P-3410

BONAFIDE CERTIFICATE

ANNA UNIVERSITY: COIMBATORE

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The report of the project work submitted by the above students in partial fulfillment for the reward of Bachelor of degree in Biotechnology of Anna University, Coimbatore was confirmed to be the report of the work done by the above students and then evaluated.

(INTERNAL EXAMINER) (EXTERNAL EXAMINER)

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ABSTRACT

The present study deals with isolation characterization of metal tolerant microorganisms from tannery effluent samples. Copper, Chromium, Nickel, Zinc and Lead were the heavy metals used for initial isolation of metal tolerant bacteria.One bacterial strain (VLB-9) which showed good tolerance against heavy metals esp Lead. Based on the biochemical tests such as catalase test, mannitol fermentation and morphological characteristics, the bacterial was identified to be *Staphylococcus aureus*. *Staphylococcus aureus* can be used for the removal of lead from the tannery effluent. Minimum Inhibitory Concentration (MIC) studies were indicated that the growth of the bacterial strain was inhibited from 1500 mg/l metal concentration. The isolated bacterial strains were capable of removing 99.70% of lead from the medium containing 100 mg of metal per litre. The various physical parameters such as pH, Temperature and Initial concentration were optimized. The bacterial isolate was also resistant to antibiotics such as Chloramphenical, Streptomycin and Tetracycline.

Key words- *Staphylococcus aureus*, Heavy metal tolerance, Lead, Antibiotic resistance and MIC.

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LIST OF ABBREVIATIONS

APIS	Air Pollution Information System
g/cm3	Gram/cubic centimeter
g/l	Gram/Litre
mg/lit	Micrograms/Litre
µg	Microgram
mg	Milligram
µl	Microlitre
mM	Millimolar
°C	Degree Celsius
ppm	Parts per million
NA	Nutrient Agar
PDA	Potato dextrose agar
LB	Luria bertani
Pb	Plumbum(Lead)
nm	Nanometer
hr	Hour

INTRODUCTION

CHAPTER-1
INTRODUCTION

Environmental pollution is one of the major problems of the modern world. On one hand industrialization is necessary to satisfy the needs of the world's overgrowing population but on the other hand, it threatens life on earth by polluting the environment. The problem of environmental pollution is increasing day by day due to the release of xenobiotic substances into water, soil and air. These substances include organic compounds and heavy metal ions. The damage caused by these pollutants to plants, animals and humans cannot be neglected and hence strategies must be developed to solve the problem of environmental pollution on priority basis.

Heavy metals are a general collective term which applies to the group of metals and metalloids with an atomic density greater than 4 g/cm^3. Although it is a loosely defined term (Duffus, 2002), it is widely recognized and usually applies to the widespread contaminants of terrestrial and freshwater ecosystems.

1.1 Metal pollution

Heavy metal pollution can arise from many sources but most commonly arises from the purification of metals, e.g., the smelting of copper and the preparation of nuclear fuels. Electroplating is the primary source of chromium and cadmium. Through precipitation of their compounds or by ion exchange into soils and mud, heavy metal pollutants can localize and lay dormant. The heavy metals which are included in APIS(Air Pollution Information System) are cadmium, chromium, copper, mercury, lead, zinc, arsenic, boron and the platinum group metals, which comprises Platinum, Palladium, Rhodium, Ruthenium, Osmium, and Iridium. Unlike almost all organic pollutants, such as organochlorines, heavy metals are elements which occur naturally in the Earth's crust. They are therefore found naturally in soils and rocks with a subsequent range of natural background concentrations in soils, sediments, waters and organisms. Heavy metals are

toxic agents and their interaction with the organisms and the environment is the subject of the discipline known as *Ecotoxicology.*

One of the largest problems associated with the persistence of heavy metals is the potential for bioaccumulation and biomagnification causing heavier exposure for some organisms than is present in the environment alone. As they are elements, they cannot be broken down, will persist in the environment. Unlike many organic pollutants, which eventually degrade to carbon dioxide and water, heavy metals will tend to accumulate in the environment, especially in lake, estuarine or marine sediments. Metals can be transported from one environment compartment to another.

1.2 Lead

Lead is a main-group element with symbol Pb (Latin: *plumbum*) and atomic number 82. Lead is a soft, malleable poor metal. It is also counted as one of the heavy metals. Metallic lead has a bluish-white color after being freshly cut, but it soon tarnishes to a dull grayish color when exposed to air. Lead has a shiny chrome-silver luster when it is melted into a liquid. Lead has the highest atomic number of all of the stable elements. Lead can be found or produced in many isotopes, with four of them being stable. The four stable isotopes of lead are ^{204}Pb, ^{206}Pb, ^{207}Pb, and ^{208}Pb with ^{204}Pb regarded as completely primordial lead, and 206, 207, 208 being formed probably from the radioactive decay of two isotopes of uranium (U-235 and U-238) and one isotope of thorium (Th 232).The one common radiogenic isotope of lead, ^{202}Pb, has a half life of about 53,000 years.

Lead is bright and silvery when freshly cut but the surface rapidly tarnishes in air to produce the commonly observed dull luster normally associated with lead. It is a dense, ductile, very soft, highly malleable, bluish-white metal that has poor electrical conductivity when compared to most other metals. This metal is highly resistant to corrosion, and because of this property, it is used to contain corrosive liquids (for example, sulfuric acid). Because lead is very malleable and resistant to corrosion it is extensively used in building construction – for example in the external coverings of roofing joints. Metallic lead can be toughened by addition of small amounts of antimony,

or of a small number of other metals such as calcium. All isotopes of lead, except for lead-204, can be found in the end products of the radioactive decay of the even heavier elements, uranium and thorium. Powdered lead burns in a flame. The flame is bluish-white. As with many metals, finely divided powdered lead exhibits pyrophoricity. Toxic fumes are released when lead is burnt. Lead is used in building construction, lead-acid batteries, bullets and shots, weights, as part of solders, pewters, fusible alloys and as a radiation shield.

1.2.1 History of Lead

During the 20th century, the use of lead in paint pigments was sharply reduced because of the danger of lead poisoning, especially to children. By the mid-1980s, a significant shift in lead end-use patterns had taken place. Much of this shift was a result of the U.S. lead consumers' compliance with environmental regulations that significantly reduced or eliminated the use of lead in non-battery products, including gasoline, paints, solders, and water systems. Lead use is being further curtailed by the European Union's RoHS directive. Lead may still be found in harmful quantities in stoneware, vinyl (such as that used for tubing and the insulation of electrical cords), and brass manufactured in China. Between 2006 and 2007 many children's toys made in China were recalled, primarily due to lead in paint used to color the product. Older houses may still contain substantial amounts of lead paint. White lead paint has been withdrawn from sale in industrialized countries, but the yellow lead chromate is still in use. Old paint should not be stripped by sanding, as this produces inhalable dust. Lead salts used in pottery glazes have on occasion caused poisoning. It is rapidly absorbed into the bloodstream and is believed to have adverse effects on the central nervous system, the cardiovascular system, kidneys, and the immune system.

