ISOLATION AND CHARACTERIZATION OF ZINC RESISTANT BACTERIA FROM ZINC CONTAMINATED SITES

A DISSERTATION

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT

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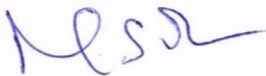
CERTIFICATE

This is to certify that the dissertation entitled "*ISOLATION AND CHARACTERIZATION OF ZINC RESISTANT BACTERIA FROM ZINC CONTAMINATED SITES*" submitted by Vikrant Mehta (Registration No. 301105022) in partial fulfillment of the requirement for the award of degree of Master of Sciences in Microbiology, to Thapar University, Patiala is a record of student's own work carried out by him under our supervision and guidance. The report has not been submitted for the award of any other degree or certificate in this or any other university or institute.



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CANDIDATE'S DECLARATION

I, hereby declare that the work which is being presented in this dissertation entitled *"ISOLATION AND CHARACTERIZATION OF ZINC RESISTANT BACTERIA FROM ZINC CONTAMINATED SITES"* submitted by the undersigned in partial fulfillment of the requirements for the award of the degree of Master of Science in Microbiology, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is an authentic record of my own independent and original research work carried out during the period of six months from Jan 2012 to July 2012, under the supervision of Dr. M. S. Reddy, Professor & Head, Department of Biotechnology & Environmental Sciences, Thapar University. The matter embodied in this dissertation has not been submitted in part or full to any other university or institute for the award of any other degree or certificate.

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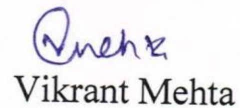

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1. INTRODUCTION

1.1 Pollution - A global problem

In present time, environmental pollution has become one of the very major concerns for the scientists and environmentalists around the globe due to inevitably injurious and deleterious effect of pollution on various forms of life including marine, human and plant biosphere. Pollution due to both natural and anthropogenic sources leads to various threats to our environment. The threats to biosphere is more due to anthropogenic sources as they pose more dangerous impacts on the environment because of our overt interference in the natural bio-geochemical cycling of such pollutants. Although we recognize this fact but subsequently we are slowly but surely harming our planet to the point where organisms are facing the consequences of ill-organized and rampant industrialization at very alarming rate (Hanif et al 2005).

1.2 Zinc and its mining

Zinc has been recognized as one of the trace element which is required in small amount for various cellular functions but becomes toxic at higher concentration leading to impairment in cellular function. Mining and power generation are among the most important developmental activities after agriculture. The natural resources are limited and non-renewable but excessive mining of natural resources (whether by open-cast or underground methods) and thermal power generation have severe and irreversible environmental implications, if proper planning and management strategies are not adopted and magnitude of these impacts depends, to a large extent, on the existing environmental conditions and socioeconomic status of the people. Open cast mining presents more serious impacts on the environment than underground operations and produce large amount of waste than the underground mines.

Industry oriented metalliferrous mining and smelting operations lead to the emission of excessive amount of zinc and other heavy metals when the runoff water and effluent is directly released into the environment without proper treatment as a result of which the toxicity in various habitats increases leading to the contamination of soil, water and air. Soil is a natural resource required for the food production and global economy and as a part of biosphere, provides nutrient-bearing environment that sustains the growth of plant and animals but its pollution has also emerged as one of the serious predicament and

challenge to the environment because of the excessive urbanization. No doubt the industrialization and associated developmental activities are important for the economic development of a nation but improper management and policies takes huge toll on the environment affecting the quality of life in various habitats because of the pollutants that are continuously released into the environment. Natural processes such as volcanic eruptions, continental dusts, forest fires, soil erosion are known to contaminate the soil by acidification and release of various gases which can lead to the generation of processes like acid rain. Anthropogenic activities like mining, spraying of agri-chemicals like pesticides and combustion of fossil fuels, military activities, metal working industries and other developmental activities like oil and gas drilling lead to emission of various pollutants and accumulation of various heavy metals such as lead and zinc and various chemicals in the ecosystem (Hooda, 2007).

1.3 Toxicity of zinc

Heavy metals such as zinc, copper, cadmium, lead, mercury have been reported as most toxic pollutants (Zovko and Romik 2011) and exposure to heavy metals has been linked with development retardation and other ailments such as cancers, kidney damage and even death in some instances and development of autoimmunity too (Brooks et al 2010).

Unlike other heavy metals such as cadmium and mercury, zinc is an essential metal and is required in trace amounts by the living organisms. However, elevated levels of zinc present a severe problem for the environment as these heavy metals are non-biodegradable by their nature hence having high toxicity patterns and long residence time in ecosystem and ubiquitous distribution worldwide. It not only decreases the soil microbial activity and crop production but also can bioconcentrate or biomagnify through the food chain (McLaughlin et al 1999). Zinc does not volatilize from soil and water but is deposited primarily in sediments through adsorption and precipitation. Severe zinc contamination tends to be confined to areas near emission sources.

The major sources of the zinc contamination are a result of increased industrialization and activities related to its mining, discharge of effluents from the industries involved in electroplating, metal processing, manufacturing of alkaline storage batteries and the use of commercial products such as fertilizers and wood preservatives that contain zinc.(El Sayed et al 2011; Ahemad 2012) .

1.4 Zinc mining in India

Udaipur district of Rajasthan has the largest deposits of zinc and its ore in the country and mining operations have been carried out for more than 40 years. Hind Zawar lead-zinc belt is an ancient base metal mining centre in India. Systematic and proper evaluation is needed to assess the impact of the mining on the environment and on the socio-economic status of the people working in and around the mines which has been in operation for almost half a century.

1.5 Impact of mining in India - A case study

Thirteen major minerals including zinc earlier reserved exclusively for public sector were thrown open for exploitation by the private sector and needs a greater attention as far as utilization and demand is concerned so that sustainable progress could be achieved. Hindustan zinc limited which was incorporated in January, 1966 as a public sector company, developed mining and smelting capacities and to substantially meet the domestic demand of zinc and lead metals and hence this belt has been continuously mined for almost 50 years and hence the impacts such as health risks to humans and animals, deforestation, increase in wasteland area pose threat to the environment.

Studies conducted by Jhanwar (1997) revealed the deleterious effect of the mining on environment and health of the workers taking place in Zaawar mines of Udaipur (Table 1.1).

Table 1.1: Comparison of the various attributes studied between 1969-1997 (Jhanwar 1997)

Attribute studied	Year 1969 (sq. km)	Year 1997(sq.km.)
Dense forest cover	45.63	6.59
Open forest cover	153.43	15.14
Area under mine/dump	0.24	1.49
Wasteland area	21.93	202.36

Air pollution in the area is small evidence of the impact of zinc mining on the environment and on the health of the people working in the belt. 16.5% workers suffered from eye related infections and 60% patients were suffering from lung related diseases such as tuberculosis (Jhanwar 1997).

Zinc is required in moderate quantities for the normal growth and development of the organism. However, elevated levels of zinc are toxic and hence both deficiency and exaggerated levels can pose adverse effects on the species. Toxicity of zinc has been documented in some texts. Toxicity towards aquatic animals starts at earlier stages of life and depends on the hardness of water, concentration of dissolved oxygen and temperature. At acutely toxic concentration zinc probably attacks the gill tissues of the fish leading to suffocation and stress is also known to be fatal at chronical levels (Skidmore 1964; Golding 2008). Severe impact on the aquatic habitats was observed when number of fishes were killed due to acid mine drainage from the Iron mountain mine near California rich in heavy metals such as zinc and copper (US department of interior 1998)

In humans, zinc toxicity is common in the welders who work on non-ferrous metals or ferrous metals alloyed with or coated with other metals. Zinc fume from galvanized coatings is a common cause of metal fume fever. Zinc in the form of zinc oxide and chloride is toxic to the lungs of rats (Cho et al 2011).

Zinc toxicity to plants results in decreased root and shoot fresh and dry mass and the physical impacts on structure resulted in the inward-rolling of the leaf edges and damage in the root system with short lateral roots (Abadia et al 2009).

1.6 Removal of zinc using conventional methods

Few conventional methods that have been used to remove heavy metals from various sites includes:-

- Filtration
- Chemical precipitation
- Electrochemical treatment
- Reverse osmosis
- Ion exchange

However all these technologies come with few drawbacks like inefficiency, high cost and labour requirement, high reagent and energy requirements, generation of toxic sludge and secondary waste. So, the main riddle before the researchers and scientists around the globe is to find an effective and eco- friendly solution to remove such heavy metals. Bioremediation of heavy metals is one of the alternatives which involve the environmental cleanup mainly by using micro-organisms and plants. Microorganisms have developed an amazing art of utilizing zinc as an necessary element for their growth when taken in and at the same time have evolved various resistance mechanisms enabling them to survive in the presence of toxic levels of zinc. Hence, the attribute of the remediation of zinc by the resistant bacteria lays the basic foundation of this project work.

OBJECTIVES

- To perform the soil analysis of the sample
- To screen and identify the efficient zinc resistant bacteria
- To study the effect of different concentrations of zinc ion on bacterial growth and determination of minimum inhibitory concentration (MIC) of zinc.

2. REVIEW OF LITERATURE

2.1 General properties of Zinc

Zinc is a common bluish white soft metal widely distributed in nature having density of about 7.13g/cm^3. It occupies the group II-B of the periodic table and has atomic weight of 65.38 and melting point of 420^{0}C. Zinc is a composite of five stable isotopes: ^{64}Zn, ^{66}Zn, ^{67}Zn, ^{68}Zn, and ^{70}Zn. It has oxidation state of II in nature and due to the amphoteric behavior, Zn forms variety of salts like chloride, sulphate, nitrate, carbonate and phosphate. Zinc forms a protective barrier in the presence of moisture by forming a carbonate salt (2 $ZnCO_3.3H_2O$) on the surface of metal thereby protecting it against corrosion (Adriano 1986).

Zinc is present not only in rock and soil, but also in air, water and the biosphere including plants, animals and humans. The most common ores of zinc with different attributes has been presented in the following table 2.1.

Table 2.1: Zinc Minerals and their Chemical Composition (IBM 2011).

Name of the zinc bearing	Composition mineral	Metal content (%)	Specific gravity
Zinc blende or sphalerite	ZnS	67.0	4.09
Smithsonite	$ZnCO_3$	52.0	4.43
Hemimorphite (Calamine)	$Zn_4SiO_7.(OH)_2.H_2O$	54.2	3.4 -3.5
Zincite	ZnO	30.3	5.68
Willimite	Zn_2SiO_4	58.5	3.9-4.2

2.2 Zinc in ancient times

Rajasthan has earliest dated lead-zinc mines in the world. India developed strong technology of mining and smelting, which is rather older than Harrapan civilization (Chauhan 2010). The use of zinc in ancient India was mainly for medicinal purposes where brass was made from zinc for wound healing purposes in the Charaka Samhita (300 BC). Archaeological evidences are witness to the utilization of zinc in ancient times as zinc alloys and brass goods have been

found in Israel and zinc objects with 87% purity have been discovered in prehistoric Transylvania. (ECIC South Africa, 2007).

