.*, 1996). The superior ability of Cd^{2+} binding by cell wall preparations demonstrated the potential recovery and decontamination of precious/toxic metal ions (Karna *et al.*, 1996). Metal accumulation by microbial cells, moderated by a number of factors such as chemical and physiological properties of the metals (Brady, 1994), cellular physiology and the ambient conditions such as pH and temperature (Johns and Gadd, 1990) is reported. In addition, the presence of competing metal cations or lack of an available energy source (Fuhrman and Roth stein, 1968) can influence metabolism-dependent internalisation of metal ions.

The concentration of metal pollutants in the environment is usually relatively low except in areas of high pollution. A gradual increase in metal concentration could possibly alter the species diversity of these environments and create an even greater problem of transference of heavy metal and antibiotic resistance. However, unpolluted environments with very low concentration of metals may still have metal-resistant

organisms present or organisms that can readily adapted to increases in metal concentration (Lemke and Leff, 1997).

In the present study emphasise was laid on cadmium resistant bacteria, although the effluent contained other metals. From the results it was inferred that substantial number of Cd^R bacteria could be isolated from metal polluted environment. These metal resistant bacteria may play a significant role in metal removal in natural environment and consequently contribute to *in situ* bioremediation. Once the metal resistant bacteria are present, it is not at all apparent that their numbers depend on metal concentration. Other multiple environmental factors which affect the ability of organisms to take up metal also influence the ability of metal to select and to maintain a metal-resistant population. The isolation of resistant microorganisms using enrichment culture techniques allow the organisms to be identified and examined for the presence of plasmid DNA which may be responsible for their resistance to metals.

Chapter - III

Role of Plasmids in Cadmium Resistant Bacteria Isolated from Industrial Effluents

3. ROLE OF PLASMIDS IN CADMIUM RESISTANT BACTERIA ISOLATED FROM INDUSTRIAL EFFLUENTS

3.1 INTRODUCTION

Bacterial resistance to toxic agents is often observed under conditions where the toxicity might select for resistance (Amit and Silver, 1998). A number of mechanisms, which impart resistance to heavy metals, have been identified in bacterial system viz.:

(i) blocking in which the toxic ions are prevented from entering the cell e.g., Cu^{2+},

(ii) active efflux of the metal ions from the cell by highly specific systems encoded by resistance gene e.g.: Cd^{2-},

(iii) intracellular sequestration of metal binding proteins to Cd^{2+} and Zn^{2+}

(iv) extracellular sequestration, often by extracellular polysaccharides on the cell wall e.g. Pb^{2-} and Cu^{2-} and

(v) enzyme conversion of metal to less toxic form e.g. CH_2 Hg and Hg^{2+} (Rouch *et al.*, 1995).

Genes located in plasmids encode most metal resistance in bacteria. The plasmid encoded metal resistance was studied in bacteria isolated from natural communities (Kelly and Reanney, 1984). Bacterial plasmids contain specific genes for resistances to toxic heavy metal ions including Ag^{+}, AsO_2^{-}, $AsO_4^{(3-)}$, Cd^{2+}, Co^{2+}, $CrO_4^{(2-)}$, Cu^{2+}, Hg^{2+}, Ni^{2+}, Pb^{2+}, Sb^{3+}, and Zn^{2+} (Silver and Ji, 1994). The isolation of resistant organisms, using enrichment culture techniques, allows the organisms to be identified and examined for the presence of plasmid DNA that may be responsible for their resistance to metals. It also allows plasmid-cured derivatives of the resistant organism to be obtained for

comparative studies on sensitive and resistant strains of the same organism (Timothy and Luke, 1983).

Cadmium ions (Cd^{2+}) are toxic to bacteria and cause a cessation of respiration by binding to sulphydril group in protein when incorporated into sensitive cells (Chopra, 1975). Resistance to Cd^{2+} in the gram positive bacterium *Staphylococcus aureus* has been shown to be caused by plasmid encoded Cd^{2+} efflux system (Tynecka *et al.*, 1981). Cadmium and zinc are related transition metals with contrasting biological roles. Zinc is an essential ion and probably has a specific transport mechanism for entering into all cells, whereas, cadmium is a toxic ion with no biological functions. Cd^{2+} needs to be excluded, if possible or to be extruded when found inside the cell (Silver and Wailderhang, 1995). Two distinct cadmium resistance determinant, *cad* A and *cad* B have been identified in a gram-positive bacteria *Staphylococcus aureus* plasmid DNA (Smith and Novick 1972). The *cad* B gene product may confer resistance by enhancing binding of cadmium to the cell, but the mechanism is not clear. The presence of the *cad* A determinant decreases the intracellular accumulation of cadmium, suggesting that resistance is due to an active efflux of the toxic cations (Kan *et al.*, 1992).

Recently, Crupper *et al.*, (1999) identified and cloned a cadmium resistance gene designated as *cad*D, identified and cloned from the *Staphylococcus aureus* plasmid pRW001. The gene is part of a two-component operon which contains the resistance gene *cad*D and an inactive regulatory gene, *cad*X. The expression of *cad*D in *S. aureus* and *Bacillus subtilis* resulted in low-level of resistance to cadmium. In contrast, *cad*A and *cad*B from *S. aureus* induced higher level of resistance. However, when the

truncated version of *cadX* contained in pRW001 is complemented in trans with *cadX* from plasmid pLUG10, resistance increased approximately 10-fold, suggesting that the cadmium resistance operons from pRW001 and pLUG10 are evolutionarily related.

In the present study, the molecular mechanism of cadmium resistance in *Pseudomonas* sp, *Alcaligenes* sp and *Staphylococcus* sp isolated from industrial effluents, was studied with reference to the role of any plasmid harboured in these strains.