1.3 Health effects

Lead is a poisonous metal that can damage nervous connections (especially in young children) and cause blood and brain disorders. Lead poisoning typically results from ingestion of food or water contaminated with lead; but may also occur after accidental ingestion of contaminated soil, dust, or lead based paint. Long-term exposure to lead or its salts (especially soluble salts or the strong oxidant PbO_2) can cause nephropathy, and colic-like abdominal pains. The effects of lead are the same whether it enters the body through breathing or swallowing. Lead can affect almost every organ and system in the body. The main target for lead toxicity is the nervous system, both in adults and children. Long-term exposure of adults can result in decreased performance in some tests that measure functions of the nervous system. It may also cause weakness in fingers, wrists, or ankles. Lead exposure also causes small increases in blood pressure, particularly in middle-aged and older people and can cause anemia. Exposure to high lead levels can severely damage the brain and kidneys in adults or children and ultimately cause death. In pregnant women, high levels of exposure to lead may cause miscarriage. Chronic, high-level exposures have shown to reduce fertility in males.

The concern about lead's role in cognitive deficits in children has brought about widespread reduction in its use (lead exposure has been linked to learning disabilities) Most cases of adult elevated blood lead levels are workplace-related. High blood levels are associated with delayed puberty in girls. Lead has been shown many times to permanently reduce the cognitive capacity of children at extremely low levels of exposure. There appears to be no detectable lower limit below which lead has no effect on cognition. Lead can also be found listed as a criteria pollutant in the United States Clean Air Act section 108. Lead that is emitted into the atmosphere can be inhaled, or it can be ingested after it settles out of the air.

LITERATURE REVIEW

CHAPTER-2

LITERATURE REVIEW

Environmental pollution occurs by deterioration of natural equilibrium of environment via various human activities. Nowadays, environmental pollution is the most important problem for all world countries. Pollution existed since the beginning of industrialization and grew by the parallel of rapidly increasing industrialization after Second World War. Precautions were taken after 1970s for preventing and reducing this pollution.

Biotechnology finds application fields in the treatment of wastewaters by biological methods and disposal of solid wastes by composting technique in environmental engineering. Biological methods are also applied to treatment of air emissions. The methods based on biotechnology in wastewater treatment are activated sludge, trickling filters, oxidation ponds, biofilters and anaerobic treatment, Furthermore, solid waste composting techniques, biotrickling filters and biosorption are the examples of biotechnology applications in environmental engineering.

In all these methods, it is essential to find suitable microorganisms that will degrade organic substances and to complete the treatment process in favorable conditions (Hanife Buyukgungor, 2009)

2.1 Metals in Ecosystem

Heavy metals are major pollutants in marine, ground, industrial and even treated wastewater (Valdman *et al.*, 2001).Toxic heavy metals are found in effluents and discharged waste water of industries like electroplating, steel, alloy, motor vehicles, aircraft, paint, chemical, textile, pigment etc.(Sani, 2001 and Saithong and Prasertsan, 2002).Metals are directly or indirectly involve in all aspects of growth, metabolism and differentiation (Beveridge and Doyle, 1989).

Raw wastewater contains significant concentration of heavy metals that are not degraded by the conventional process of wastewater treatment. The main source of heavy metals is the industrial activities such as metal processing, mining and electroplating, tanning, carpet washing and dyeing. Presence of high concentration of

toxic heavy metals in wastewater directly lead to both contamination of receiving water bodies and deleterious impact on aquatic life (Moten and Rehman, 1998).

Heavy metal releases to the environment have been increasing continuously as a result of industrial activities and technological developments, posing a significant threat to the environment and public health because of their toxicity, accumulation in the food chain and persistence in nature. It is therefore important to develop new methods for metal removal from dilute solutions and for the reduction of heavy metal ions to very low concentrations. The use of conventional treatment technologies such as ion exchange, chemical precipitation, reverse osmosis and evaporative recovery is often inefficient and/or very expensive (Chong and Volesky, 1995; Leusch *et al.*, 1995).
The most commonly occurring metals at these sites are lead, chromium, arsenic, zinc, cadmium, copper, and mercury. Presence of these metals in groundwater and soils may cause a significant threat to human health and ecological systems (Evanko and Dzombak, 1997).

Lead, a major pollutant that is found in soil, water and air is a hazardous waste and is highly toxic to humans, animals, plants and microbes (Low and Lee , 2000)

Most heavy metals are essential for microbial growth, but some (Au, Ag, Cd, Pb, Hg and Al) have no essential biological function, and even can be extremely toxic for living cells, thereby making their removal by living biomass extremely difficult. In this situation, dead biomass or derived products can avoid the problem of toxicity, and relieve the economic burden of nutritive supplements and the maintenance of cultivation (Rivera-Utrilla.J *et al.*, 2002)

Traces of these heavy metals are necessary as Co-factors of enzymatic reactions, but high levels of them may cause extreme toxicity to living organisms due to inhibition of metabolic reactions. The micro organisms respond to these heavy metals by several

processes; including transport across the cell membrane, biosorption to the cell walls and entrapment in extracellular capsules, precipitation, complexation and oxidation-reduction reactions.(Virender singh *et al.*, 2010)

2.2 Mechanism of accumulation

Microbes are capable of accumulating toxic metal ions by two well defined processes viz:(1) biosorption; an energy-independent binding of metal ions to cell walls and (2) bioaccumulation: energy dependent process of metal uptake into the cells. Both live and inactivated microbial mass of bacteria, fungi and algae are utilized for removing toxic metal ions (Volesky, 1994; Karna *et al.*, 1996; Li *et al.*, 2004)

Bacteria that are resistant to and grow on metals play an important role in biogeochemical cycling of those metal ions (Gadd, 1990).

The heavy metal resistant microorganisms have significant role in wastewater treatment system. The detoxifying ability of these resistant microorganisms can be manipulated for bioremediation of heavy metals in wastewater. Effluents having heavy metals can be treated with these microorganisms by the processes like biosorption, bioaccumulation and bioprecipitation

2.3 Microbial removal of metal

Metal ions uptake by biosorption is complex and may involve the contribution of diffusion, adsorption, chelation, complexation, coordination or microprecipitation mechanisms, depending on the specific biomass or substrate (Veglio *et al.*, 1997). Therefore this biological phenomenon can be affected by many chemical and physical variables such as pH, ionic strength, biomass concentration and presence of different heavy metals in solution. All these factors have to be investigated in order to understand how this phenomenon takes place and to optimize operating conditions (Mulaba-Bafubiandi *et al.*, 2009).

Among the microorganisms, bacteria, yeast and protozoa are generally the first category to be exposed to heavy metals present in the environment. Microorganisms have acquired a variety of mechanisms for adaptation to the presence of toxic heavy metals. Among the various adaptation mechanisms, metal sorption, mineralization, uptake and accumulation, extra cellular precipitation and enzymatic oxidation or reduction to a less toxic form and efflux of heavy metals from the cell has been reported (Rajbanshi, 2008)

2.4 Biosorption

The alternative use of microbe-based biosorbents for the removal and recovery of toxic metals from industrial effluents can be an economical and effective method for metal removal. The metal-removing ability of microorganisms including bacteria, microalgae and fungi has been studied extensively. Their capacity for heavy metal removal is apparently higher than for conventional methods and the uptake of heavy metals can be selective. Microbial cells can also be supplied inexpensively as waste from industrial fermentation processes as well as biologic wastewater treatment plants. (WA C. Leung *et al.*, 2001).