2.3 Sources of Zinc in environment

The increasing load of urbanization and industrialization has resulted in an increased level of pollutants like heavy metals which are non-destructible and are toxic at even low concentration (Nasrazadini et al 2010). Zinc is natural component of earth's crust and several natural as well as anthropogenic sources are responsible for the presence of zinc in the atmosphere (El Sayed et al 2011). Usage and dispersion of the zinc and related products has seen an excessive increase in the last decade and hence the increased level in the environment is a matter of concern (Nriagu 1990).

2.3.1 Natural sources of zinc emission

The average natural level of zinc in the earth's crust is 70 mg/kg, but it ranges between 10 and 300 mg/kg. Zinc in rivers varies from less than 10 micrograms per litre to over 200 micrograms. Similarly, falling leaves in autumn lead to a seasonal increase in zinc levels in soil and water. In some areas, zinc has been concentrated to much higher levels by natural geological and geochemical processes (5-15% or 50,000-150,000 mg/kg). Approximately 45,000 tons of zinc is released per year through natural processes like forest fires. (Courtesy: http://www.safe.nite.go.jp/english/risk/pdf/03_summary/001sum.pdf.)

Zinc is constantly transported around our environment by nature through a process called natural cycling. Rain, snow, ice, solar heat and wind erode zinc-containing rocks and soil. Wind and water carry minute amounts of zinc to lakes, rivers and the sea, where it collects as sediment or is transported further. Sea salt and the movement of soil dust particles in the air are the principal sources of natural zinc emissions in the atmosphere (Richardson 2001). Forest fires and volcanoes also contribute in a minor way to zinc's natural cycling. It is estimated that these natural emissions of zinc amount to 5.9 million metric tonnes each year (IZA 1997).

Forest fires have the potential to release toxic industrial and agricultural pollutants previously immobilized on soil. After adsorbing onto smoke particles, these pollutants can travel over large-distance rides sometimes across oceans before they are grounded again and contaminate some new region. Tropical forest and savannah forest burning in Africa and Brazil resulted in the enrichment of aerosols containing elements like zinc (Echalar et. al 1995).

Emissions of metals from volcanic eruptions and production of toxic aerosols of such metals like zinc, mercury, cadmium, copper and selenium exceeding the permissible air concentration was estimated using dispersal model for 1976 eruption of Mount Etna (Weinstein and Cook 2005).

3.3.2 Anthropogenic sources of zinc emission

As stated above both natural and anthropogenic activities continue to deposit zinc and other heavy metals in the environment, however emission of such chemicals from anthropogenic sources is far more dangerous and uninterrupted. Zinc is the 23rd most abundant element in the Earth's crust and its concentrations are rising unnaturally, due to addition of zinc through hominid activities (Gakwisiri et al 2012). Zinc contamination in soil aids in the global pollution by the unfair and excessive use of the natural resources made available to humans. Various developmental, industrial works are responsible for toxic levels of zinc emission. Mining, manufacturing, and the use of synthetic products (e.g. pesticides, paints, batteries, industrial waste, and land application of industrial or domestic sludge) can result in heavy metal contamination of urban and agricultural soils (USDA NRCS 2000).

Non- ferrous metal production is major source of anthropogenic emission of trace elements like that of zinc. Asian continent releases the largest amount of such trace elements in the atmosphere evidencing the impact of growing population levels and industrialization in the region (Pacyna and Pacyna 2001).

Major sources of zinc emission from anthropogenic sources into the environment can be summarised under following:-

- Industries such as electroplating, steel, paper mills, batteries
- Mining and smelting operations
- Municipal solid waste
- Unrighteous agriculture practices

(Zhang et al 2008; Mehta 2002; Rehmani 2012; Cameron 1992; Arora 2008)

Heavy metals other than zinc which are dispersed in nature due to anthropogenic sources along with their sources have been presented below

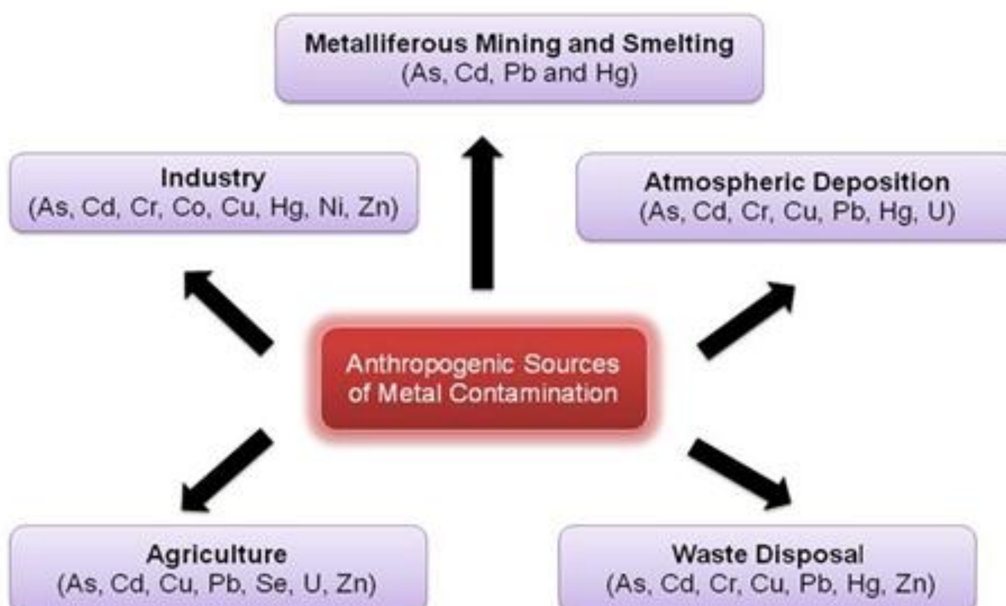


Figure 2.1: Anthropogenic activities leading to the contamination of soils with heavy metals (Ahemad 2012)

Industrial activities

India, as we all know is the second largest populous country of the world and hence to support the basic requirements of such a large population in continuum requires fast production and hence to meet the rate of requirement, production processes have been massively improved. The harshest impact on environment is left by the various industrial activities. Industrialization in India gained a momentum with initiation of five year developmental plan in the early 50's (Ramachandra et al 2003). Metals leaching from eating utensils and cookware lead to metallic contamination of food and water (Verma and Singh 2005).Zinc in environment is released from industries dealing in smelting, electroplating, (Sahni 2011), rubber and tire work (Landa et al 2004), oxides and chemicals, galvanization and diecasting alloys (IBM 2011).

Zinc which is an excellent protector of steel has seen an unprecedented growth. The galvanized steel has longer life and hence increased uses of brass and galvanized products have been employed in buildings, airports and railways (IBM 2011).

As a pigment, zinc is used in plastics, cosmetics, photocopier paper; wallpaper, printing inks etc, whereas in rubber production its role is to act as a catalyst during manufacture and as a heat disperser in the final product (Gakwisiri et al 2012). Due to excessive usage the level of zinc in soil increases mainly from the disposal of wastes of metal manufacturing industries

and coal ash from electric utilities. Waste streams from zinc and/or other metal manufacturing chemical industries, domestic waste water, and run-off from soil containing zinc can discharge it into waterways (ASTDR 2005) leading to accumulation and toxicity in various aquatic animals such as fish, frog and crabs (Al-Weher 2008; Itow et. al 1998).

Mining activity

Mine wastes have been generated for several centuries, and mining activity has accelerated significantly during the 20th century (Chaalal et al 2003). In some cases, mining operations have been carried out without concern for the 'carrying capacity' of the environment.
Corruption among higher officials promotes illegal mining in many sensitive sites causing deleterious impact on the environment while additionally causing loss of public revenues (ISID 2012). Mining sector contributed around 4% to country's GDP occupying 36 lakh hectare (0.11%) of total land area (329 million hectare) and providing employment generation (4 %) for 1.1 million people of the country (Mehta 2002; Saviour 2012).

In India the mining of coal is carried out on a large scale covering 80% of the the total mining activity for being the basic fuel for a majority of industries like steel, power plants, railways. The mining of zinc, copper, lead etc. account for the rest 20% of mining industry (ASA 2012).The extraction of the mineral from natural sources with the help of mining is an essential destructive development activity where ecology suffers at the altar of economy. By far the greatest impact of mining on the nations soil resources is due to open cast mining, which is having a very much potential for the deterioration of soil quality than underground operations and produce large amount of waste than the underground mines (Srivastava 1997).Underground mining usually causes little surface disturbance and rehabilitation is restricted to tailings dumps, removal of buildings and equipment, and making the area safe. (Saviour 2012)

Mining companies have to face the challenge of ensuring minimal impact on the environment but the current patterns of mineral usage raises concerns like recycling, and/or minimization of the generated waste, sustainable development. There are several areas of environmental concerns in Indian mining such as cyanide discharges, acid mine drainage, disposal of metal-containing wastes and organic-ladden effluents (Natarajan 2008). Zinc containing ore like sphalerite (ZnS) is hazardous sulfide minerals present in many of the nonferrous ore deposits and tailing dumps (Natarajan 2008). . By far the greatest impact of mining on the nations soil resources is due to open cast mining, which is having a very much potential for the

deterioration of soil quality than underground operations and produce large amount of waste than the underground mines. Underground mining usually causes little surface disturbance and rehabilitation is restricted to tailings dumps, removal of buildings and equipment, and making the area safe. (Saviour 2012).

Sewage and municipal solid waste

Municipal Solid Waste (MSW) is complex refuse consisting of various materials with different properties. Leachate resulting from this is hazardous pollutant and can lead to the contamination of soil and ground water underlying (Alhassan 2012). Sludge and municipal waste also contribute to increased levels of zinc in the soil. Zn^{2+} can arrive in agricultural soils with municipal sludge at once or by compost based on municipal sludge (Smaranda et. al 2005). The production of waste is an unavoidable consequence of our day to day activity. Huge quantities of waste piles can be seen lying along the roads, market place, rivers and other water sources leading to disturbance in the ecosystem.Indian cities which are fast competing with global economies in their drive for fast economic development have so-far failed to effectively manage the huge quantities of waste generated (Zia and Devadas 2007). The components of kitchen waste, ash, plastic and paper have high universality in municipal solid waste (MSW) and these four components are responsible for the majority of zinc contamination in the environment (Long et al2011)

Metals are found naturally in the soil and are required in trace amounts by plants and soil habituating organisms. Metals found in waste dumps exist in various forms either as the pure metal or alloyed with various other metals. The disposal of materials contaminated with heavy metals occurs with garbage dumps and with polluters dumping waste on the side of a road pose dangers to people in contact with the contaminated soils. The occurrence of zinc contamination of the soil around the solid waste dumping site was found in the Nigeria (Ideriah et al 2010).

Zinc and lead contamination is in the largest amounts in MSW-compost. Zinc in sewage sludge-treated agricultural soil has been identified as the main element of concern in relation to potential impacts on soil microbial activity (Smith, 2009).