3.2 MATERIALS AND METHODS

3.2.1 Large Scale Isolation of Plasmid DNA

3.2.1.1 Bacterial Strains and Growth Conditions

Cadmium resistant bacteria, *Pseudomonas* sp, *Alcaligenes* sp and *Staphylococcus* sp, isolated from metal polluted environment were used. All the cultures were grown in LB broth (Lauria Britani) at 37^0C under agitated condition. Strains were maintained in LB agar plates containing ampicillin (50 µg/ml) and cadmium (10 µM $CdSO_4$). Chemicals and reagents used are listed in Appendix-II.

3.2.1.2 Isolation of Plasmid DNA

Large-scale isolation of plasmid DNA was done by modified alkali lysis procedure (Susan, 1995).

1. 2.0 ml of LB broth with ampicillin (50 µg/ml) was inoculated with the strains and incubated at 37^0C overnight, in an environmental shaker.

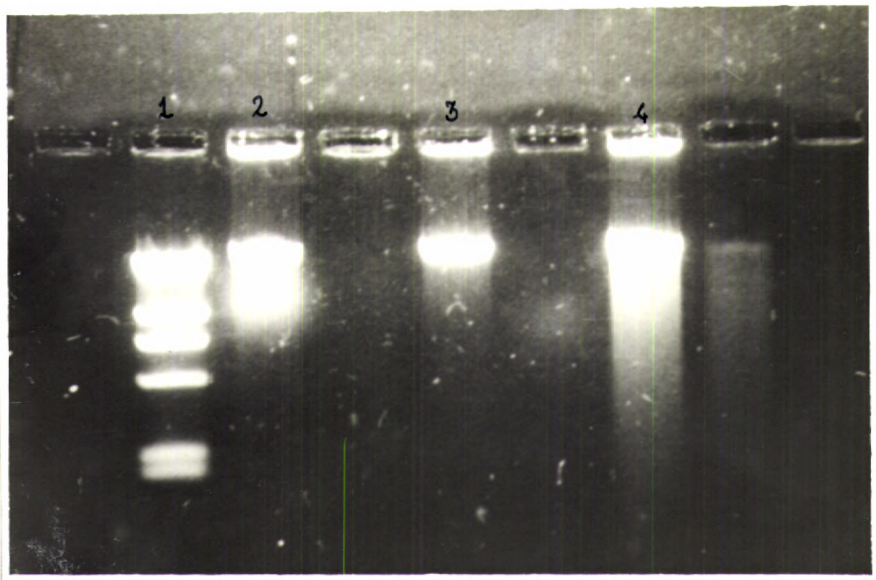


Figure .3.1

Figure 1
Agarose gel electrophoresis of plasmid isolated from bacterial strains
Lane 1-λ-HindIII DNA digest; Lane 2-*Pseudomonas* sp *p*BP-100; Lane 3-*Alcaligenes* sp *p*BA-400
Lane 4-*Staphylococcus* sp *p*BS-900

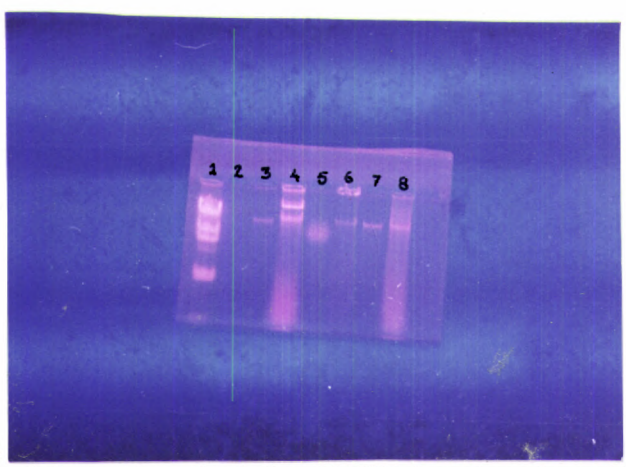


Figure .3.2

Figure 2
Agarose gel electrophoresis of plasmid DNA from transformed *E.coli* cells
Lane 1-λ-HindIII DNA digest;Lane 2-*E.coli* (Krgl$^-$); Lane 3-*p*BP-100; Lane 4-*tran* (Krgl$^+$1);
Lane 5-*p*BA-400; Lane 6-*tran E.coli* (Krgl$^+$4); Lane 7-*p*BS-900 and Lane 8-*tran E.coli* (Krgl$^+$9).