Staphylococcus, *Bacillus*, *Pseudomonas*, *Citrobacteia*, *Klebsilla*, and *Rhodococcus* are organisms that are commonly used in bioremediation mechanisms (Connor *et al.*, 1996).

MATERIALS AND METHODS

CHAPTER-3
MATERIALS AND METHODS

3.1 Sample collection

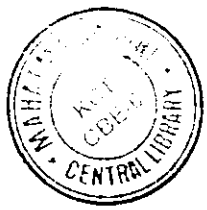
The tannery effluent samples were collected from Ezar Tanneries,Vaniyambadi.The untreated samples were used for the isolation of Bacterial and Fungal colonies.

3.2 Isolation of bacterial and fungal colonies from the collected effluent samples

The bacterial and fungal colonies were isolated from the effluent samples collected from the tannery. These were then studied for their ability to show heavy metal tolerance.

3.2.1 Isolation of bacterial colonies from the effluent samples

1. The effluent samples were serially diluted to a dilution factor of 10^{-5} with sterile distilled water. About 1gm of the sludge sample was dissolved in 10 ml of sterile distilled water and is then serially diluted to a dilution factor of 10^{-5}.
2. The Nutrient Agar (HiMedia) plates were then prepared.
3. 100 µl of the diluted effluent sample is added to the centre of the Nutrient Agar plates and using the L-rod spread the sample to the whole surface of the Agar plate.
4. The plates were then incubated at 37 °C for 24 hours.
5. A mixture of bacterial colonies was obtained on these plates after incubation. From these distinct colonies were chosen and were then pure cultured through streak plate method.
6. The pure cultured bacterial colonies were then maintained as slant for further studies.



3.2.2 Isolation of fungal colonies from the effluent samples

1. The effluent samples were serially diluted to a dilution factor of 10^{-4} with sterile distilled water. About 1gm of the sludge sample was dissolved in 10 ml of sterile distilled water and is then serially diluted to a dilution factor of 10^{-4}.
2. The Potato dextrose Agar (HiMedia) plates were then prepared.
3. 100 µl of the diluted effluent sample is added to the centre of the potato dextrose agar plates and using the L-rod spread the sample to the whole surface of the Agar plate.
4. The plates were then incubated at 30 °C for 48 hours.
5. A mixture of fungal colonies was obtained on these plates after incubation. From these distinct colonies were chosen and were then pure cultured through streak plate method.
6. The pure cultured fungal colonies were then maintained in broth for further studies.

3.3 Screening for metal resistant bacterial strains

1. The isolated bacterial strains were inoculated in test tubes containing 5 ml of nutrient broth medium.
2. The individual isolated strains were screened with different metals (copper, chromium, nickel, zinc and lead) in 100 mg/L concentration.
3. Test tubes were incubated at 37 °C.
4. Samples were taken at different time intervals (0^{th}, 12^{th}, 24^{th} hours) for finding the cell density at 600 nm in spectrophotometer.

3.4 Screening for metal resistant fungal strains

1. The isolated bacterial strains were inoculated in test tubes containing 5 ml of potato dextrose broth medium.
2. The individual isolated strains were screened with different metals (copper, chromium, nickel, zinc and lead) in 100 mg/L concentration.
3. Test tubes were incubated at 30°C.

4. Samples were taken at different time intervals (0^{th}, 12^{th}, 24th hours) for finding the cell density at 600 nm in spectrophotometer.

3.5 Screening for lead-resistant isolates

The numbers of lead-resistant organisms were determined by plating the isolated cultures onto minimal medium agar.
The pH of the medium was 6.4.Lead was added as Pb $(NO_3)_2$ at a concentration of 1 mM. (Roan, 1998)

3.6 Growth curve of Bacteria with metal Induction

In 100 ml of LB broth 100 µl of culture was inoculated (control).In 100ml of Luria broth 100 µl of culture was inoculated and 100mg/lit Pb $(NO_3)_2$ was added (test).Culture was incubated at 37°C on shaker and growth was monitored as a function of biomass by measuring the absorbance at 600 nm against blank.(Qurat-ul-Ain Affan *et al.*,2009).

3.7 Determination of Antibiotic sensitivity and resistance

Antibiotic sensitivity of the lead resistant bacteria was determined by the disc diffusion method. Antibiotic-impregnated discs (6mm diameter, HiMedia) were placed on LB agar plates spread with culture and incubated at 37 °C for 24 hours. The diameter of the inhibition zones around the discs was measured. The antibiotic concentrations of the disc used were Ampicillin (10 µg),Bacitracin (0.05 µg),Chloramphenicol (50 µg),Penicillin (10 µg),Streptomycin (10 µg) and Tetracycline (10 µg) respectively.(Edward Raja, *et al.*,2006)

3.8 Determination of MIC

Maximum resistance of the bacteria against increasing concentrations of lead on LB broth was evaluated until the strains unable to grow in the broth. The bacterial growth was measured as cell density using spectrophotometer. The initial concentration used (100 ppm) was added from 100 mg/100ml of stock solution. Concentration of metal up to 3000 mg/L were taken. The stock solution of Pb $(NO_3)_2$ (Ranbaxy) was prepared in distilled water and sterilized by autoclaving at 121 °C, 15 psi for 15 min.(Edward Raja,C *et al.*,2006)

3.9 Identification of the Bacterial strain

The bacterium was identified using the biochemical characterization process involving 3 different tests as follows: Gram staining, Catalase test and Mannitol fermentation. (Claus and Berkeley,1986).

3.10 Biosorption of lead by *Staphylococcus aureus*

Removal of lead by *Staphylococcus aureus*

The isolated Staphylococcus aureus was used for further removal studies by treating with different concentrations of lead. The physical parameters such as pH, and temperature for the bacteria were optimized.

$$\% \text{ REMOVAL} = \frac{\text{initial concentration} - \text{final concentration}}{\text{Initial concentration}} \times 100$$

3.10.1 Effect of Temperature on biosorption of lead by *Staphylococcus aureus*

The effect of temperature on the growth of bacteria in the presence of lead was analyzed at three different temperatures 30 °C, 37 °C and 45 °C.

3.10.2 Effect of pH on biosorption of lead by *Staphylococcus aureus*

The effect of pH on the growth of bacteria in the presence of lead was analyzed at five different pH 4.5, 5.5, 6.5, 7.5 and 8.5 .

3.10.3 Effect of Concentration on biosorption of lead by *Staphylococcus aureus*

The effect of concentration on the growth of bacteria was studied. The bacterium was allowed to grow in both an orbital shaker and incubator. Then the effect of shaking and static condition on bacterial growth was compared and an optimum condition for bacterial growth was determined.

RESULTS AND DISCUSSION

CHAPTER-4
RESULTS AND DISCUSSION

The bacterial and fungal strains were isolated from tannery effluent sample and the isolated strains were treated with metal for the identification of metal tolerance. The metal tolerant microbial strains were isolated based on their growth in the presence of different metal ions including copper, chromium, lead, nickel, and zinc. The microbial strains isolated from the contaminated site can easily adapt to the adverse environment and thus can be used for metal removal studies.