2.4 Toxic effects of zinc

Zinc is required in trace amounts by the living organisms for the normal growth and development of the organs (Stefanidou et al 2006) Compared to several other metal ions with

similar chemical properties, zinc is relatively harmless (Haase et al 2010) Essential functions of zinc includes its role as cofactor in the activity of more than 300 enzymes such as alcohol dehydrogenases (EC 1.1.1.1), RNA polymerases (EC 2.7.7.6), Carbonic anhydrases (4.2.1.1) and DNA binding proteins such as zinc finger protein (Chou et al 1998; WHO 2004; ASTDR 2005).

However, the elevated levels of zinc are toxic to the living organisms and its accumulation in soil pose toxic effect to the human, animals and plants (Cameron 1992; Smaranda et al 2005). Adventitious zinc in water from contaminated wells and from galvanized cooking utensils could also lead to high zinc intakes (WHO 2004).Dietary reference values for zinc vary according to the dietary pattern of the country, assumptions on the bioavailability of dietary zinc, and age, sex and physiological status.

Table 2.2: Dietary reference intake of zinc for various ages (WHO 2001)

Category	Dietary reference value(mg/day)
Infants(0-12 month)	3.3 to 5.6
Children(1-10 years)	3.8 to 10.0
Adolescents(11-18 years)	8.7 to 15
Adult (19-50 years)	6.7 to 15
Females (during pregnancy)	7.3 to 15
Females (during lactation)	11.7 to 19

After ingestion of 4-8g (60-120mmol) of zinc the toxicity symptoms are nausea, vomiting, diarrhoea, fever and lethargy (WHO 2004).

Poisoning incidents with symptoms of gastrointestinal distress, nausea and diarrhoea have been reported after a single or short-term exposure to concentrations of zinc in water or beverages of1000–2500 mg/litre. Similar symptoms, occasionally leading to death, have been reported following the inadvertent intravenous administration of large doses of zinc.

Zinc fumes have corrosive effects on skin and could lead to damage in nervous membrane (Singh et al 2011).

Toxic effect of zinc is also induced because of the inhalation of zinc fumes present because of zinc chloride or zinc oxide present in bombs. Acute respiratory distress syndrome (ARDS) was observed in case of two soldiers upon exposure to a zinc chloride-containing smoke bomb (Reid et al 1992). Similar respiratory problem was also reported in other text (Zerahn et al 1999). People inhaling zinc chloride or zinc oxide present in bombs suffered effects that include interstitial oedema, interstitial fibrosis, pneumonitis, bronchial mucosal oedema, ulceration and even death under extreme exposure conditions in confined spaces (WHO 2001).

The most widely known effect of inhaling zinc-containing smoke is the so-called metal fume fever (MFF), which is mainly caused by inhalation of zinc oxide. This acute syndrome is an industrial disease which mostly occurs by inhalation of fresh metal fumes with a particle size less than 1μm in occupational situations such as zinc smelting or welding, cutting, or soldering with galvanized metal (Vogelmier et al 1987). Symptoms of this reversible syndrome begin generally a few hours after acute exposure and include fever, chills, thirst, muscle aches, nausea, fatigue, vomiting, gastrointestinal pain and respiratory effects like chest pain, cough, and dyspnea (American welding society 2002).

Whereas several other metals like cadmium, lead, arsenic are well-known carcinogens, zinc is not generally considered to be a causative agent for cancer development.In contrast, displacement of zinc from zinc-binding structures, e.g., finger structures in DNA repair enzymes, may even be a major mechanism for carcinogenicity of other metals (Beyersmann and Hartwig 2008). Men with moderate to higher zinc intake may have a lower risk for prostate cancer, but the opposite may be true at extremely high doses and long-term supplementation (Jarrard 2005).

Zinc chloride can produce significant lung and renal damage in rats when instilled directly into the lung; zinc oxide, by contrast, does not produce lung damage even when administered at relatively high concentrations (WHO 2001; Mizari et al 2012). Toxic effect of zinc on the neurotransmitter of male rats has also been documented and the levels of dopamine, norepinephrine, and epinephrine were reported to be altered in the male albino rat, *Rattus norvegicus* (Kumar et al 2010). Excessive exposure to zinc may produce toxic effects on various tissues and organs including the hematopoietic system, cytogenetics, biochemistry and endocrine system function (Piao et al 2003).

Several factors such as water hardness, salinity, temperature, and the presence of other contaminants influence zinc toxicity in aquatic environments (US department of interior 1998). This modification in zinc toxicity is the result of an effect on zinc availability and on sorption or binding of available zinc to biological tissues (BC EPD 1999). Acutely lethal concentrations for freshwater fish are in the range 0.066-2.6 mg/litre while the range for marine fish is 0.19-17.66 mg/litre (WHO 2001). Zinc concentrations exceeding 20 μg/litre have been shown to have adverse effects on aquatic organisms. Fish can collect zinc in their bodies from the water they swim in and from the food they eat (ASTDR 2005).

Zinc concentrations in liver increased during exposure to a high zinc diet on thecoho salmon (*Oncorhynchus kisutch*) and the U.S. federally threatened steelhead trout (*Oncorhynchus mykiss*). Iron concentrations in liver increased during simultaneous exposure to high zinc diet, and growth was reduced in this experimental treatment (Bowen et al 2006).

Streams in the Boulder River watershed in southwestern Montana receive drainage from abandoned mine adits and runoff from old tailings piles. Elevated concentrations of Zn along with Cd and Cu in the water column were associated with increased mortality of trout at sites located near mine waste sources. The hypertrophy (swelling), degeneration (dying), and necrosis of epithelial cells were observed in the gills supporting conclusion that the cause of death was related to metals in the water column (Farag et al 2003). Similar symptoms were reported when different levels of zinc (0, 200, and 400 ppb as Zn SO4) were treated in freshwater containing Atlantic salmon *Salmo salar*. The infection intensity was highest when 400 ppb zinc was amended (MacKinnon et al 2000).

Zinc is an essential trace element for growth and enzyme activities of microrganisms like bacteria. However, excess load of Zn shows toxicity and inhibition to microbial enzymes like aminopeptidases (Bong et al 2010) and by the use of various chemical compounds the environment has been negatively altered imposing threat on the organisms with shortest generation times like bacteria (Sevgi et al 2010). Microbial diversity is severely reduced by high level of zinc and only a very limited number of resistant bacteria can survive (Kelly et al 2003). The inhibition of microbial growth because of zinc could be dangerous as heavy metals can damage the cell membranes, alter enzymes specificity, disrupt cellular functions and damage the structure of the DNA (Rathanayake et al 2010; Zhou et al 2008). Figure 2.2 represents the various toxic effects of zinc on the microorganisms.

However bacteria like *Pseudomonas, Bacillus, Streptomyces*have been isolated from the metal contaminated sites. Table 4 represents the Minimum Inhibitory Concentrations of zinc to some of the bacteria.

The influences of Zn and Cu on soil enzyme activities and microbial biomass carbon were investigated in agricultural soils amended with municipal sewage sludge or compost since 1978. Due to long term application of sludge, soil enzyme activity was found to be adversely affected by zinc than copper and dehydrogenase, urease, and beta-D-glucosidase activities were reduced by the $KNO_3 + H_2O$ extractable fraction of Zn in the soils (Kunito et al 2001).

Legume plants are able to fix nitrogen in the soil through a symbiotic relationship with *Rhizobium* bacteria in their root nodules. Such crops (including beans, lentils, peas, and peanuts) are often used to replenish nitrogen levels in depleted soils. However, these bacteria are sensitive to zinc contamination, which can disrupt the nitrogen fixation process. Nitrogen, a key nutrient for plant growth, may then no longer be available to the plant or to the rest of the system (Shayler et al 2009). The effect of metal to inhibit the N_2-fixation by free living heterotrophic bacteria initiates when the soil metal concentration of zinc approaches about 127 mg/kg and N_2-fixation by free-living cyanobacteria was reduced by 50% at 114 mg/kg of Zn (McGrath et al 1995).

Table 2.3: Minimum Inhibitory Concentrations (MICs) of zinc metal on various bacteria isolated from different sources.

Bacteria	Source of isolation	MIC value(mM)	Reference
Pseudomonas	Soil	5	Sevgi et al 2010
Bacillus	Soil	3	Sevgi et al 2010
Pseudomonas	Soil	12	Bhojiya and Joshi 2012
Pseudomonas	Water	18	Jasmine et al 2012
Pseudomonas	Water	24	Malik and Jaiswal 2000; Ahemad and Malik 2012
Sphingomonas	Soil	35	Liu et al 2010
Streptomyces	Soil	35	Wei et al 2011

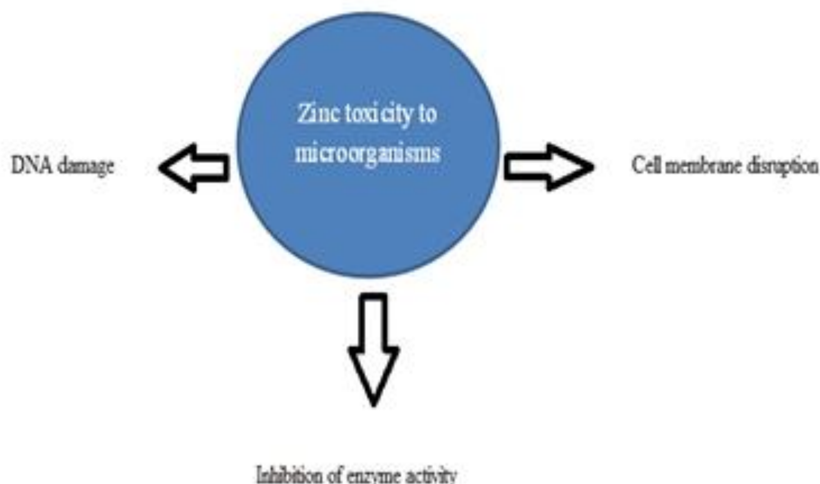


Figure 2.2 : Toxic effects of zinc on bacteria (Khan et al 2009; Zhou et al 2008)

2.5 Zinc resistance in bacteria - Mechanisms

Zinc actually, displays comparatively less toxicity to bacterial cells than other heavy metals and generally occurs in higher concentrations within bacterial cells. That is why bacteria in heavy metal polluted environment accumulate zinc by a fast but unspecific uptake mechanism (Nies 1999). Zinc, however at higher concentration is known to produce toxic effects on the microbial flora and hence there is a need for the existence of effective resistance mechanisms in bacteria to evade the detrimental effects of zinc and proliferate in zinc contaminated environment (Ahemad 2012).

Generally, uptake of zinc ions by bacterial cells is coupled with magnesium, and both ions may be transported by similar mechanism (Spain 2003; Nies and silver 1995). Zn^{2+} is accumulated by the fast and unspecific CorA(MIT) Mg^{2+} transport system in some bacterial species, and by the fast and unspecific MgtE system in others (Nies 1999).