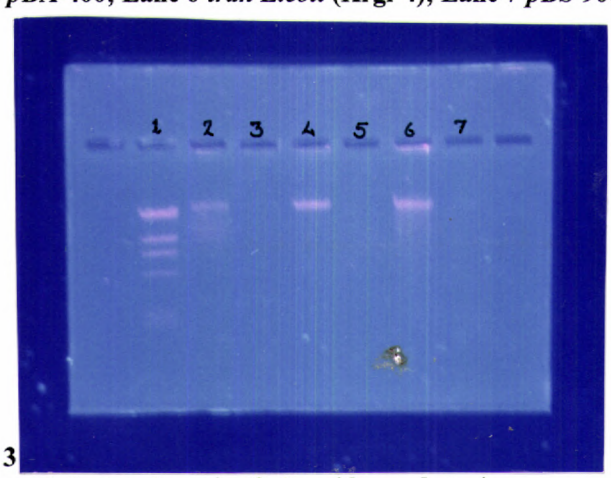
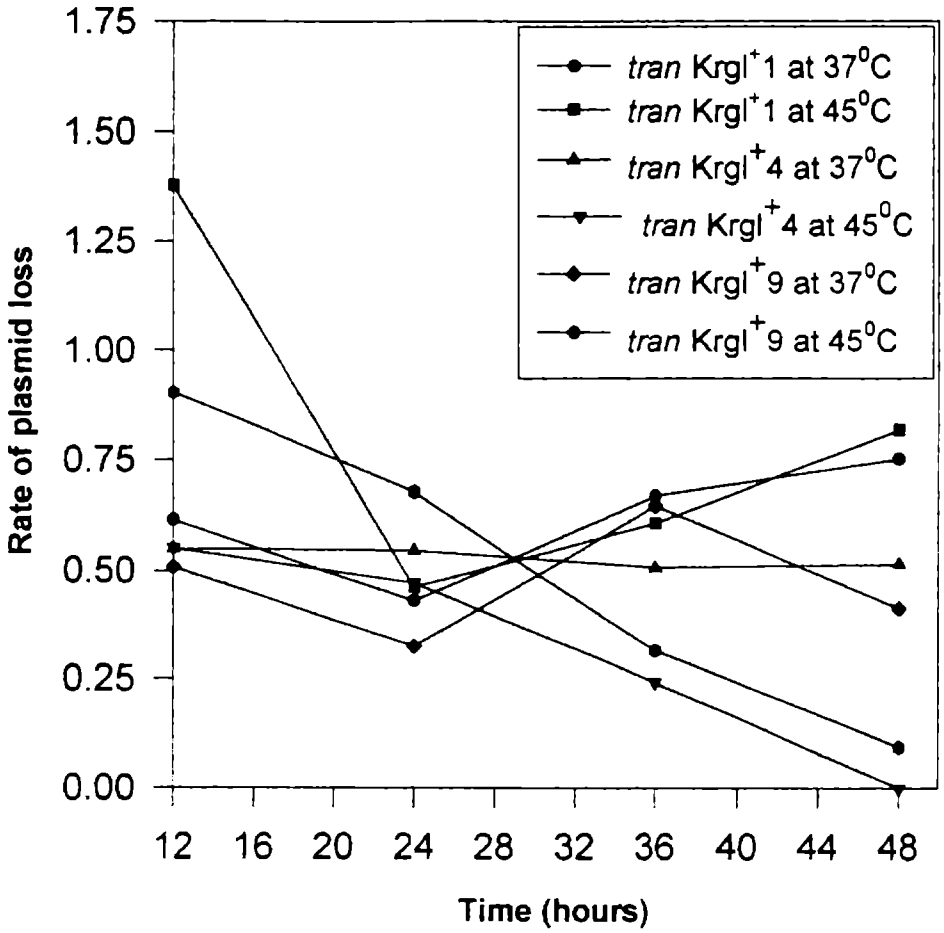


Figure. 3.3

Figure 3
Agarose gel electrophoresis of plasmid cured strains.
Lane 1-λ-HindIII DNA digest; Lane-2 *p*BA-100; Lane 3-cur *p*$^-$1; Lane 4-*p*BA-400;
Lane 5-*cur* A$^-$1; Lane 6-*p*BS-900 and Lane 7-*cur* S$^-$9

Figure 3.4

Rate of plasmid loss in transformed *E.coli* cells at different temperatures



3.4 DISCUSSION

Bacteria have evolved different mechanisms either by chromosomal or plasmid coded genes to overcome cadmium toxicity. These include reduced cadmium transport, enhanced efflux of metal and cadmium complexation by bacterial components. Cadmium resistant bacterial isolates have approximately 23 Kb covalently closed circular plasmid. The plasmids isolated from *Pseudomonas* sp, *Alcaligenes* sp and *Staphylococcus* sp were designated as *p*BP-100, *p*BA-400 and *p*BS-900, respectively. The transformation efficiency was high in plasmid (*p*BP-100) bearing recipient *E.coli* cells (*tran* $Krgl^{+}1$).

Earlier investigations of metal resistance determinants described the Cd^{R} genes as primarily plasmid borne. The transfer of cadmium resistant phenotypes from wild isolates to *E.coli* ($Krgl^{-}$) was detected in the present study. Bacterial transformation gives heritable changes in the properties of recipient bacterial cells caused by the uptake of naked DNA from wild type bacterial system having Cd^{R} phenotypic character. It is considered to have a wide host range since it was readily introduced into an enteric relative such as *E.coli*. The plasmid preparation of the transformed recipient cells also showed the same molecular weight DNA bands indicating that the plasmids were the same that was introduced. The specific observation on the growth of transformants in the presence of Cd^{2+} suggests that cadmium resistant phenotypic character could be considered as plasmid encoded and is transferred through transformation process. Recent findings have revealed chromosomal based cadmium resistance in *Alcaligenes eutrophus* CH 34 (Dietrich *et al.*, 1987). Present results also indicate a similar nature for *Alcaligenes* sp isolated during the course of study. Although this *Alcaligenes* had high

degree of Cd^{2+} resistance, its plasmid (*p*BA-400) on transformation to *E.coli* cells (i.e. *tran* $Krg^{-}4$) could not support growth in high concentration of cadmium supplemented selective media, even though it was highly resistant to ampicillin. Two distinct genes such as *cad*A and *cad*B for cadmium resistance determinants have been analysed to date as in molecular level of *Staphylococcus* sp by Silver *et al.*, (1990).

The loss of cadmium resistance of cured clones (*cur* $P^{-}1$, *cur* $A^{-}4$ and *cur* $S^{-}9$) might be associated with the loss of plasmids, which were present in the uncured parental strains. Inga *et al.*, (1986) tried to cure plasmid DNA by acridine orange but the cured clones were resistant to cadmium. George *et al.*, (1990) observed the loss of ability to degrade 2,4 D in plasmid cured strains of *Variovorax paradoxus* population. Pakniker *et al.*,(1996) reported that the chromium resistant phenotypic character could be eliminated by the treatment of bacterial culture with ethidium bromide and acridine orange and the cured clones were unable to survive under chromium supplemented medium. Whereas, in the present study ethidium bromide in combination with nalidixic acid was efficient to cure plasmid in *Pseudomonas* sp and *Alcaligenes* sp. However in the case of *Staphylococcus* sp, acridine orange was also required in addition to ethidium bromide and nalidixic acid, for curing of plasmids.