4.1. Isolation of microbial strains from Vaniyambadi Leather Industry

The microbial colonies were isolated by pour plate method. Eleven bacterial and five fungal strains were purified using streak plate method .The colonies were named based on the source from which it was isolated

Table 4.1 Isolation of microbial strains from Vaniyambadi Leather Industry

S.No	MICRO - ORGANISM	DILULTION	DILUTON FACTOR	N0.OF COLONIES		MEAN(CFU/ml)
1.	FUNGI (VLF)	R1	10^{-3}	116	40	78×10^{-3}
2.		R2	10^{-4}	44	40	42×10^{-4}
3.	BACTERIA (VLB)	R1	10^{-5}	24	12	18×10^{-5}
4.		R2	10^{-6}	32	12	22×10^{-6}

VLB-Vaniyambadi Leather industry Bacteria

VLF-Vaniyambadi Leather industry Fungi

CFU-Colony forming unit

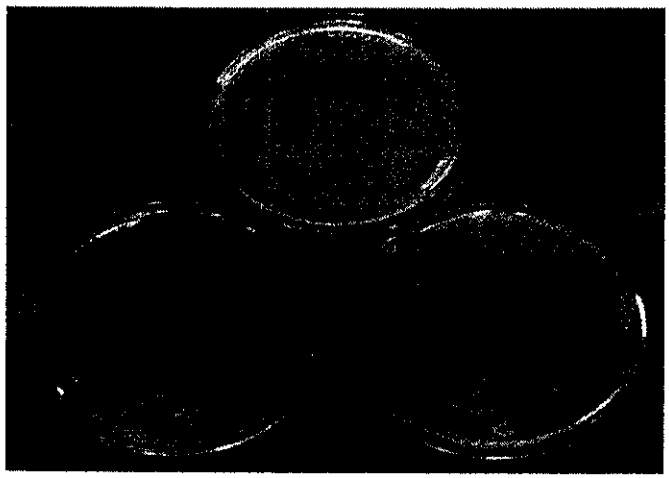


Fig 4.1.1 Isolated bacterial colonies from the effluent sample

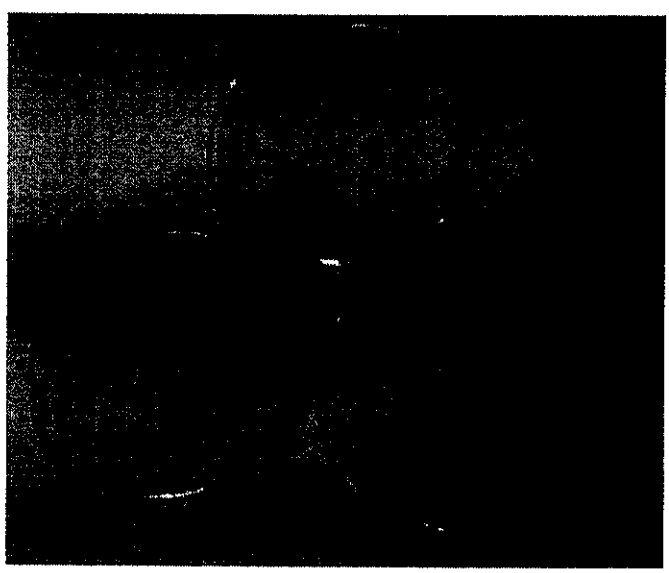


Fig 4.1.2 Isolated fungal colonies from the effluent sample

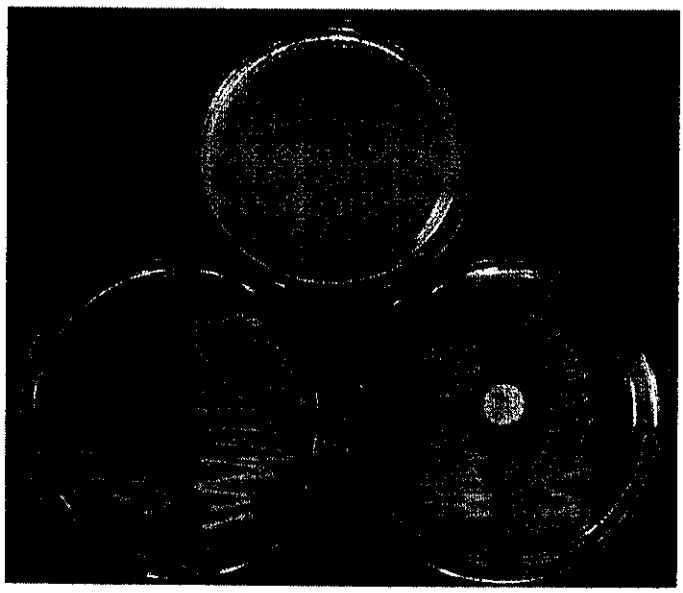


Fig 4.1.3 Purified bacterial strains by streak plate method

4.2 Screening of metal resistant bacterial and fungal strains

The isolated bacterial and fungal colonies were screened with the metal concentration of 100 mg/l and the metal resistant colonies were isolated based on their cell density values measured at 600 nm.

Table: 4.2.1 Screening of metal resistant bacterial strains

S.No	METALS (100 mg/lit)	DAYS	MICROBIAL STRAINS										
			VLB-1	VLB-2	VLB-3	VLB-4	VLB-5	VLB-6	VLB-7	VLB-8	VLB-9	VLB-10	VLB-11
1.	Cr	0	+	+	+	+	+	+	+	+	+	+	+
		1	+	+	+	+	++	++	++	+	++	++	+
		2	++	+	+	+	++	++	+++	+++	+++	+++	+
2.	Cu	0	+	+	+	+	+	+	+	+	+	+	+
		1	+	+	+	+++	++	+	++	+	+	++	+
		2	+	+	+	+++	++	+	++	++	+	++	+
3.	Ni	0	+	+	+	+	+	+	+	+	+	+	+
		1	+	+++	+	++	++	++	++	+++	++	++	+
		2	+	+++	++	++	++	+++	++	+++	+++	++	+
4.	Pb	0	+	+	+	+	+	+	+	+	+	+	+
		1	++	++	+	+++	++	++	++	+	++	++	+
		2	++	++	+	+++	++	++	++	+++	+++	++	+
5.	Zn	0	+	+	+	+	+	+	+	+	+	+	+
		1	+	++	+	+++	++	+	+	++	+	+	++
		2	+	++	+	+++	++	++	+	++	+	+++	+++

The microbial strains VLB-4, VLB-9, VLB-10 were resistant to most of the metal and thus selected for further studies.

Table: 4.2.2 Screening of metal resistant fungal strains

S.No	METALS (100mg/lit)	DAYS	Microbial strains				
			VLF-1	VLF-2	VLF-3	VLF-4	VLF-5
1.	Cr	0	+	+	+	+	+
		2	+	+	+	+	+
		4	+	+	+	+	++
2.	Cu	0	+	+	+	+	+
		2	+	+	+	+	++
		4	+	++	+	+	++
3.	Ni	0	+	+	+	+	+
		2	+	+	+	+	+
		4	+	+	+	+	+
4.	Pb	0	+	+	+	+	+
		2	+	+	+	+	++
		4	+	+	+	+	++
5.	Zn	0	+	+	+	+	+
		2	+	+	+++	+	+
		4	+	+	+++	+	+

The microbial strain VLF-5 was found to be more resistant the heavy metals.

Next to chromium, lead is the second most abundant metal found in the tannery effluent and is toxic to the environment. Further studies were carried out for the lead resistant bacteria.