A DNA fragment conferring resistance to zinc and cobalt ions was isolated from a genomic DNA of *Staphylococcus aureus* RN450. The DNA sequence analysis revealed two consecutive open reading frames, designated zntR and zntA. The predicted ZntR and ZntA showed significant homology to members of ArsR and cation diffusion families, respectively. However, the zinc uptake studies suggested that the *zntA* product was involved in the export

of zinc ions out of cells. The 2.9-kb *Eco*RI fragment containing *zntR* and *zntA* was cloned into vector pTZ18R. The resulting plasmid pTZ18R-ZC (5.8 kb) containing 2.9-kb *Eco*RI fragment conferred resistance to zinc and cobalt (Jayaswal and Xiong 1998).

Similar chromosomal gene *znt*A was isolated from *E.coli* K-12 and was found to be responsible for the ATPase that transports zinc and other cations across cell membrane (Beard et al 1997). Three types of Zn^{2+} export systems that protect cells from high toxic concentrations of Zn^{2+} have been identified:

- RND multi-drug efflux transporters,
- P-type ATPases, and
- Cation-diffusion facilitators (Nies 1999; Hantke 2001).

The mechanism involved in zinc efflux from high efficiency transenvelope RND-driven transporters like Czc transports zinc across the cell wall of gram-negative bacteria and is powered by a proton gradient and not ATP. Slow efflux of zinc is catalysed by cation-diffusion facilitator (CDF) transporters. P-type ATPases may transport zinc in both directions, bringing about its uptake as a by- product of Mg2+-uptake again, and its efflux as detoxification (Nies 1999). Besides providing resistance to Zn^{2+}, Cd^{2+} is also known to be eliminated by the gram positive bacteria by P-type ATPases (Nies and silver 1995).

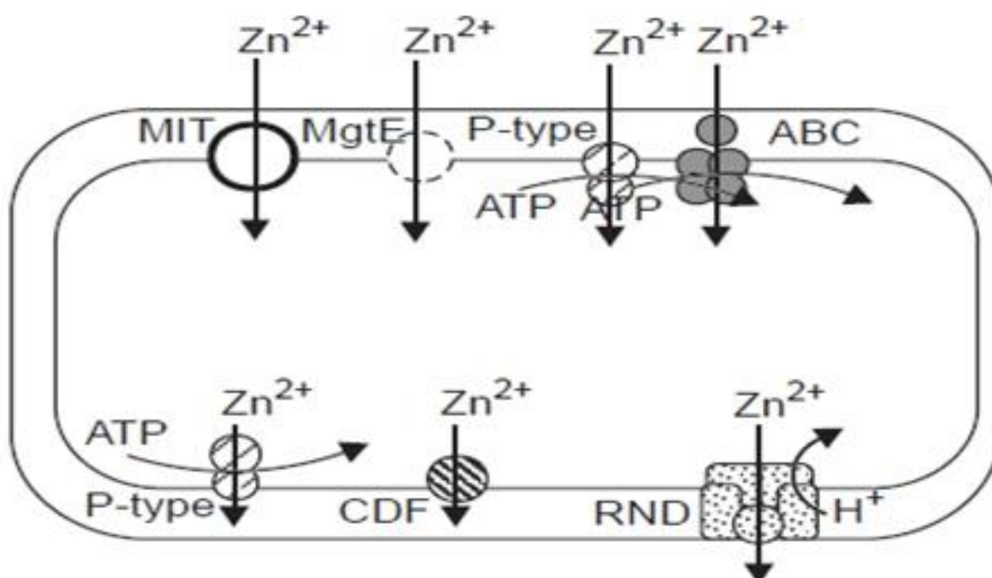


Figure 2.3: Different protein families involved in the transport of zinc in bacteria (Nies 1999)

Bacteria over the course of evolution have developed various mechanisms to thrive in adverse conditions of high acidity/alkalinity/toxicity and high temperature. They can develop biological resistance against any toxic substance in the environment due to special ‘jumping genes’ (Sinha et al 2010). Actually, the microbiological properties are more sensitive than the chemical and physical properties of soils to the presence of pollutants (Ramakrishna 2012).

Conventional technologies like reverse osmosis, ion exchange, adsorption, electrochemical treatment, ultrafiltration and chemical precipitation are commonly used procedures for removing metal ions from aqueous streams. However disadvantages like incomplete metal removal, high energy requirements, expensive cost, generation of toxic sludge or other waste products are associated with these technologies (Ramachandra et al 2003; Zouboulis et al 2004) and hence an eco-friendly alternative to these techniques is required. Moreover the issue with cost becomes even more expensive when the contaminant contents are in the range of 10-100 mg/l (Chaalal et al 2005).

The cost effective choice to the above enigma is decontamination through microbial functions. The microorganisms can use the chemical substances as sources of energy and nutrients in soils, which are an important sink for many pollutants (Ramakrishna 2012). Bioremediation using microorganisms is a sustainable solution to clean up the pollutants from the contaminated sites. Both plants and microbes play leading roles in bioremediation processes but compared to phytoremediation, microbial bioremediation is paid more attention and has wide applications since microorganisms are ubiquitous in the environment and they are able to function under various conditions, especially at really extreme environmental conditions where plants cannot survive (GuangshuZhai and Jincai 2012). Bacteria, waste fungal biomass derived from several industrial fermentations are considered the cost-effective and efficient sources of biosorptive materials (Ahemad 2012). Hence the removal of zinc or any other heavy metal from contaminated sites using microorganism could be an efficient approach to reduce and maintain the optimum levels of zinc in the environment.

3. MATERIALS AND METHODS

3.1 Zinc mines in Udaipur

Rajasthan is one of the leading zinc producing state of the country. Along with zinc, copper and lead deposits are also found in the state. The district of Udaipur and Bhilwara comprise the major mining districts of Rajasthan and are well known for their Lead-Zinc deposits and related mining and smelting industry. Zawar area of Udaipur district in Rajasthan has been recognized by the American Institute of Metallurgy as the first site where zinc was smelted.

3.2 Collection of soil sample

The soil sample was collected from the area near Zawar Lead-Zinc mine belt, Udaipur district, Rajasthan in sterile plastic bags and kept at 4^0C until further use. The Metalliferous Zawar area in Udaipur district is about 67 sq. km. and is situated between the latitudes $24^0 18^{'} 48^{''}$ and 24^0 $22^{'}48$” N, and longitudes 73^0 $40^{'}$ and 73^0 $45^{'}24$” E, at a distance of about 43 km to the south of main city of Udaipur and has an elevation of 377.7 m above the mean sea level.

3.3 Physiochemical analysis of soil

The soil sample was sieved through a 2 mm mesh sieve before the analysis. After sieving, properties like pH, electrical conductivity (EC), total dissolved solids (TDS), organic carbon content, available phosphorus content, total phosphorus content and total nitrogen content were determined.

3.3.1 Determination of pH

pH of soil sample was measured potentiometrically in a 1:5(w/v) soil-water suspension.

Procedure

1. 20g of air dried soil samples were weighed and taken in a 100 ml beaker.
2. Added 100 ml of distilled water and thoroughly stirred for 2-3 min using a glass rod.
3. Further, it was kept in shaking condition (120 rpm) for 30 minutes.
4. Suspension was allowed to settle down.
5. The connections of the analysis kit were switched ‘on’ and its knob was fixed on the pH parameter.

6. The pH of sample was measured by immersing the electrode in supernatant solution and recorded when the reading was stabilized (usually after 30 seconds).
7. The electrode was rinsed with distilled water and carefully wiped with tissue paper after use.

3.3.2 Determination of Electrical conductivity and Total dissolved solids

Electrical conductivity (EC) and Total dissolved solids (TDS) in soil sample was measured in a 1:5 soil-water suspension.

Procedure

1. 20 g of air dried soil samples were weighed and taken in a 100 ml beaker.
2. Added 100 ml of distilled water and thoroughly stirred for 2-3 min using a glass rod.
3. Further, it was kept in shaking condition (120 rpm) for 30 minutes.
4. The suspension was allowed to settle down.
5. The connections of the analysis kit were switched 'on' and its knob was fixed onto electrical conductivity/ total dissolved solids parameter.
6. Both the parameters were measured by immersing the electrode in supernatant solution and recorded when the reading was stabilized.
7. The electrode was rinsed with distilled water and carefully wiped with tissue paper after use.

3.3.3 Available phosphorus (P)

Reagents for the estimation of available phosphorus

1. **0.5M $NaHCO_3$ extracting solution -** 84g of sodium bicarbonate was added in distilled water and volume was made upto 2 litre. The pH was adjusted to 8.5 with 1M or 1N NaOH
2. **Reagent A -** 12.0g ammonium molybdate in 250ml distilled water and 0.2908g antimony potassium tartarate in 100ml distilled water was added to 1000ml of 2.5M H_2SO_4 ,mixed thoroughly and volume was made upto 2 litre with distilled water
3. **Reagent B (freshly prepared) -** 1.058 g of ascorbic acid was added in 200ml of reagent A and mixed
4. **Sulphuric acid (2.5M) -** 140 ml of conc. H_2SO_4 was diluted to 1 litre
5. **Stock standard P solution (50 ppm) -** 0.2917 KH_2PO_4 was dissolved in distilled water to a final volume of 1 litre
6. **Working standard P solution(1 ppm) -** 20ml of 50 ppm solution was diluted to 1 litre

Procedure for estimation of available phosphorus (Olsen *et al*, 1954)

1. 2.5g soil was weighed and 50 ml of extracting solution was added to it.
2. Kept on a shaker for 30 minutes and was filtered through whatman filter paper no. 42 3. 10ml aliquot of filterate was transferred to a 100ml beaker.
4. 1ml of 2.5M H_2SO_4, 15.5ml distilled water, 8ml reagent B and again 15.5ml of distilled water was added.
5. After 10 minutes, the intensity of the colour was measured at 882 nm against blank
6. Blank was prepared as above without the soil.
7. To prepare standard curve, 0, 2, 5,10, 15 and 20 ml of 50 ppm standard stock solution was measured in 50 ml volumetric flask separately and followed the steps as above.
8. The Phosphorus concentrations of these solutions were 0.04, 0.1, 0.2, 0.3 and 0.4 ppm respectively. After 10 min read the P concentration at 882 nm.

Calculation

Available P in soil (ppm): P in extract (ppm) × 20 (standard soil to solution ratio)

3.3.4 Total phosphorus (P)

Reagents for the estimation of total phosphorus

Vanadomolybdate solution -

1. **Solution A -**25 g ammonium molybdate [$(NH_4)_6 Mo_7O_{24}.4H_2O$] was dissolved in 300 ml water in a 500 ml beaker
2. **Solution B -**1.25 g ammonium vanadate (NH_4VO_3) was dissolved in 300 ml boiling water, cooled and 250 ml concentrated HNO_3 was added and cooled again. Solution A was added to solution B and was made upto 1000ml in a volumetric flask.
3. **Phosphorus stock standard solution (50 mg/l) -**0.2195 g of dried KH_2PO_4 was dissolved in distilled water and mixed thoroughly. Acidified with 25 ml of 7N H_2SO_4 and made the volume up to 1litre to get 50 mg/ml P solution. 4 to 5 drops of toluene was added to prevent microbial activity.

Sample preparation for elemental analysis

For the release of mineral elements from soil and sediments, di acid (HNO_3- $HClO_4$) oxidation of sample was carried out.