Alcaligenes eutrophus CH 34 is the first gram-negative bacterium which showed plasmid bound resistance to cadmium and Zinc and the same result was observed in *Staphylococcus aureus* (Dietrich, 1992). An ever increasing range of gram positive

bacteria is being found to share a single mechanism of Cd^{2+} resistance. The Cd^{2+} efflux P-type ATPase was first studied and sequenced by Silver *et al.*, (1989). The *Cad*A Cd^{2+} ATPase of *Staphylococcus* is an outward directed transport system whose synthesis is induced when resistant cells are exposed to Cd^{2+} (Tsai and Linnet, 1993).

The metabolic burden associated with plasmid DNA replication, gene expression, transcription and synthesis of proteins are responsible for imposition of P^-cells in culture of transformed bacteria (Glick, 1995). The stability of a set of plasmids has been explored in *E.coli* at different growth temperatures and in the absence of antibiotic selection. Plasmid maintenance has been explored at low growth temperature supporting different level of gene expression and 37^0C was found to be optimum for their stability from generation to generation. The temperature dependant evaluation of correlation is indicative of negative pressure acting on P^+ cells when the gene expression is stimulated, which can over shadow the mild effect of size or other parameters. General influence of plasmid size on P^+ cell replications have been reported (Chaeh *et al.*, 1987), but according to our results the size of the plasmid rather than the competent cells influence the fitness of P^+ cells. The increase in the biosynthesis burdens associated with replication of additional DNA segment seems to be too low to account for the decline of P^+ fitness in cultures carrying derived plasmid.

Results obtained in the present study with reference to presence of plasmid in *Staphylococcus* sp and its cadmium resistance is in agreement with earlier report on

Staphylococcus aureus (Crupper *et al.*, 1999). Whereas, in the case of *Pseudomonas* sp it is a new information, although *Pseudomonas* sp one known to harbour plasmids and demonstrate wide range of activities from biodegradation of rare organic and inorganic compounds to metal and pesticide resistance. Specific results on plasmid curing experiment evidence the fact that metal resistance, particularly cadmium is plasmid mediated. However further studies are wanted for utilisation of these results for environmental applications.

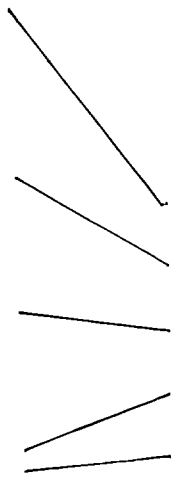
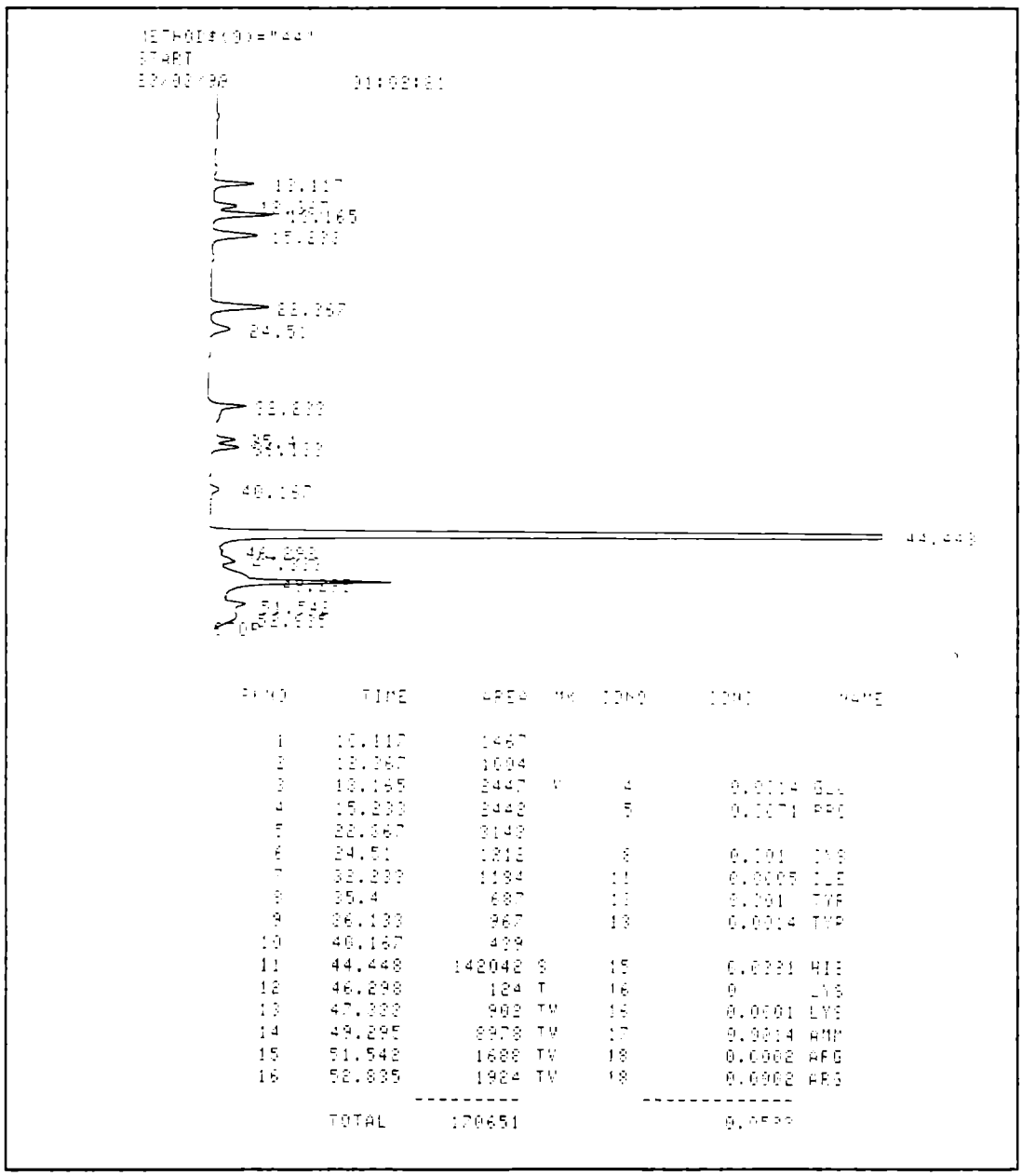


Figure 5.2
Separation and detection of metal binding protein by SDS-polyacrylamide gel electrophoresis.
Lane-1 control,
Lane-2 test (Cd treated) and
Lane-3 marker proteins.