4.3 Screening of selected bacterial isolates in minimal media

The selected bacterial strains were subjected to stress condition by using minimal media. The strains which was able to grow in minimal media were selected for further removal studies

Table: 4.3 Bacterial colonies in minimal media

S.no	Bacterial strains	Colonies observed
1.	VLB-4	-
2.	VLB-8	-
3.	VLB-9	+

Colonies were observed in plate containing VLB-9 and hence VLB-9 was selected for bio removal studies.

4.4. Growth curve of Bacteria with Metal Induction

The effect of metals on microbial growth was studied at the concentration of 100 mg/l. the bacterial culture was grown in LB media (10 to 15 ml) at 37°C for 24 hr. the culture grown in absence of metal was treated as control. The metal concentration was measured using AAS(Atomic Absorption Spectroscopy).

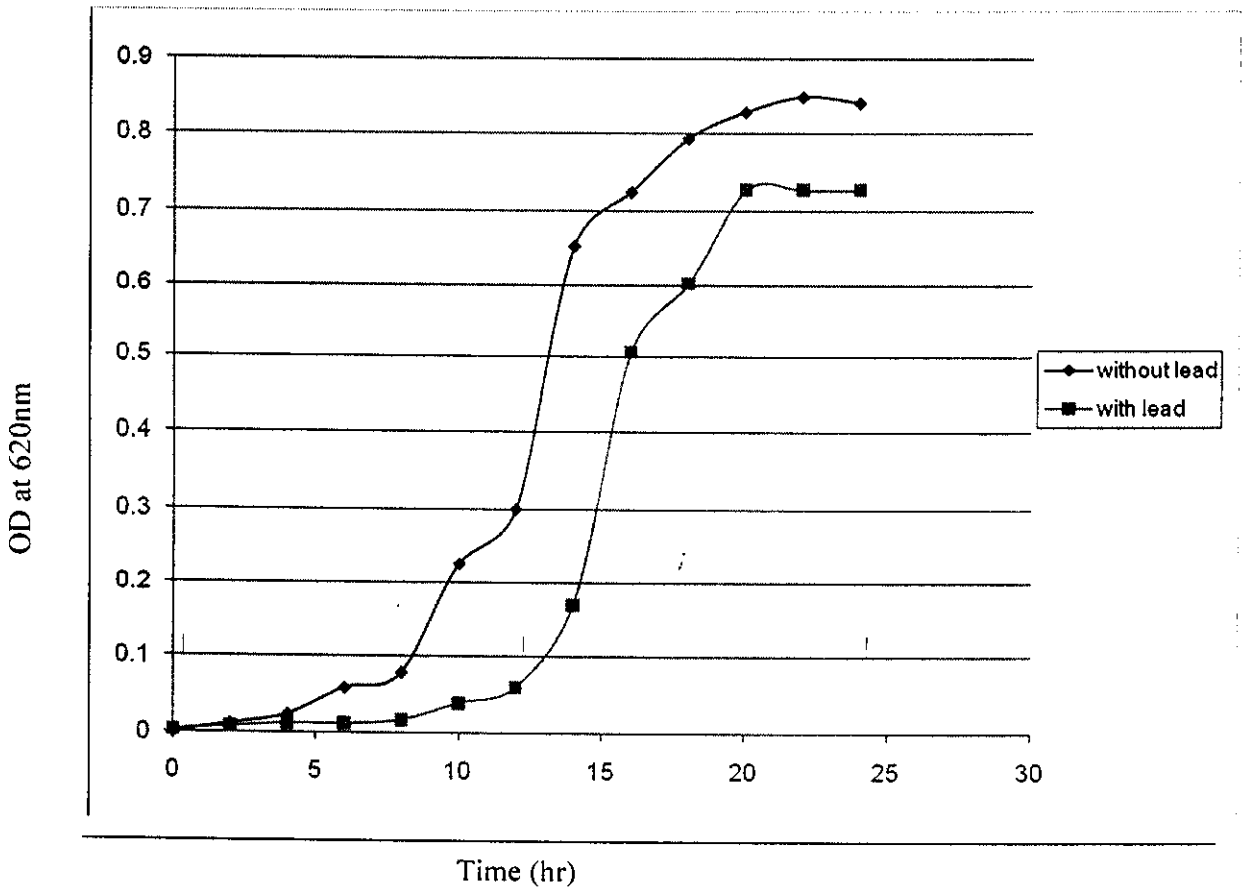


Fig.4.4.1 Growth curve of Bacteria with metal Induction

Growth rate of bacterial isolates in the presence of heavy metal was consistently slower than the control (Edward Raja, *et al.*, 2006). From the fig. 4.4.1.The bacterial growth was increased exponentially from the 10 – 20 hours.

4.5 Determination of Antibiotic sensitivity and resistance

The antibiotic sensitivity of the lead resistant bacteria was determined for Ampicillin, Bacitracin, Chloramphenical, Penicillin, Streptomycin and Tetracyline and the zone of inhibition was observed.

Table: 4.5 Antibiotic resistance of VLB-9

S.No	Antibiotic discs	Concentration(μg)	Diameter of inhibition zone(mm)
1.	Ampicillin	10	No zone(R)
2.	Bacitracin	0.05	No zone(R)
3.	Chloramphenical	50	3.6(S)
4.	Penicillin	10	No zone(R)
5.	Streptomycin	10	2.2(S)
6.	Tetracycline	10	2.2(S)

Fig. 4.5(a)

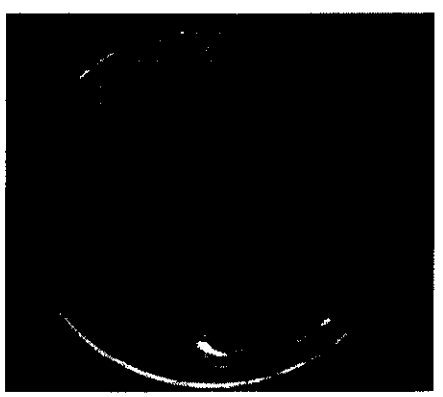


Fig. 4.5(b)

Fig. 4.5 Antibiotic resistance

1-Tetracycline 2-Streptomycin 3-Chloramphenical 4-Penicillin

From the figure, the zone of inhibition was observed for Chloramphenicol, Streptomycin and Tetracyclin which shows that VLF-9 was sensitive. No zone was observed for Ampicillin, Bacitracin and Penicillin and was inferred as resistant based on the Kirby-bauer chart.

4.6 Determination of Minimum Inhibitory Concentration (MIC)

Microbial strain VLB-9 was highly resistant for various concentration of metal. The concentration varies from 100 mg/l to 3000 mg/l. Visible growth was observed in the broth containing up to 1000 mg/l concentration of lead as shown in Fig 4.6.1. The growth of the isolated bacteria was inhibited from 1500 mg/l concentration of metal. Thus the minimum inhibitory concentration of the isolated bacteria was found as 1500 mg/l.