HNO_3/ $HCLO_4$ digestion

1. 1 g sample of air dried soil was weighed in digestion tube and added 10 ml concentrated HNO_3 digest on electric heater for 1hr at 145°C in acid proof digestion chamber having fume exhaust system.
2. Allowed to cool it and 5 ml $HClO_4$ was added and heated to about 100°C for the first one and then raised the temperature to about 200°C.
3. Continued the digestion until the contents become colourless and only white fumes appeared.
4. Reduced the acid contents till white matter remains left in the digestion tube.
5. After this removed from the heating mental and cooled and 50% diluted HCl was added and filtered through whatman filter paper no. 42.
6. 2 or 3 washings with 50% diluted HCl were given and final volume made was 50 ml.

Procedure for the estimation of total phosphorus in soil and plant samples (Kitson and Mellon, 1944)

Ammonium molybdate reacts under acidic conditions to form a heteropoly acid and molybdophosphoric acid. In the presence of vanadium, yellow vanadomolybdate acid is formed. The intensity of colour is propotional to phosphorus concentration.

1. 10 ml of acid digests of soil sample was placed in 50ml volumetric flask, 10 ml of the vanadate molybdate reagent was added and diluted to 50 ml
2. Mixed well and read the phosphorus concentration after 10 minutes using spectrophotometer at 420 nm.
3. Blank was prepared by taking 10 ml of distilled water in place of 10 ml of acid digests of soil sample
4. For standard readings, 0, 1, 2, 3, 4 and 5 ml of 100 mg per litre stock phosphorus solution was taken in 50 ml volumetric flask and the colour was developed as mentioned above
5. Calibrated the spectrophotometer with known phosphorus concentration and read the concentration of the sample.

Calculation*P (mg/kg):*

$$\frac{Volume\ make\ up\ after\ acid\ digestion}{Weight\ of\ sample\ (s)\ to\ develop\ colour\ (ml)} \times \frac{50}{volume\ of\ digest\ used} \times P\ (mg)\ in\ 50\ ml\ solution$$

3.3.5 Organic carbon

Reagents for the estimation of organic carbon

1. **1N potassium dichromate -** 49.04 g was added in distilled water and volume was made upto 1 litre
2. **0.5N ferrous ammonium sulphate -** 198 g was added in distilled water and volume was made upto 1 litre
3. **Diphenyl amine indicator -** 0.5 g of diphenyl amine indicator (DPA) was dissolved in a mixture of 200ml water and 100ml concentrated H_2SO_4

Procedure for estimation of organic carbon and organic matter (Walkley and Black, 1934**)**

1. 1 g of soil was taken in 500 ml conical flask and 10 ml of 1 N $K_2Cr_2O_7$ was added.
2. The flask was swirled for mixing the soil and reagent.
3. 20 ml of concentrated H_2SO_4 was added and the flask was allowed to stand undisturbed for 30 minutes after which 200ml of distilled water was added
4. 1 ml of diphenylamine indicator was then added
5. Ultimately the contents were titrated with freshly prepared 0.5N ferrous ammonium sulphate till the end point is observed from blue violet to green.
6. Run a blank without soil sample and followed the steps as above

Calculation

Organic carbon (%): $\frac{10(B - T) \times 0.003 \times 100}{B \times weight\ of\ soil\ (g)}$

Where

B is volume of ferrous ammonium sulphate solution for blank titration T is volume of ferrous ammonium sulphate solution for soil sample Because organic matter contains 58% carbon, so

Organic matter (%): Organic carbon (%) × 17.24 (van bemmelen factor)

3.3.6 Total nitrogen

Reagents for the estimation of total nitrogen in soil

1. **Concentrated H_2SO_4**
2. **0.02 N H_2SO_4**
3. **Sulphuric salicylic acid -** 1g salicylic acid was mixed with 30 ml sulphuric acid

4. **Sodium thiosulphate**
5. **4% boric acid -** 4g of boric acid was dissolved in 100 ml of distilled water
6. **Mixed indicator -** 0.066 g of methyl red and 0.099 g of bromo cresol green was dissolved in 100 ml of ethyl alcohol
7. **50 % NaOH**
8. **Digestion mixture -** 10 g HgO , 5 g $CuSO_4$ and 100 g K_2SO_4 (2:1:20)

Procedure for estimation of total nitrogen in soil (Kjeldahl method given by Piper, 1960)

1. 5 g soil was mixed thoroughly with sulphuric salicylic acid and followed by 5 g of sodium thiosulphate. Heating was carried out for 5minutes followed by cooling and addition of 10 g of digestion mixture. The contents were mixed well in a kjeldahl flask
2. The flask was kept in a digestion chamber at 100°C for two hours
3. The colour change was monitored from dark brown to greenish white after which the contents were cooled and 300 ml distilled water was added.
4. 20 ml of the digested sample, 15-20 ml NaOH and glass beads were added to the distillation flasks through the open end of the condenser attachment and stoppered. Water flow was maintained through the condenser.
5. The distillate was collected through a receiver tube in a beaker containing 15 ml boric acid and 2 drops of mixed indicator was added till the end point colour changes from pink to green.
6. The distillate was titrated against 0.02 N H_2SO_4 until the colour changed from green to pink.

Calculation

$$\text{Total N (\%)}: \frac{(T - B) \times \text{normality of } H_2SO_4 \times 14 \times 300}{\text{Weight of sample } (g)}$$

T is titre value for sample

B is for blank

3.4 Isolation of the zinc resistant bacteria from soil sample

1 gm of soil sample was amended in 50 ml each of Minimal broth media (MB) for 3 days at 37^0C; 120 rpm. Standard serial dilutions upto 10^{-6} were made for the isolation of preliminary bacterial population from two different flasks as per following procedure:-

1. 14 different test tubes were filled with 9 ml of distilled water and autoclaved. These were then differentiated into two different sets of 7 test tubes each.
2. 1 ml aliquot from Minimal broth media was then transferred to the first test tube labelled as 10^{-1} and vortexed.
3. Subsequent transfer of 1 ml suspension from each test tube to the next dilution was done in the same manner upto 10^{-7} for both the sets of test tubes.
4. 100 µl of the suspension from each test tube was spreaded onto Minimal agar medium (5.6pH) plates containing 6mM of zinc as $ZnSO_4.7H_2O$.

5. Plates were incubated at 37^0C for 48-72 hours.

3.4.1 Screening for the most efficient zinc resistant bacteria

The different isolated bacteria which could grow in the presence of 6mM of zinc were then screened for their resistance at higher level of zinc. The selected isolates were streaked on higher concentration of zinc (10 mM, 12 mM, 15 mM, 18 mM, 20 mM, 25 mM, 28 mM, 30 mM and 40 mM) and the selection of the bacteria to be further studied upon was based on these results.

3.4.2 Purification of the most efficient zinc resistant bacteria

The isolated species able to grow in the presence of the highest concentration of zinc was purified on the nutrient agar slants by streaking method which were stored at 4^0C until further use.

3.5 Co resistance of the selected isolate towards lead

The selected strain was also tested for its co resistance to lead at different concentration (15mM, 20mM, 25mM, and 30mM) provided in form of $(PbNO_3)_2$.

Biochemical tests for the characterization of selected bacteria

Bacteria are identified routinely on the basis of various morphological and biochemical tests. Various biochemical as well as morphological features of the selected bacteria was studied.

3.6.1 Colony morphology

Various aspects of colony morphology like form, elevation, surface, consistency and margin were observed. Gram staining of a bacterial culture is a very important preliminary step in the initial characterization and classification of bacteria.Gram staining characteristics of the selected strain was observed according to the standard procedure.

3.6.2 Gram staining characteristics

Gram staining of the bacteria was done with the help of Himedia K001-1KT gram staining kit. To study the gram character of the selected strain, smears from 2-5 days of diluted suspensions of the bacteria were fixed on clean slides.The slides were flooded with crystal violet solution for one minute, washed with water and flooded with Gram's iodine for one minute. The slide were washed with water and decolorized with 95% ethyl alcohol with the help of dropper until no violet colour was visible from drain off solution. The slides were washed with water and counter stained with safranin stain for about 30 second and washed with water. The slides were air dried and examined under a microscope using 100x objectives using a daylight filter. Cells were then identified by the colour observed purple for Gram positive and pink or red for Gram negative cells.

3.6.3 Indole production test

Requirements: Broth culture of test organism, Tubes containing 1% tryptone broth, Kovac's reagent, Inoculating loop, Dropper bottle/1 ml pipette.

Procedure:-

1. Prepared 1% tryptone broth by dissolving 10g of peptone in one litre of distilled water. Sterilized in the autoclave at 15 psi and 121^0C for 15 minutes.
2. Inoculated one tube with the test organism, second with *E.coli* and other one uninoculated as control.
3. The tubes were then incubated at 37^0C for 48 hours.
4. Added 1 ml of kovac's reagent to each tube and shake them after intervals for 10-15 minutes
5. Allowed the tubes to stand to permit the reagent to come to the top.
6. Development of cherry red colour in the top layer is detected by adding Kovac's reagent (dimethylaminobenzaldehyde) which produces a cherry red reagent layeron the top of medium in the tube and indicates a positive test while the absence of this colour indicates negative test.

3.6.4 Methyl-Red and Voges-Proskauer (MRVP) tests

Requirements: Broth culture of test organism, MRVP broth tubes, Methyl red pH indicator, V-P reagent 1 (Napthol solution), V-P reagent 2 (40% Pottasium hydroxide), Inoculating loop.

Procedure:-

1. Prepared MRVP broth (pH 6.9) tubes and sterilized in the autoclave at 15 psi and 121^0C for 15 minutes.
2. Inoculated one tube with the test organism, second with *E.coli* and other one uninoculated as control.
3. The tubes were then incubated at 37^0C for 48 hours.
4. For methyl red test, added 5 drops of methyl red indicator to the each set of the tube.
5. For Voges-proskauer test added 12 drops of V-P reagent I and 2-3 drops of V-P reagent II.
6. Shook the test tubes gently for 30 seconds with caps off to expose the media to oxygen.
7. Allowed the VP reaction to complete for 15 minutes.
8. The formation of red color in methyl red test indicates positive result while turning of methyl red to yellow is a negative test.
9. The development of crimson to ruby pink colour is a result of production of organic acid from glucose after adding methl red and hence indicates a positive test while no change in colour indicates a negative result for VP test.

3.6.5 Citrate utilization test

Requirements: Culture of test organism, Simmon's citrate agar slants, Inoculating loop, test tubes.

Procedure:-

1. Prepared Simmon's citrate agar slants (pH 6.9) in tubes and sterilized in the autoclave at 15 psi and 121^0C for 15 minutes.
2. Inoculated one tube with the test organism, second with *E. aerogens* and other one uninoculated as control.
3. The tubes were then incubated at 37^0C for 48 hours.
4. Change in the medium from green to blue is a result of metabolism of citric acid present in the medium, producing CO_2 which further reacts with sodium and water to form

sodium carbonate an alkaline product which changes the colour of the medium indicating positive result while no change in colour is negative result.