Figure 5.3

Amino acid analysis of metal binding protein using high performance liquid chromatography (HPLC)



PEAK	TIME	AREA	MM	IDNO	IDN	NAME
1	10.117	1467				
2	13.367	1004				
3	13.165	2447	V	4	0.0014	GLU
4	15.233	2442		5	0.0071	PRO
5	20.367	3149				
6	24.51	1818		8	0.101	[illegible]
7	32.233	1194		11	0.0005	ILE
8	35.4	687		11	0.101	TYR
9	36.133	967		13	0.0014	TYR
10	40.167	499				
11	44.448	142042	S	15	0.0331	HIS
12	46.298	124	T	16	0	[illegible]
13	47.332	902	TV	16	0.0001	LYS
14	49.295	8978	TV	17	0.0014	AMM
15	51.542	1688	TV	18	0.0002	ARG
16	52.835	1924	TV	18	0.0002	ARG
	TOTAL	170651			0.0533	

Elution was carried out with a gradient mixture of sodium citrate, pH 3.25 & 10. Post column derivatisation of the amino acids were done using Orthophthalaldehyde (OPA). The detector used was FLD-6A fluorescence meter. Base line corresponds to elution time.

Figure 5.4

Specific activity of Alkaline phosphatase in presence of Cd^{2+} ions in *Staphylococcus* sp

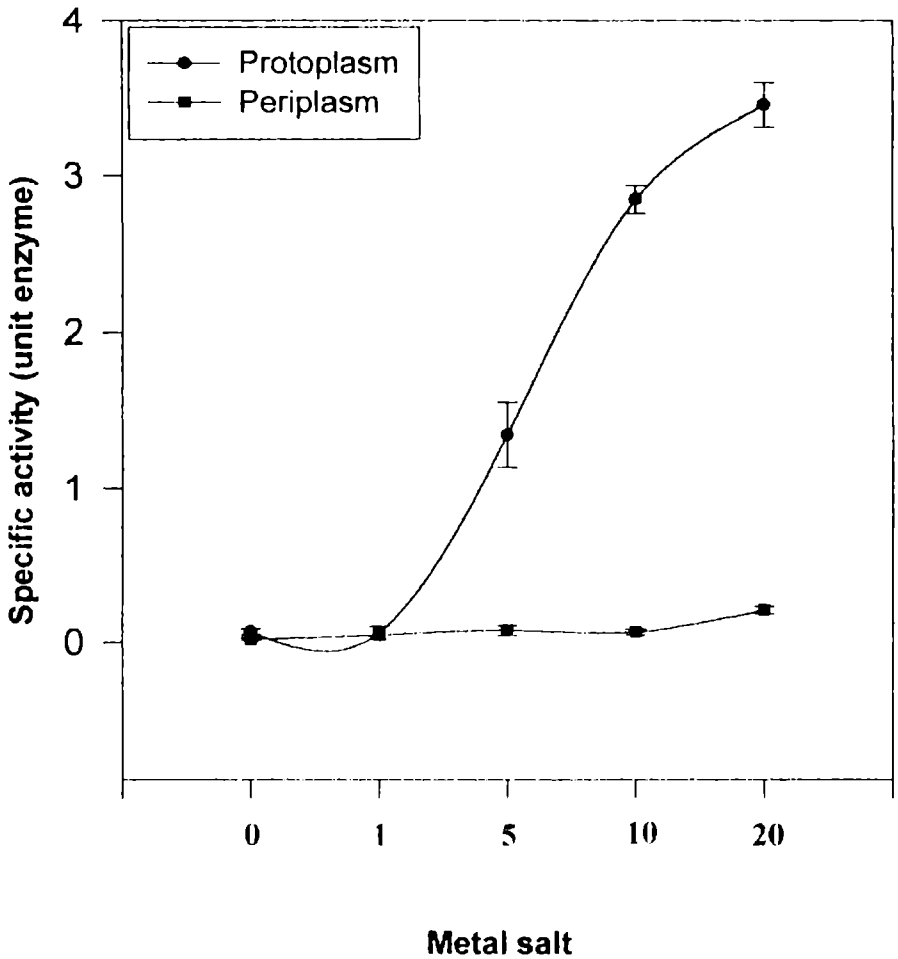
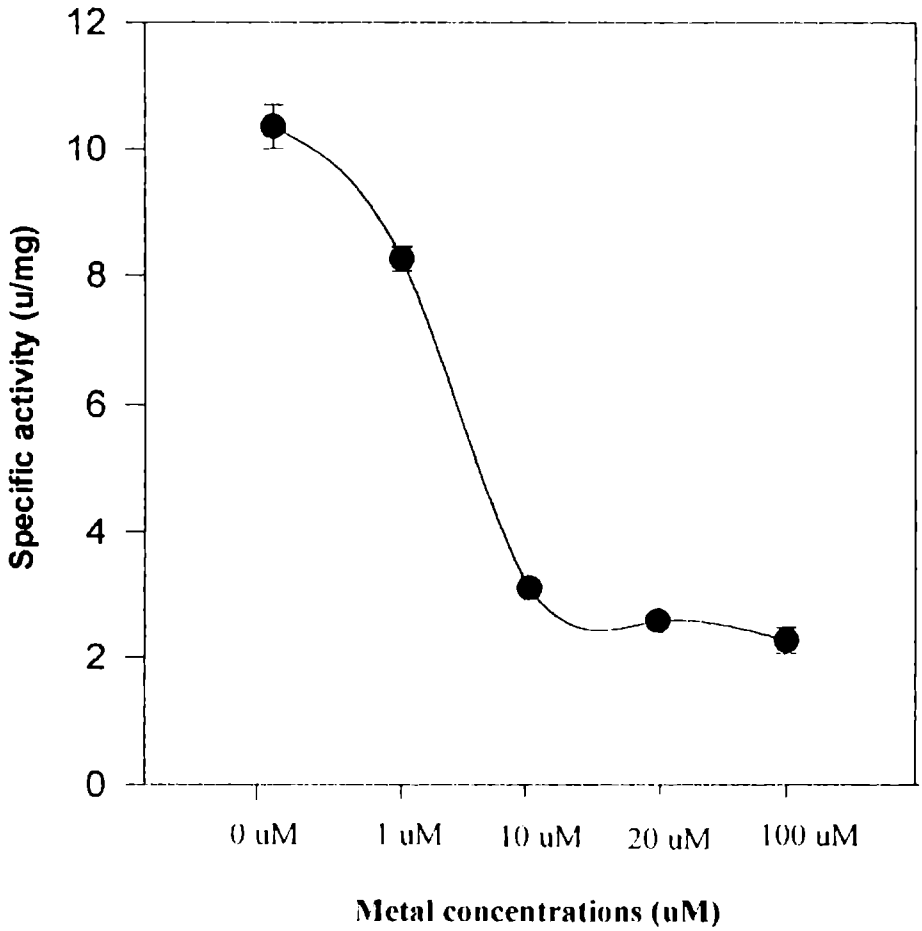