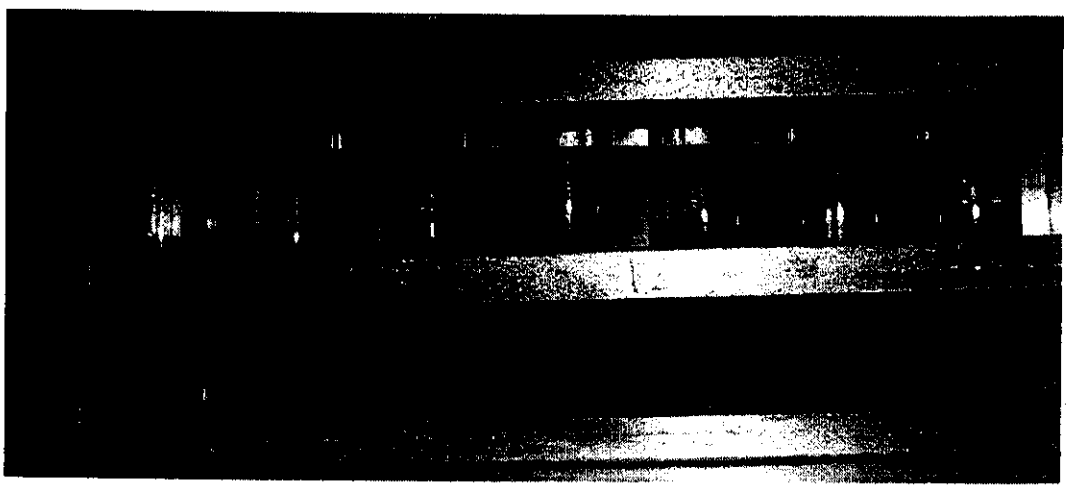


Fig 4.6.1 Minimum Inhibitory Concentration of VLB-9

1 – Control	2 - Positive Control
3 - 500 ppm lead	4 - 1000 ppm lead
5 – 1500 ppm lead	6 – 2000 ppm lead
7 – 2500 ppm lead	8 – 3000 ppm lead

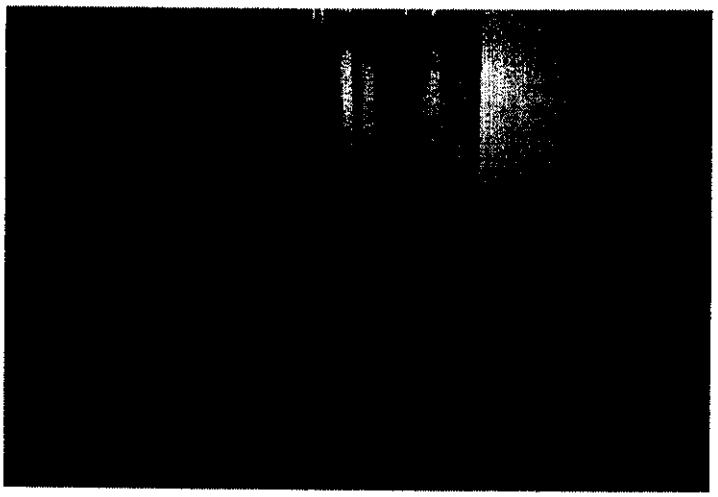


Fig4.6.2 Minimum Inhibitory Concentration of VLB-9 (Inhibition in 1500 mg/l)

1 – No inhibition at 1000 ppm of lead

2 – Inhibition at 1500 ppm of lead

4.7 Identification of the Bacterial strain

Fig. 4.7.1 Microscopic view of Gram staining

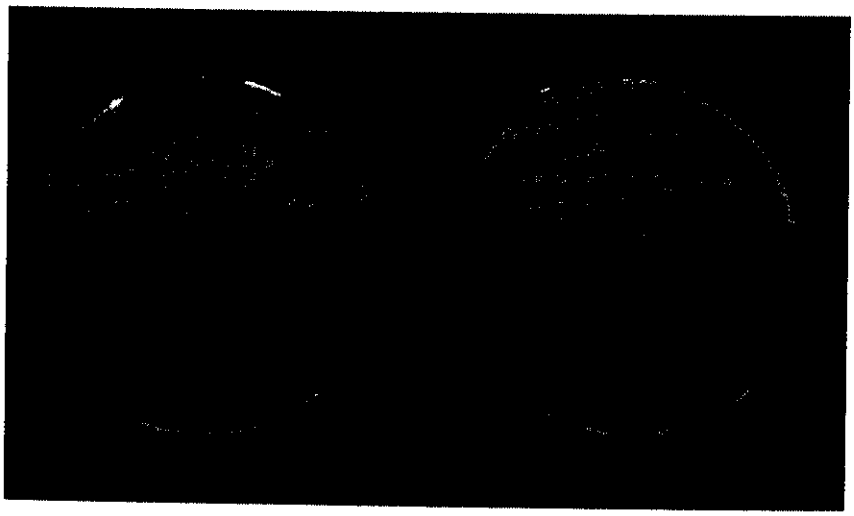


Fig. 4.7.2 Cultural characteristics- QS and CS

QS- Quadrant streaking

CS- Continuous streaking

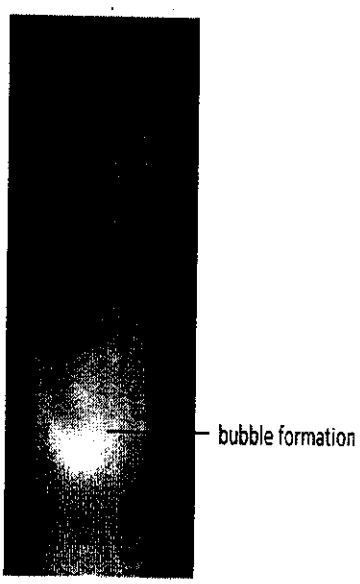


Fig.4.7.3 Catalase test

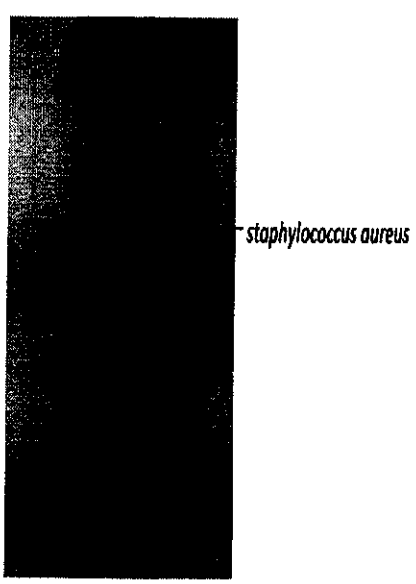


Fig. 4.7.4 Mannitol sugar fermentation

Table: 4.7.1 Characterization of (VLB-9)

S.no	Cultural characteristics	
1.	Gram staining	Positive
2	Form	Circular
3.	Color of colony	Yellow
4.	Elevation	Slightly elevated
5.	Turbidity	Uniform dispersed growth

Table: 4.7.2 Biochemical characterization of VLB-9

S.no	Test	Result
1.	Catalase	+
2.	Mannitol sugar fermentation	+
3.	Oxidase	-
4.	Coagulase	+

From the gram-staining procedure it is found that the microbial strain was a gram positive spherical bacterium that occurred in microscopic clusters formed yellow colonies in nutrient medium. Further biochemical tests resulted positive for catalase, mannitol sugar fermentation and coagulase test confirmed that the isolated microbial strain VLB-9 was *Staphylococcus aureus.*

4.10.1 Effect of Temperature on biosorption of lead by *staphylococcus aureus*

The effect of temperature on biosorption was studied at 3 different temperatures 30 °C, 37 °C and 45 °C.