3.6.6 Hydrogen Sulphide production test

Requirements: Culture of test organism, SIM (Sulphide indole motility) medium, inoculating loop, test tubes.

Procedure:-

1. Prepared SIM medium (pH 7.3) in tubes and sterilized in the autoclave at 15 psi and 121^0C for 15 minutes.
2. Stab inoculated the test organism into the medium.
3. The tubes were then incubated at 37^0C for 48 hours.
4. Blackening of the culture medium occurs when ferrous sulphate reacts with hydrogen suphide indicating a positive result while no change in the medium represents a negative result.

3.6.7 Motility test

Requirements: Culture of test organism, motility test medium, triphenyltetrazolium chloride (TTC), inoculating loop, test tubes.

Procedure:-

1. Motility test medium (pH 7.3) was prepared in the test tubes and sterilized in the autoclave at 15 psi and 121^0C for 15 minutes.
2. Stab inoculated the test organism into the medium.
3. The tubes were then incubated at 37^0C for 48 hours.
4. Growth of the culture dispersed away from the line of inoculation indicates positive test which is due to the reduction of TTC creating a diffuse red color while growth confined only to the line of inoculation indicates a negative test.

3.6.8 Nitrate reduction test

Requirements: Culture of test organism, sulfanilic acid, α-naphthylamine, Nitrate broth, inoculating loop, test tubes.

Procedure:-

1. Nitrate broth medium (pH 7.0) was prepared in the test tubes and sterilized in the autoclave at 15 psi and 121^{0}C for 15 minutes.
2. Inoculated the test organism into the medium upon cooling.
3. The tubes were then incubated at 37^{0}C for 48 hours.
4. Added drop full of sulfanilic acid and α-naphthylamine to the test tubes.
5. Formation of red colouroccurs when sulfanilic acid is added which reacts with the nitrous acid to produce diazotized sulfanilic acid. This furthur reacts with α-naphthylamine to form a red-colored compound indicating a positive test while absence of formation of red colour indicates a negative test.

3.6.9 Starch hydrolysis (amylase) test

Requirements: Culture of test organism, Starch agar medium, Gram's iodine solution, Inoculating loop.

Procedure:-

1. Starch agar media was prepared and sterilized in the autoclave at 15 psi and 121^{0}C for 15 minutes.
2. Streaked the test organism on the plates of starch agar media.
3. The plates were then incubated at 37^{0}C for 48 hours.
4. Added gram's iodine solution with the help of dropper onto the plates.
5. Formation of clear zone around the growth of bacteria after flooding the agar's surface with gram's iodine due to the production of amylase enzyme indicates a positive result while no clear zone formation indicates a negative result. Iodine complexes with starch to produce a clear zone of hydrolysis.

3.6.10 Cellulase production test

Requirements: Culture of test organism, Cellulose yeast peptone agar medium, Inoculating loop, 1M NaCl, 1% congo red.(15 min)

Procedure:-

1. Cellulose yeast peptone agar media was prepared and sterilized in the autoclave at 15 psi and 121^{0}C for 15 minutes.

2. Streaked the test organism on the plates of cellulose yeast peptone agar media.
3. The plates were then incubated at 37^0C for 48 hours.
4. Added 1% congo red solution for 15 minutes in the plate followed by addition of 1M NaCl solution.
5. Formation of clear zone around bacterial growth due to the reaction of congored with carboxymethylcellulose(CMC) present in the medium shows cellulase production and thus a positive resultwhile no clear zone formation indicates negative test.

3.6.11 Casein (Caseinase) hydrolysis test

Requirements: Culture of test organism, Skim milk agar medium, inoculating loop.

Procedure:-

1. Skim milk agar media was prepared and sterilized in the autoclave at 15 psi and 121^0C for 15 minutes.
2. Streaked the test organism on the plates of skim milk agar media.
3. The plates were then incubated at 37^0C for 24-48 hours.
4. Formation of clear zone around bacterial growth due to hydrolysis of casein shows positive result while no clear zone formation indicates negative test.

3.6.12 Gelatin hydrolysis (gelatinase) test

Requirements: Culture of test organism, Gelatin agar medium, inoculating loop, refrigerator, Mercuric chloride solution.

Procedure:-

1. Gelatin agar media was prepared and sterilized in the autoclave at 15 psi and 121^0C for 15 minutes.
2. Streaked the test organism on the plates of gelatin agar media.
3. The plates were then incubated at 37^0C for 48-72 hours.
4. After incubation, the plates were kept at 4^0C for 15 minutes.
5. Flooded the incubated agar plates with mercuric chloride solution and allowed the plate to stand for 5-10 minutes.
6. The liquefication of agar media even after refrigeration indicates a positive test while the solidification of media indicates a negative test.

3.6.13 Urease production test

Requirements: Culture of test organism, Urea agar medium, inoculating loop.

Procedure:-

1. Urea agar media was prepared by sterilizing all the components except urea in the autoclave at 15 psi and 121^0C for 15 minutes while urea in the media was added after filter sterilization.
2. Streaked the test organism on the plates of Urea agar media after solidification.
3. The plates were then incubated at 37^0C for 48 hours.
4. Pink colour appearance of media is positive test for the production of urease which degrades the urea present in the medium and because of the increase in pH while no change in colour is negative result.

3.6.14 Phosphatase test

Requirements: Culture of test organism, Pikovskaya agar medium, inoculating loop.

Procedure:-

1. Pikovskaya agar media was prepared by sterilizing in autoclave at 15 psi and 121^0C for 15 minutes.
2. Streaked the test organism on the plates of Pikovskaya agar.
3. The plates were then incubated at 37^0C for 48 hours.
4. Formation of clear zone around bacterial growth due to the degradation of Phosphate present in the medium shows positive result while no clear zone formation indicates negative test.

3.6.15 Carbohydrate fermentation test

The selected isolate was tested for the fermentative degradation of various carbohydrates like sucrose, fructose, dextrose, lactose, mannitol, arabinose, cellobiose, ribose, xylose, galactose, mannose, raffinose, rhamnose were tested.

Requirements: Bacterial culture, Durham tubes, phenol red, carbohydrate source, test tubes, inoculating loop.

Procedure:-

1. Carbohydrate fermentation medium (pH 7.3) was prepared in the test tubes by sterilizing in autoclave at 15 psi and 121^0C for 15 minutes.
2. Inoculated the test organism into different test tubes containing different carbohydrate sources.
3. Incubated the test tubes at 37^0C for 72 hours.

4. Change in the colour of medium from red to yellow indicates acid production by the respective bacteria and formation of gas bubble at the top of durham tube suggests gas production by the bacteria.

3.7 Physiological studies of the selected zinc resistant bacteria

3.7.1 Temperature profile of the isolated strain

The growth of the selected strain at different temperatures was checked (4^0C, 25^0C, 28^0C, 37^0C) according to following procedure:-

1. The culture was grown in minimal broth and kept on shaking at 37^0C; 120 rpm for 72 hours.
2. A loopful of this broth was taken and streaked onto 10 different minimal agar media plates amended with 20mM of zinc.
3. The plates were then incubated at different temperatures for 96 hours and hence optimum temperature profile on solid media was determined.

3.7.2 NaCl level tolerance profile of the isolated strain

The salt tolerance level of the selected strain was checked on YPG (Yeast extract, peptone, glucose) media supplemented with different concentrations (%w/v) of sodium chloride according to following procedure:-

1. The culture was grown in minimal broth and kept on shaking at 37^0C; 120 rpm for 72 hours.
2. 100 µl aliquot from this flask was transferred into 5 different test tubes containing 20 ml of YPG media in each test tube and amended with 2%, 4%, 6%, 8% and 10% NaCl.
3. The tubes were then incubated at 37^0C for 96 hours.

3.7.3 Production of diffusible pigment and growth of the isolated strain on different media:-

The selected isolate was screened for the determination of substrate mycelium colour and production of diffusible pigment, on various media likeActinomycete isolation agar, Starchcasein agar, ISP1 media(Tryptone-yeast extract agar), ISP2 media (Yeast extract-Malt extract agar), ISP4 media (Inorganic salts-starch agar), ISP5 media (Glycerol-asparagine agar) and ISP7 media (Tyrosine agar).

3.7.4 Production of acid in liquid medium by selected strain:-

One of the mechanism in which bacteria shows resistance towards heavy metals is the production of organic acids which could chelate the metal ions thus enabling the bacteria to survive in high concentrations of heavy metals. (Hall 2002) The production of organic acids by the isolate after amendment of different concentration of zinc in liquid media was checked by the reduction in pH which was taken after 5 and 10 days of incubation at 37^0C and 120 rpm. The following procedure was adopted:-

1. 100 ml of minimal broth media was prepared in 5 different flasks and pH was adjusted at 5.6 prior to autoclaving.
2. After sterilization of the minimal media, different concentrations of zinc (0mM, 5mM, 10mM, 15mM, 20mM, and 25mM) were amended into different flasks.
3. 100µl of the aliquot from a previous flask into which no zinc had been amended was taken and transferred into each flask.
4. Incubation of these flasks was done at 37^0C; 120 rpm.
5. Reduction in pH was measured after 5 days and 10 days of incubation.

3.7.5 Estimation of Biomass production and Zinc uptake analysis of selected strain by Atomic absorption spectroscopy (AAS)

The estimation of biomass and zinc uptake was done according to following procedure:-

1) Pure culture from one of the plates was inoculated in a flask containing 10mM of zinc and was incubated at 37^0C; 120 rpm for 24 hours to obtain a uniform turbidity.
2) 100µl aliquot from the overnight incubated flask was transferred to 5 different flasks containing 100 ml of minimal medium broth having5mM, 10mM, 15mM, 20mM, and 25mM of zinc.
3) The cultures were allowed to grow for 10 days at 37^0C; 120 rpm.
4) Cultures were centrifuged at 8000 rpm for 10 minutes and the resulting pellets were washed thrice with 25mM Tris EDTA buffer (pH 8.0)
5) The biomass was estimated by drying the obtained pellets to a constant weight at 100^0C and biomass produced in mg/ml was calculated.
6) Acid digestion of the obtained biomass was carried out and obtained filtrate was then used for the Atomic absorption spectroscopy analysis.

4. RESULTS AND DISCUSSION

4.1 Physico-chemical properties of the soil sample

Various physico-chemical properties of the soil sample like organic carbon content, Total Phosphorus content, Available Phosphorus content and Total nitrogen content was determined by methods previously described. The results of the physiochemical properties of soil samples are given in Table 4.1.

Microorganisms have limits of tolerance for particular environmental conditions, as well as optimal conditions for pinnacle performance. Factors that affect success and rate of microbial biodegradation are nutrient availability, moisture content, pH, and temperature of the soil matrix. Inorganic nutrients including nitrogen and phosphorus are necessary for microbial activity and cell growth.

Table 4.1: Physiochemical properties of soil sample

Soil sample	pH	EC	TDS	Organic carbon (%)	Total nitrogen (%)	Available P (mg kg^{-1})	Total P(mg kg^{-1})
Zinc mine soil	8.06±0.04	1.46±0.04	0.92±0.025	1.131±0.09	0.06±0.02	1.80±0.003	105.27±0.004

Values are Mean ± SD (*n*=3).