Figure 5.5

Effect of metal concentrations on B galactosidase activity of *Staphylococcus* sp



5.4 DISCUSSION

Staphylococcus sp isolated from metal polluted environment, showed potential to survive in the presence of cadmium, a toxic metal. When grown in LB medium containing cadmium (1.0 mM), there was an accumulation of cadmium ions in the cytosolic fraction of the bacterial cells. This observation suggests that there may be some kind of mechanism, which protects the cells from toxic cadmium ions. A low molecular weight protein was induced in the presence of cadmium ions in *Staphylococcus* sp. The cytoplasmic Cd^{2+}ions were found to bind low molecular weight proteins (Denise *et al.*, 198). The binding capacity of cadmium in these forms significantly affects its availability and potential toxicity to organisms and may be of environmental importance.

The heavy metal binding protein from *Staphylococcus* sp contains large amount of histidine (93.1%), which is not found in mammalian metallothioneins, and other amino acids were recorded at a lesser percentage. Probably heavy metal binding protein, in *Staphylococcus* sp, may use histidine residue to bind metal ions. The tolerance of *Staphylococcus* sp for cadmium was coincident with an increased level of the heavy metal and the heavy metal binding protein, induced by cadmium (Kissling *et al.*, 1977).

Denise *et al.*, (1984) isolated a cysteine-rich prokaryotic metallothionein protein molecule from *Pseudomonas putida* which grow in cadmium supplemented media. Olafson *et al.*, (1979) isolated low molecular weight cysteine-rich protein molecules from a cyanobacteria, *Synechococcus* sp. Koji *et al.*, (1998) constructed a cyanobacterial strain with enhanced heavy metal ion tolerance by introducing an exogenous

cyanobacterial metallothionein gene *smt*A, from a fresh water unicellular cyanobacterium *Synechococcus* sp PCC 7942. An unusual feature of *smt*A is the presence of three histidine residues, and these contribute metal co-ordination (Daniel *et al.*, 1998).

The effect of cadmium ions on the expression of alkaline phosphatase was studied using *Staphylococcus* sp. The results indicated that cadmium ions induced the expression of alkaline phosphatase under the stress conditions. The activity of alkaline phosphatase was more in protoplast fraction.

Some toxic metals can induce the production of enzymes. Although the resulting metabolites may prove to be more toxic than the original chemical, enzyme induction is regarded in general as a defence mechanism often being not specific (Bolton and Dean, 1972). A similar observation on the activity of alkaline phosphatase, where a four fold increase in the enzyme activity, in the presence of Cd^{2+} ions, was reported in *Escherichia coli* (Cohen *et al.*, 1991).

The biogenesis and secretion of over produced alkaline phosphatase depends on the gene located on their chromosomes. The assembly of alkaline phosphatase usually includes several steps of its post-translational modifications. The metal ions bind to the signal peptides and trigger the synthesis of alkaline phosphatase, which get released into the cell membrane (Inouye *et al.*, 1982). According to Karen *et al.*, (1996) the bacterial cells produce inorganic phosphate ligands (HPO^{2}_{4}) in the presence of heavy metals. Deposit of metals as polycrystalline cell bound metal precipitation is via the production

of high concentrations of phosphate ligands locally which exceed the solubility product of the metal phosphate in juxtaposition to nucleation sites of the cell surface.

Higher concentrations of cadmium resulted in a sharp decrease of β-Galactosidase activity in *Staphylococcus* sp.

The present result clearly evidence induction of alkaline phosphatase and repression of β-galactosidase activity in *Staphylococcus* sp in response to increase in the concentration of cadmium in the environment. Probably the observed results suggest a phosphate dependent transport of cadmium, through binding with metalloprotein of cell wall at higher conc. of cadmium in the environment as it was repeated earlier (Macaskie *et al.*, 1994). Where as the observed results for β-galactosidase are in this agreement with that reported for *Alcaligenes* by Dietrich (1992). Probably the nature of response to heavy metal concentration. in the environment in terms of enzyme induction and repression and metal-microbe interaction is species specific and vary from species to species.