Table 4.10.1 Effect of Temperature on biosorption of lead by *Staphylococcus aureus*

S.No	Temperature (°C)	Initial concentration of lead (mg/l)	Final concentration of lead (mg/l)	% removal
1.	30	100	2.24	97.76
2.	37	100	4.44	95.56
3.	45	100	5.82	94.18

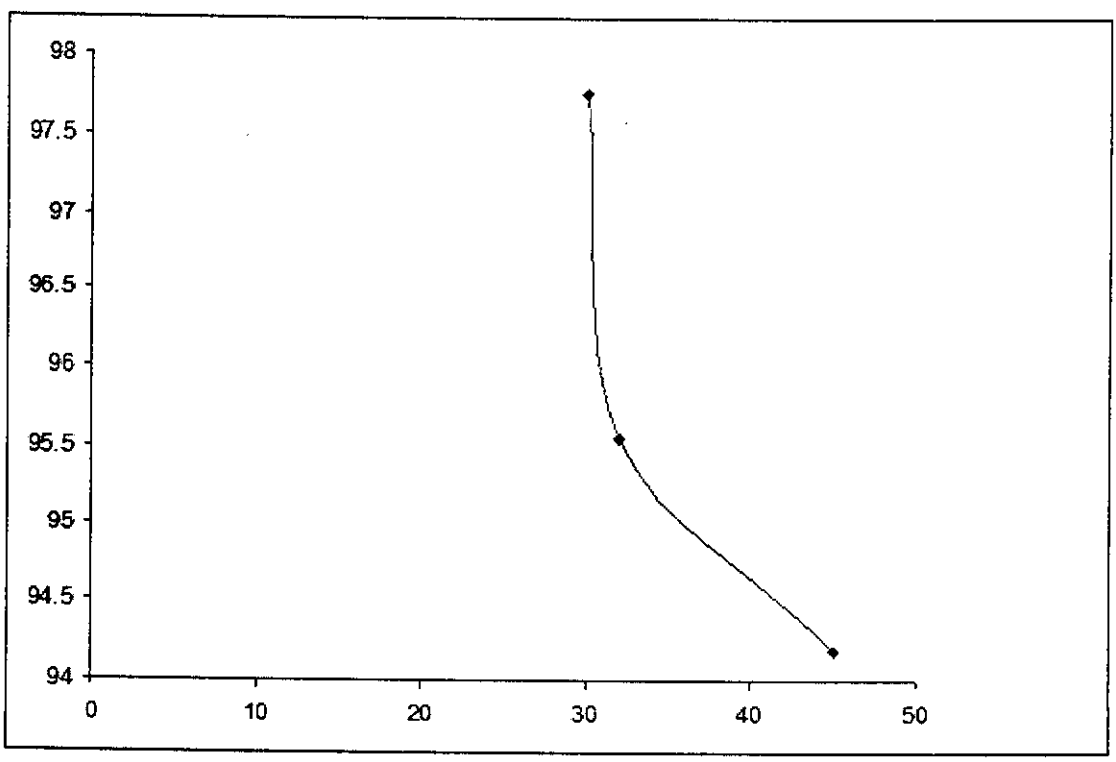


Fig:4.10.1 Effect of Temperature on biosorption of lead by *Staphylococcus aureus*

The maximum removal of the metal was found to be at 30 °C. Thus the optimum temperature was found as 30°C.

4.10.2 Effect of pH on biosorption of lead by *Staphylococcus aureus*

The effect of pH on biosorption was studied in various pH ranging from 4.5 to 8.5

Table 4.10.2: Effect of pH on biosorption of lead by *Staphylococcus aureus*

S.No	pH	Initial concentration of lead(mg/l)	Final concentration of lead (mg/l)	% removal
1.	4.5	100	19.17	80.83
2.	5.5	100	6.56	93.44
3.	6.5	100	1.84	98.16
4.	7.5	100	1.94	98.06
5.	8.5	100	2.92	97.08

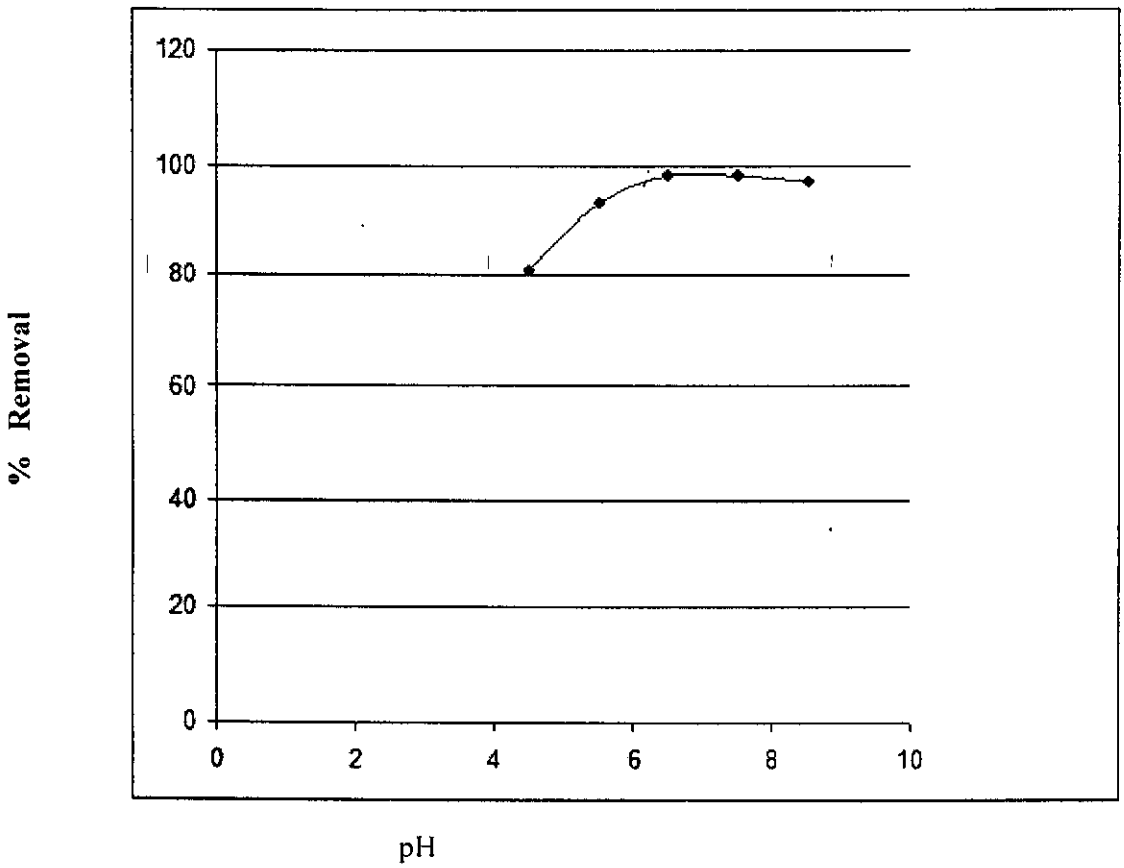


Fig: 4.10.2 Effect of pH on bio removal of lead by *Staphylococcus aureus*

The maximum removal of the metal was found to be at 6.5. Thus the optimum pH was found as 6.5.