Importance of Organic carbon content: Accumulation of zinc in resistant or tolerant species may lead to toxicity in organisms present higher in the food chain. The main process that occurs in the soil is the organic matter decomposition. The bacterial population needs organic carbon to grow and proliferate in such contaminated environment. The organic carbon content (%) in the zinc mine soil was found out to be 1.131±0.09.

4.2 Total microbial count in soil sample

Serial dilution technique for the isolation of zinc resistant bacteria was used to and after incubating the plates for 48-96 hours cfu/gm at different zinc concentration was calculated (Table 4.2). A significant decrease in the cfu/gm was observed as the concentration of zinc was raised. Initially, at 6 mM concentration of zinc the total bacterial count was highest. The decrease in the cfu/gm is assisted by the findings of Abbas and Edwards 1989 who studied the effects of zinc metal on a range of *Steptomyces* species and found that distance of growth inhibition increased as higher concentrations of zinc were amended in growth medium.

Table 4.2: Total bacterial count in terms of CFU/g of soil sample (after incubation at 37^0C for 96 hours)

Zinc concentration	CFU/gm
6 mM	4.6×10^3
12 mM	3.1×10^3
18 mM	5×10^2

4.3 Screening of efficient zinc resistant bacteria

Initially 7 different isolates with different colony morphology exhibited resistance towards zinc (6 mM). These were designated as ZRB 1, ZRB 2, ZRB 3, ZRB 4, ZRB 5, ZRB 6 and ZRB 7. These isolates were then tested for resistance at higher concentration of zinc (upto 18mM) and visible growth was observed upto 25mM of zinc in solid media. The bacteria reporting highest resistance was ZRB 7 which could tolerate upto 25 mM of zinc in solid media (Table 4.3).

Table 4.3 Resistance of various isolates (ZRB 1-ZRB 7) towards different concentrations of zinc

Strain	Concentration of zinc		
	12 mM	18 mM	25 mM
ZRB1	+	+	-
ZRB2	+	+	-
ZRB3	-	-	-
ZRB4	-	-	-
ZRB5	-	-	-
ZRB6	-	-	-
ZRB7	+	+	+

High levels of zinc resistance has been found in *Streptomyces zinciresistens* which has been isolated recently (Wei et al. 2011) from the soil of a copper and zinc mine in China. This bacterium could tolerate up to 35 mM of zinc. In a similar study (Ahemad and Malik 2012) heavy metal tolerant *Pseudomonas* species from waste water was isolated which could tolerate upto 24mM of zinc.

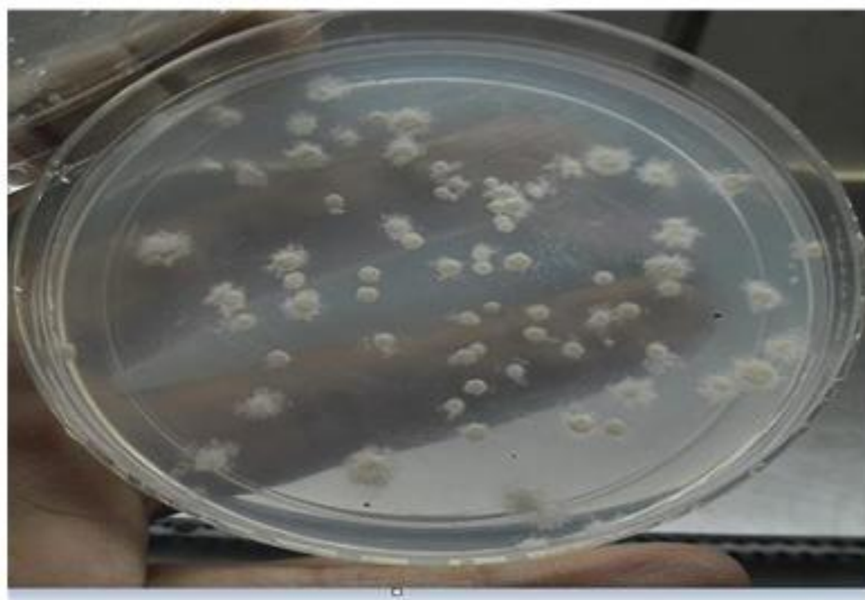


Figure 4.1: ZRB 7 strain showing growth on Minimal agar media amended with 25 mM Zinc.

4.4 Co resistance of the selected isolate towards lead

Strain ZRB 7 showed resistance against lead along with zinc. The maximum tolerable concentration for lead on solid media was found out to be 28 mM. Table no. 4.4 shows the level of resistance of ZRB 7 towards different concentration of lead. Resistance towards multiple heavy metals has been described by *Streptomyces* sp that showed co resistance against chromium, cadmium and lead (Kannabiran and Deepika 2010). Zinc and lead resistant population was isolated from Otamiri river of Nigeria by Mgbemena et al. 2012. In another study heavy metal resistance towards lead, zinc and cadmium in bacteria was isolated from the industrial wastewater samples of Iran.(Nasrazadani et al 2010).

Table 4.4 Represents growth of ZRB7 at different lead concentrations

S.No.	Lead concentration	Growth of ZRB7
1	5 mm	+
2	10 mm	+
3	15 mm	+
4	20 mm	+
5	25 mm	+
6	28 mm	+
7	30 mm	-

Based on above findings strain ZRB 7 was further purified and various morphological and biochemical tests were carried out for its characterization.

4.5 Biochemical characterization of ZRB 7

4.5.1 Morphological characteristics of strain ZRB 7

The morphological characteristic of the selected strain was observed on the agar surface and various attributes like colony colour and the elevation on the agar surface were studied. Table 4.5 shows the various morphological aspects of ZRB 7 showing white-yellowish colonies on the agar surface; forming entire to irregular margin. The strain ZRB 7 showed cottony or velvety growth and was completely opaque. Same morphological characteristics of an unknown bacteria were reported by Gupta et al 2009 which was later on identified as a morphotype of *Streptomyces* species.

Table 4.5: Morphological characteristics of strain ZRB 7

Colony morphology	Results
Colour of colony	Whitish
Form	Filamentous
Elevation	Umbonate
Edges/margin	Entire to irregular
Surface	Smooth
Consistency/texture	Velvety/cottony/Sticking into agar medium
Opacity	Opaque

4.5.2 Gram staining characteristics of ZRB 7.

Gram staining of ZRB 7 showed gram positive nature and long hyphae in the form of straight and rectiflexible structures and bear spiral or rod like spores at its end.

Table 4.6: Staining characteristics of strain ZRB 7

Gram character	+ ive (long, branching, rectiflexible hyphae which differentiate into rod shaped spores at the end of hyphae
Length of the spore chain(100X magnification)	3-10
Spore bearing hyphae morphology	Rectiflexible

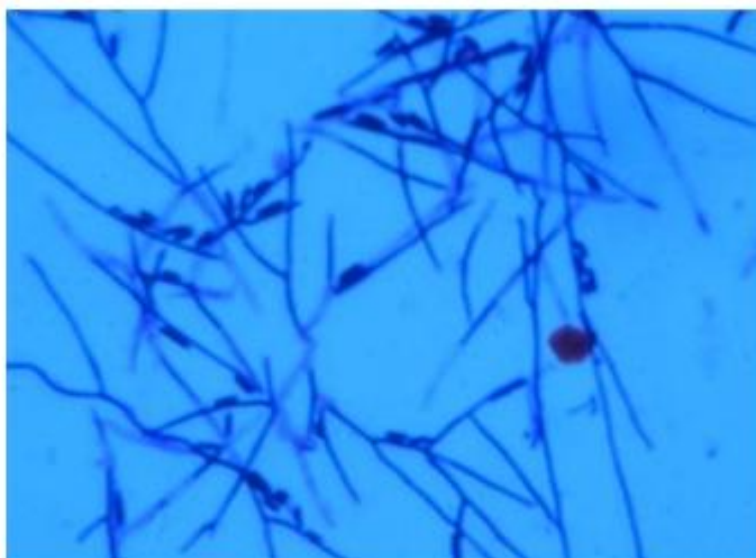


Figure 4.2: Gram staining of ZRB 7 showing highly branched rectiflexible hyphae and spores

Based on the morphological and gram staining characteristics, the strain ZRB 7 was closely related to actinomycetes species as the formation of hyphae and long branching structure is rarely reported to be ever shown by a bacteria. The results of the gram staining were closely related to the findings of Wei et al 2011 in which one of the strain showed rectiflexible to spiral spore chain morphology. Result of the gram staining also showed resemblance with the work of Panbangred et al who reported similar spore chain morphology and the length of spores per chain was also 3-10.

Various biochemical tests were performed for the identification of ZRB 7. The biochemical data provides a more efficient basis of characterizing an unknown organism and it consists of about 10-15 different tests. Table 4.7 indicates the various biochemical tests that were performed for the identification of ZRB 7.

The results of the biochemical tests performed were closely related to the findings of Wei et al 2011, Al-Kadeeb et al 2012, Reddy et al 2011 and Rao et al 2012. The result showing similarity to their findings were namely gelatin liquefication, non ability to produce H_2S gas, reduction of nitrate, ability to degrade urea and starch , negative reaction of MRVP and Indole test, non motile character of the spores, utilization of citrate. Table 4.7 presents the results of various biochemical tests.

Table 4.7: Biochemical characteristics of strain ZRB 7

Test	Results
Indole Production	(-ive)
Citrate utilization	(+ive)
Lactose utilization	(+ive)
Methyl Red Test	(-ive)
Voges Proskauer Test	(-ive)
Urea hydrolysis	(+ive)
Starch hydrolysis	(+ive)
Casein hydrolysis	(+ive)
Gelatin liquification	Weakly(+ive)
Nitrate reduction	(+ive)
H_2S production	(-ive)
Motility Test	Non-motile
Cellulase activity	(+ive)
Phosphatase activity	(-ive)

4.6 Carbohydrate source utilization test

Various sugars like glucose, mannitol, xylose, arabinose, etc were provided as sole carbon source and production of acid, gas and growth was recorded. It was found that D-rhamnose, D-arabinose, D-mannitol and D-fructose were least utilized. Modest growth in case of D-raffinose, D-galactose, D-mannose, D-cellobiose, D-xylose, D-galactose and L-rhamnose was seen and acid was also produced by the bacteria when these carbon sources were utilized. The results obtained were comparable with the results obtained by Wei et al 2011 who reported zinc resistant actinomycete *Streptomyces zinciresistens*.

Table 4.8: Utilization of different carbohydrates by strain ZRB 7

Sugar	Growth	Acid production	Gas production
D-glucose	+++	W	(-ive)
D-lactose	++	(-ive)	(-ive)
D-sucrose	++	(-ive)	(-ive)
D-fructose	+	(-ive)	(-ive)
D-mannitol	+	(-ive)	(-ive)
D-raffinose	++	(+ive)	(-ive)
L-rhamnose	++	(+ive)	(-ive)
D-xylose	++	(+ive)	(-ive)
D-galactose	++	(+ive)	(-ive)
D-mannose	++	(+ive)	(-ive)
D- cellobiose	++	(+ive)	(-ive)
D- arabinose	+	(-ive)	(-ive)
D- rhamnose	+	(-ive)	(-ive)

+++: good; ++: moderate; +: low; W: weakly positive

4.7 NaCl (w/v) tolerance level of ZRB 7

Good growth was observed upto 6% NaCl concentration and growth decreased after this concentration. The results so obtained were similar to the findings of Al-Kadeeb et al 2012.