Summary & Conclusions

6. SUMMARY AND CONCLUSIONS

6.1 SUMMARY

Cochin, commercial capital of Kerala, located on the west-coast of South India has a large number of chemical and sea food industries. Earlier studies in the past indicated that these industries contribute to heavy metal pollution, particularly mercury, copper, and cadmium, in Cochin backwater. Hence, in the present study, it was desired to isolate cadmium resistant bacteria from effluent discharged by chemical industry with a view to develop an ideal bioremediation process for safe discharge of industrial effluent in to the nearby aquatic environment.

Effluent from three industries, located in the industrial belt of Cochin, were collected from the discharge point and cadmium resistant bacteria were screened using standard microbiological techniques. Twelve isolates were obtained which showed cadmium resistance at a considerable level. They were further reconfirmed and three isolates which showed significant levels of cadmium resistance, were selected for further studies. They were identified as, *Alcaligenes* sp, *Pseudomonas* sp and *Staphylococcus* sp. Their growth conditions were optimised.

All the three isolates were evaluated for the presence of plasmids, if any, which may attribute to their cadmium resistance. It was observed that all the three isolates harboured plasmids (*P*BP-100, *P*BA-400, *P*BS-900 isolated from *Pseudomonas* sp, *Alcaligenes* sp, and *Staphylococcus* sp. respectively). The size of the plasmids was about 23 kb, in low copy number. In order to evaluate the role of plasmids in cadmium

resistance, the isolated plasmids were transformed using *E.coli* (Krgl$^-$) as host. Three transformants, namely *E.coli tran* Krgl$^+$1, *E.coli tran* Krgl$^+$4, and *E.coli tran* Krgl$^+$9 respectively for the parental strains *Pseudomonas* sp, *Alcaligenes* sp, and *Staphylococcus* sp, also showed cadmium resistance at a concentration of 5-10 ppm, indicating that the efficiency was plasmid mediated. Further, in order to reconfirm that cadmium resistance was plasmid mediated, wild strains were subjected to plasmid curing with ethidium bromide, nalidixic acid and acridine orange reagent. Both *Pseudomonas* sp, and *Alcaligenes* sp could be cured for plasmids with a combination of ethidium bromide, and nalidixic acid (1.0:0.25 and 1.0:1.5 respectively), while *Staphylococcus* sp, needed a reagent including ethidium bromide, nalidixic acid and acridine orange (1.0: 1.5 :1.0). Plasmid cured strains failed to show cadmium resistance. Plasmids lost stability at 45°C while they were stable at 37°C. The results of this study suggest that gene coding for cadmium resistance could be plasmid encoded.

When tested for their metal resistance profile, it was observed that host *E.coli* (Krgl$^-$) could not show resistance against the metals tested. Whereas, the *E.coli* transformants with plasmids received from native bacteria *Pseudomonas* sp and *Staphylococcus* sp showed resistance to metals such as zinc, cobalt and lead at 1.0 mM concentration. Whereas, *E.coli* transformants, with plasmids received from native bacteria, *Alcaligenes* sp, were sensitive to these metals. Further, all the tested transformants could not grow at 1.0 mM concentration of cadmium, mercury and copper.

Cadmium biosorption experiments were carried out with bacterial biomass to determine the microorganism's biosorbent properties under different conditions. At pH

6-8 there was maximal biosorption of cadmium. Temperature.15-40°C did not influence the rate of biosorption of cadmium by the three strains. Maximal uptake of cadmium was observed by 60 min and contact time beyond 60 min did not enhance accumulation of cadmium. Glucose, zinc and manganese, when present in the medium along with cadmium, led to inhibition of cadmium sorption. Possibly there may be a competitive binding of metals during biosorption. EDTA effected maximal desorption of cadmium. Not only the native strains, but also the *E.coli* transformants with plasmids of *Pseudomonas* sp and *Staphylococcus* sp showed biosorption of cadmium at a significant level, compared to that of *Alcaligenes* sp.

Among the three strains, *Staphylococcus* sp showed comparatively better efficiency to accumulate cadmium through biosorption as well as resistance. Hence, this strain was selected for further studies on isolation of metal binding protein and metalloenzymes associated with cadmium sorption. On isolation and analyses with SDS-PAGE, 5 bands of protein were obtained. Of them a low molecular weight protein showed greater affinity for cadmium compared to the others. On amino acid analysis of the protein it was observed that the cadmium protein is rich in histidine residue, unlike human metallothionein II.

Present results evidenced induction of alkaline phosphatase activity and repression of β galactosidase in response to increased metal concentration in *Staphylococcus* sp. Probably the observed results suggest a phosphate dependent transport of cadmium, through binding with metalloprotein of cell wall at higher conc. of cadmium in the environment.

6.2 CONCLUSIONS

From the present study it is concluded that native bacteria present in the effluent have the property of cadmium resistance and ability to accumulate cadmium from environment through the process of biosorption. *Staphylococcus* sp and *Pseudomonas* sp are efficient in removal of cadmium from the environment. It was observed that plasmids harboured by these strains have probably the genes encoded on them, which regulate cadmium resistance and biosorption process. Further, results obtained for analysis of the metal binding protein and alkaline phosphatase suggest that these native bacteria are potential biotools for bioremediation of metal pollutants in the environment.