4.10.3 Effect on Concentration of Bioremoval of lead by *Staphylococcus aureus*

S.No	Initial concentration(C_0) mg/l	Final concentration(C) mg/l	% removal
1.	50	1.78	96.44
2.	100	0.30	99.7
3.	150	6.21	95.86
4.	200	11.44	94.28

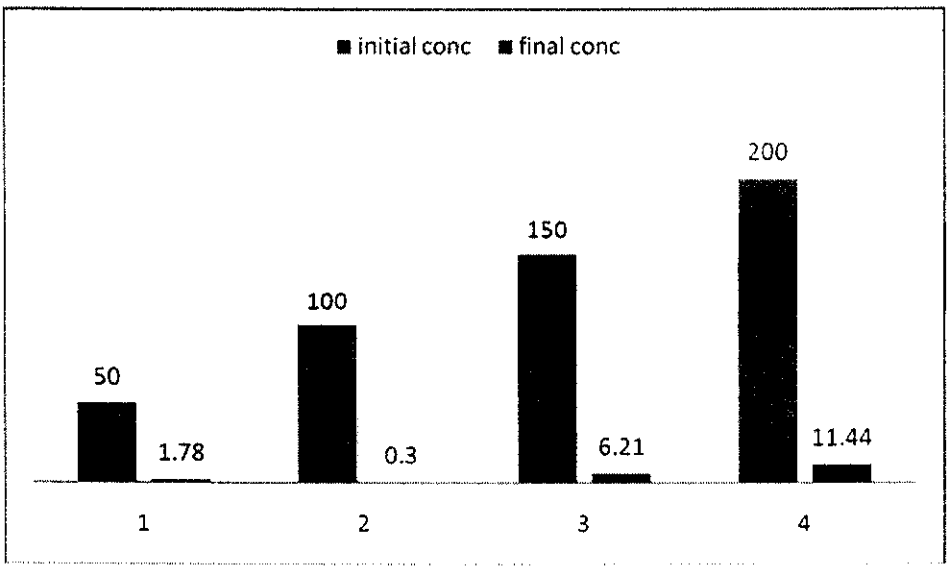


Fig.4.10.3(a) Comparison between Initial and Final lead concentrations

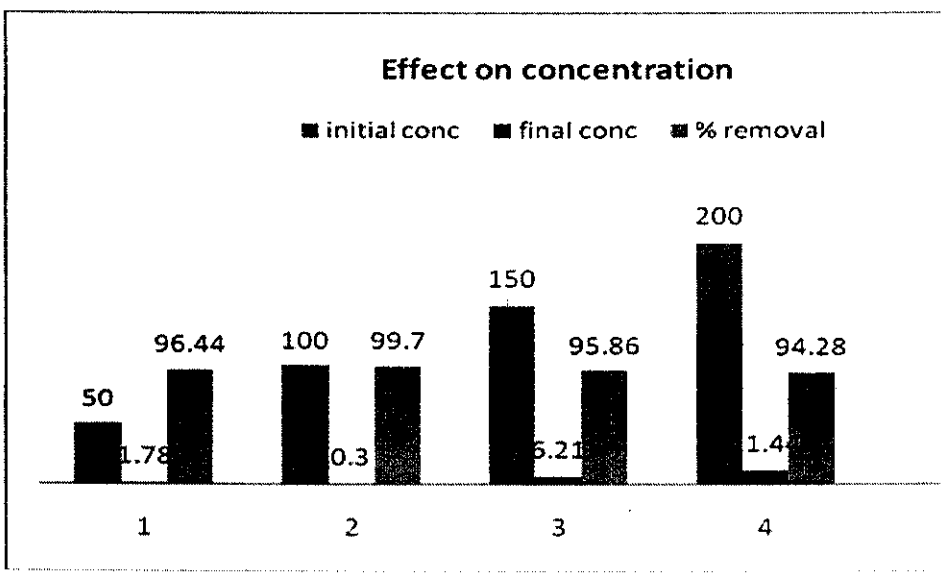


Fig.4.10.3(b) Percentage removal of Lead

1.50 mg/lit lead 2. 100 mg/lit lead 3. 150 mg/lit lead 4. 200 mg/lit lead

The effect of concentration was observed by various concentrations ranging from 50 to 200 mg/l. Since the percentage of removal of lead in the broth was high in 100 mg/l concentration, the optimum value can be around 100 mg/l. The suggestion is that adsorption in dilute solutions is mainly through ion exchange processes whereas in concentrated solutions adsorption occurs mainly through precipitation which is much slower in comparison to ion exchange.

CONCLUSION

CHAPTER-5
CONCLUSION

Heavy metal contamination in the environment has become a serious problem due to the increase in the addition of these metals to the environment. Removal of metals using microorganisms is an environmental friendly and cost-effective process.

The microbial strains including bacterial and fungal strains were isolated from the effluent sample from the contaminated site. The strains were subjected to initial screening through the metal tolerant studies. After screening one bacterial strain (VLB-9). The bacterial strain (VLB-9) was identified as *Staphylococcus aureus* by using various morphological and biochemical test. The identified *Staphylococcus aureus* was then tested for its ability to remove the lead. The minimum inhibitory concentration was found to be from 1500 mg/l metal concentration (lead).

The various physical parameters such as Temperature, pH and concentration were optimized for the removal of lead by *Staphylococcus aureus*. The ability to remove lead at high concentration was found in 100 mg of lead per litre. The removal of lead was found high at pH of 6.5 and at 30°C.

APPENDIX

APPENDIX

NUTRIENT AGAR

Composition per litre

Peptic digest of animal tissue	- 5.00 g/l
Beef extract	- 1.50 g/l
Yeast extract	- 1.50 g/l
Sodium chloride	- 5.00 g/l
Agar	- 15.00 g/l
pH (at 25 °C)	- 7.4 ± 0.2

NUTRIENT BROTH

Composition per litre

Peptic digest of animal tissue	- 5.00 g/l
Beef extract	- 1.50 g/l
Yeast extract	- 1.50 g/l
Sodium chloride	- 5.00 g/l
pH (at 25 °C)	- 7.4 ± 0.2

POTATO DEXTROSE AGAR

Composition per litre

Potatoes, infusion from	- 200 g/l
Dextrose	- 20 g/l
Agar	- 15 g/l
pH (at 25 °C)	- 5.1 ± 0.2

POTATO DEXTROSE BROTH

Composition per litre

Potatoes, infusion from	- 200 g/l
Dextrose	- 20 g/l
pH (at 25 °C)	- 5.1 ± 0.2

MINIMAL MEDIA

Composition per litre

Sodium citrate	- 0.5 g/l
Magnesium sulphate	- 0.1 g/l
Ammonium sulphate	- 1.0 g/l
Glucose	- 1.0 g/l
Disodium hydrogen phosphate	- 0.1 g/l
pH	- 6.4 ± 0.2

LURIA BERTANI BROTH

Composition per litre

Casein enzymatic hydrolysate	- 10.00 g/l
Yeast extract	- 5.00 g/l
Sodium chloride	- 5.00 g/l
pH (at 25 °C)	- 7.1 ± 0.2

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