Table 4.9: Growth of ZRB 7 under different concentrations of Sodium chloride

Concentration of NaCl %(w/v)	Growth
2	+++
4	+++
6	+++
8	++
10	-

+++: good, ++: moderate, -: absent

4.8 Growth at different temperature

Temperature profile of the strain ZRB 7 was optimized at different temperatures and Table 12 suggests that the optimum growth range of the ZRB 7 strain was 28^0C - 37^0C.

Table 4.10: Growth of ZRB 7 under different temperatures

Temperature	Growth
4^0C	-
25^0C	++
28^0C	+++
37^0C	+++
45^0C	-

+++: good, ++: moderate, +: low; -: absent

4.8 Growth characteristic on different media :

After having identified the ZRB 7 strain as an actinomycete species, growth on various media designed particularly for the *Streptomyces* species was evaluated for the detection of any diffusible pigment produced and to determine the color of substrate mycelium. Diffusible pigment was not produced on any of the media. The color of substrate mycelium produced was mainly pale yellow. However, Yellow brownish color on tyrosine agar was produced.

Table 4.9: Growth of ZRB 7 on various media

Name of media	Color of colony	Color of substrate mycelium	Diffusible pigment
ISP-1[a]	White	Pale yellow	No
ISP-2[b]	Whitish-yellow	Pale yellow	No
ISP-4[c]	Yellow-brownish	Pale yellow	No
ISP-5[d]	White	White-yellowish	No
ISP-7[e]	Yellow- brownish boundaries	Yellow brownish	No
Starch casein nitrate agar	Bright yellow	Pale yellow	No
Actinomycete isolation agar	Yellow	White-yellowish	No

a: Tryptone-yeast extract agar
b: Yeast extract-malt extract agar
c: Inorganic salts-starch agar
d: Glycerol-asparagine agar
e: Tyrosine agar

4.9 Assessment of pH reduction by ZRB 7.

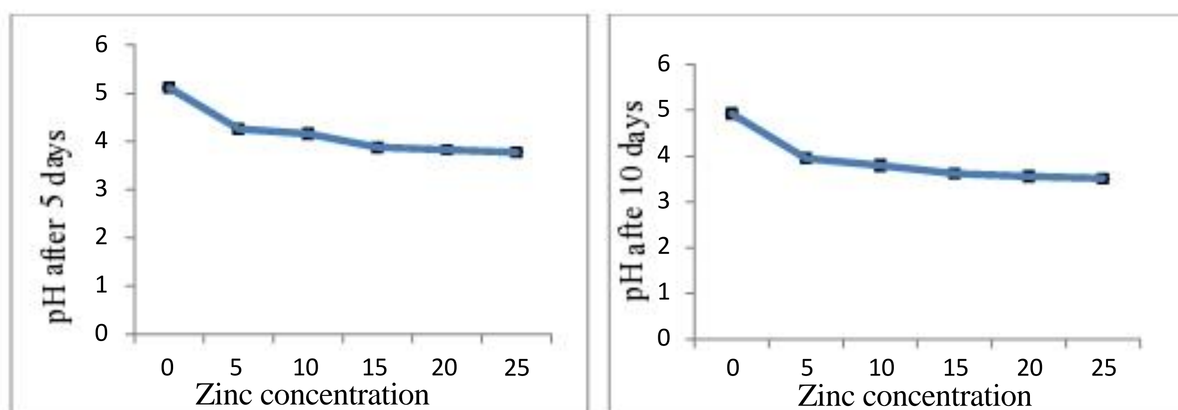
Production of organic acid in response to different concentrations of zinc was assessed by inoculating the organism into Minimal medium broth amended with different concentrations of zinc and then subsequent reduction in the pH of the medium was observed after 5 days and 10 days of incubation shaking. Hall 2002 describes the reduction in pH or production of an organic acid as a mechanism to tolerate and proliferate in the presence of heavy metals and Table 4.10 provides the reduction data in the pH of the media and it was found that reduction in the pH was maximum at 25 mM concentration of zinc and reduction in the pH was observed even when no zinc was amended in the media.

Table 4.10 Reduction in pH of medium after 5 and 10 days at different concentrations of zinc

Concentration of zinc (mM)	Initial pH	Final pH mean ± SD	
		After 5 days	After 10 days
0	5.60	5.12 ± 0.02	4.92 ± 0.04
5	5.60	4.26 ± 0.03	3.95 ± 0.03
10	5.60	4.16 ± 0.04	3.79 ± 0.04
15	5.60	3.88 ± 0.03	3.62±0.02
20	5.60	3.83 ± 0.01	3.55 ± 0.03
25	5.60	3.78 ± 0.02	3.51 ± 0.02

Values are Mean ± SD (*n*=3).

Figure 4.3 graph showing reduction in pH of medium after 5 and 10 days at different concentrations of zinc



4.9 Biomass estimation produced by ZRB 7

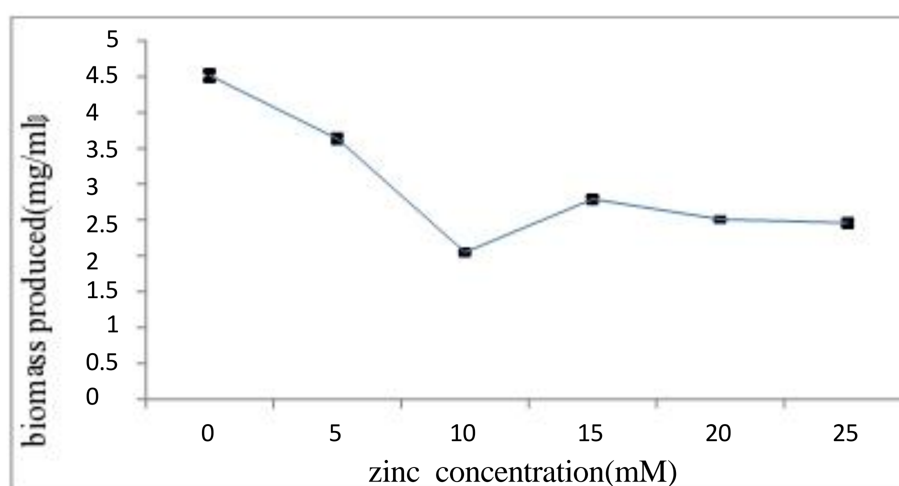
Biomass production by the strain ZRB 7 was calculated by inoculating the organism in liquid medium. The pellets obtained were dried in an hot air oven till a constant weight was gained and biomass produced was calculated in mg/ml. The biomass produced was maximum when no zinc was amended in the medium providing the evidence of metal toxicity on the ZRB 7. The biomass production was interestingly larger in the presence of 15 mM zinc than 10 mM and then kept on decreasing with the higher concentrations of zinc.

Table 4.11 Biomass produced at different concentrations of zinc

Concentration of zinc (mM)	Biomass produced (mg/ml) Mean±SD
0	4.51±0.10
5	3.63±0.07
10	2.05±0.03
15	2.79±0.05
20	2.51±0.02
25	2.46±0.06

Values are Mean ± SD (*n*=3).

Figure 4.4 Graph showing biomass productions at different zinc concentration



4.10 Zn uptake studies by strain ZRB 7 with Atomic absorption spectroscopy

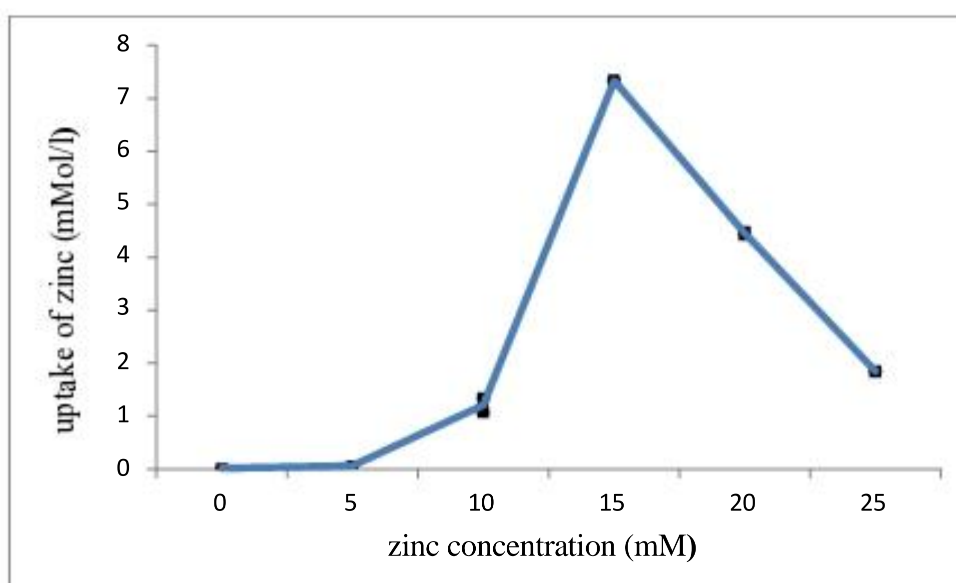
Atomic absorption spectroscopy helps in determining the zinc uptake by the biomass which is produced by the bacteria. The zinc uptake was determined at six different concentrations of zinc and the correlation of biomass produced in case of 15 mM zinc concentration was found to be highest suggesting that 15 mM is the optimum concentration of zinc which could be utilized by the *Streptomyces* species.

Table 4.12 Uptake of zinc by ZRB 7 at different concentration of Zinc

Concentration of zinc (mM)	Uptake of zinc (mmol/l) Mean ± SD
0	0.013±0.01
5	0.07±0.04
10	1.05±0.06
15	7.32±0.15
20	4.47±0.10
25	1.88±0.09

Values are Mean ± SD (*n*=3).

Figure 4.5 Graph showing uptake of Zinc at different zinc concentration



5. CONCLUSION

Heavy metals such as zinc are toxic to a variety of organisms and pose various environmental threats. Conventional methods to detoxify zinc are often costly and have high energy requirements. Microorganisms however have developed various mechanisms that lead to resistance against zinc. Actinomycetes are known to be present in stressful conditions and hence resist the effect of toxicity of zinc. Various biochemical test confirmed the isolated species ZRB 7 to be *streptomyces* species, which have been earlier reported in various articles to exhibit the property of resistance against heavy metals. The species showed maximum tolerance to 25 mM of zinc on solid media and 28 mM on liquid media. Moreover it showed coresistance to lead which is another toxic heavy metal and could grow in the higher presence of lead than zinc on solid media and hence the identified *streptomyces* species is a good future prospect for bioremediation of heavy metal contamination in soil.

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