APPENDIX-I

Chemicals and Reagents

1. TGP Medium

Solution-I	(gl^{-1})
40 mM Tris –HCl (pH 7.6)	: 4.846
0.6 mM $MgSO_4.8H_2O$	: 0.1479
5mM K_2SO_4	: 0.872
10 mM Glucose	: 1.8016
10mM Na_2 glycerophosphate	: 3.0612
2mM $(NH_4)_2SO_4$	: 3.304

Solution -II (Trace metal)	mg l^{-1}
$MnSO_4$	: 120.00
$CaCl_2$	: 110.01
$ZnSO_4.7H_2O$	: 287.50
$CoCl_2.6H_2O$	: 237.80
$FeSO_4.7H_2O$	: 278.00
MoO_3	: 200.00

Solution-III	g l^{-1}
Yeast Extract (Himedia)	: 1

2. Nutrient Broth Medium

Peptone	: 5000 mg
Yeast extract	: 2500 mg
NaCl	: 500 mg
Water	: 100 ml

3. Luria Broth medium

Tryptone	: 1000 mg
Yeast extract	: 500 mg
NaCl	: 500 mg

4. Mineral Base medium

KH_2PO_4	: 700 mg
KH_2PO_4	: 300 mg
$(NH_4)_2SO_4$	: 100 mg
$MgSO_4$	: 10 mg
Glucose	: 200 mg
Water	: 100 ml

5. TY medium

Tryptone	: 500 mg
Yeast extract	: 500 mg
NaCl	: 500 mg
Sodium- gluconate	: 200 mg
Water	: 100 ml

6. CY medium

Yeast Extract	: 500 mg
Casamino acid	: 500 mg
Water	: 100 ml

7. MG medium

Mannitol	: 500 mg
Sodium-gluconate	: 200 mg
Yeast extract	: 500 mg
Water	: 100 ml

8. Stock solutions (1000 ppm) concentrations

   $CdSO_4$

   $HgCl_2$

   $CuSO_4$

   $Pb(NO_3)_2$

   $Co(NO_3)_2$

   $ZnSO_4$

9. Antibiotic Stock Solutions (50 mg/ml) concentrations.

   Ampicillin

   Chloramphenicol

   Gentamycine

   Kanamycine

   Streptomycine

   Tetracycline

APPENDIX-II

Chemicals and Reagents

Large - scale isolation of plasmid

1. Solution-I

   50 mM Glucose

   25 mM Tris HCl

   10 mM EDTA

   Adjusted the pH to 8.0 with NaOH or HCl.

2. Solution-II

   0.2 M NaOH

   1% SDS

   Freshly prepared solution II each time. For 100 ml of solution II, mix 10 ml of 2M NaOH, 5 ml of 20% SDS and 85 ml sterile distilled H_2O.

3. Solution- III

   3 M potassium acetate solution pH 5.5

4. LB broth (Himedia)
5. LB Agar (Himedia)
6. Agarose (Biorad)
7. Lysozyme (10mg/ml in solution-I)
8. Phenol:Chloroform (1:1) use phenol crystals (melt, colourless) 44 ml phenol, 44 ml chloroform. Mix well, add 2.64 g of NaCl mix, add 44 ml of 2 M Tris buffer (pH 7.0). Mix and remove the aqueous (top) phase. Add 44 ml of 50 mM Tris HCl (pH 8.0). The solution is now ready for use, store at 4 ^{0}C.
9. 3 M NaOAc (sodium acetate)

10. Ethanol

11. RNase (10mg/ml, pre-boiled)

12. Isopropanol saturated with 20 × SSC (1× SSC is 0.15 M NaCl, 0.015 M Na citrate)

13. 1mM Tris-HCl, 0.5 mM EDTA (pH8.0)

14. TAE (50X), 50 ml

    Tris base 12.1 g.

    Glacial acetic acid (AR) 2.9 ml.

    EDTA 0.5 M 5 ml.

    Distilled water 50 ml.

15. Ampicillin stock solution (50 μg/ml)

16. 30 mM $CaCl_2$

17. 10 mM NaCl

18. Ethidium bromide (50mg/ml) stock solution.

19. Nalidixic Acid (50mg/ml) stock solution.

20. Acridine orange (50mg/ml) stock solution

APPENDIX - III

Chemicals and Reagents

1. TGP Medium.

Solution- I	gl^{-1}
40 mM Tris–HCl (pH 7.6)	: 4.846
0.6 mM $MgSO_4.8H_2O$	: 1479.
5 mM K_2SO_4	: 0.872
10 mM Glucose	: 1.8016
10 mM Na_2 glycerophosphate	: 3.0612
2 mM $(NH_4)2SO4$	: 3.304

Solution -II (Trace metal)	mg l^{-1}
$MnSO_4$	: 120.00
$CaCl_2$	: 110.01
$Zn\ SO_4.7H_2O$	: 287.50
$CoCl_2.6H_2O$	: 237.80
$FeSO_4.7H_2O$	: 278.00
MoO3	: 200.00

Solution-III	g l^{-1}
Yeast Extract(Himedia)	: 1

2. Nitric acid:Picric acid solution: 9:1 ratio.
3. 1.0 N NaOH solution
4. 1.0 N HCl solution

5. Cadmium (1000 ppm.)Stock solution

   $Cd(NO_3)_2$: 2.7442 g l-1

6. 20mM Tris buffer (pH 7.2)

7. 1.0 mM EDTA

8. 1.0 mM HCl

9. 10.0 mM Glucose solution

10. 10.0 mM Zinc solution

11. 10.0 mM Manganese solution

12. Double distilled water

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LIST OF PUBLICATIONS

- Cadmium tolerance and metal accumulation by bacterial strains isolated from industrial effluents. **M.K. Suresh Kumar**, G.S. Selvam and P.S Jasmine. Journal of Scientific and Industrial Research, Vol.57, 1998. (817-820).
- Cadmium resistant bacteria *Pseudomonas* species isolated from Cochin environment. **M.K.Suresh Kumar**, H.H.Krishnan and G.S.Selvam.(accepted for book publication).

SCIENTIFIC MEETINGS ATTENDED

1) National conference of seaweed utilization and research. December 26^{th} –29^{th} 1995, conducted by CMFRI (ICAR) ,Madurai, Tamilnadu.
2) International conference in frontiers in Biotechnology, November 27^{th} –30^{th} 1997, conducted by Regional Research Laboratory, Trivandrum, Kerala.
3) International conference on environmental science, December 2^{nd} - 4^{th} 1998, Conducted by Centre for marine analytical reference and standards, RRL, Trivandrum, Kerala.