ISOLATION AND CHARACTERIZATION OF BACTERIA FROM A HEAVY METAL POLLUTED ENVIRONMENT

J. Joonu* and Horne Iona Averal

*Department of Zoology, Bishop Heber College, Tiruchirapalli, Tamilnadu
Department of Zoology, Holy Cross College, Tiruchirapalli, Tamilnadu.
jean_joonu@yahoo.co.in

ABSTRACT

Pollution in industrial areas is of serious environmental concern and has significant implications for human health. The present study focuses on isolation and characterization of bacteria from a heavy metal polluted environment. The contaminated soil samples were collected from around the BHEL (Bharath Heavy Electrical Limited), Tiruchriappalli Tamilnadu. The isolation of bacteria from soil sample was done by nutrient agar plate method. The colonies isolated were confirmed by morphological and biochemical tests like IMVIC, Catalase, Citrate Utilization, Coagulase, Oxidase, Starch hydrolysis,(Table-1,2,3) etc., The colonies identified as *Pseudomonas aeruginosa, Enterobacteriacea, Staphylococus aureus, and Bacillus* sps. Thus the total heavy metals present in the sample were also analysed both in the soil and water of the effluent sample.

Keywords: Bacteria, heavy metal, polluted soil

INTRODUCTION

The heavy metal and radionuclide pollution from nuclear power plants, mining industries, electroplating industries and agricultural runoffs is a major cause of concern to public health, animals and ecosystems (Rehman *et al.,* 2007). Persistent toxicants (heavy metals) in water and sediments affected by heavy metal pollution can have serious affects on the aquatic ecosystem and can make water unsuitable for human consumption (DPIW). Several studies have also demonstrated heavy metal induced changes in specific parts of the soil microbial community. These metals have peculiar characteristics including that (i) they do not decay with time (ii) they can be necessary (or) beneficial to plants at certain levels but can be toxic when exceeding specific thresholds (iii) they are always present at a background level of non-anthropogenic origin, their inputs in soils, being related to weathering of parent rocks and pedogenesis and (iv) they often occur as cations which strongly interact with the soil matrix. Thus the heavy metals in soil can become mobile as a result of changing environmental conditions. This situation is referred to as 'Chemical timing bomb' (Facchinelli *et al*., 2001).

The bioremediation of heavy metals using microorganisms has received a great deal of attention in recent years, not only as a scientific novelty but also for its potential application in industry. Metal accumulative bioprocess generally falls into one of two categories, bisorptive (passsive) uptake by nonliving, non growing biomass or biomass products and bioaccumulation by living cells (Macaski and Dean, 1989; Aksu and Kutsal, 1990; Huang *et al*., 1990; Volesky *et al*., 1992; Avery and Tobin, 1993; Brady and Duncan, 1994; Aksu, 1998; Doenmez and Aksu, 1999;2001). Biosorption can be defined as the ability of biological materials to accumulate heavy metals from waste water through metabolically mediated (or) physico chemical pathways of uptake (Fourest and Roux, 1992). Algae, bacteria and fungi, yeasts have proved to be potential metal biosorbents (Volesky, 1986). The complex structure of microorganisms implies that there are many ways for the metal to be taken up by the microbial cell. According to the dependence on the cell's metabolism, biosorption mechanisms can be divided into: (i) Metabolism dependent and (ii)Non -metabolism dependent. According to the location where the metal removed from solution is found, biosorption can be classified as (i) Extra cellular accumulation/ precipitation (ii) Cell surface sorption/ precipitation (iii) Intracellular accumulation (Ahalya, *et al*., 2003).

Thus the present aim of this study is to isolate the microorganisms that survive in heavy metal polluted environment and to detect the presence of various heavy metals.

MATERIALS AND METHODS

The samples were collected using pre-cleaned polythene bags. The effluent collected was diluted with distillery water up to 10^{-9} dilutions.

Identification of microorganisms

The isolated bacterium was subjected to identification by staining and biochemical tests. Gram staining procedure was carried out to identify the gram reaction of the organism. The motility test was performed by hanging drop method to identify motility of the organism. The biochemical tests such as indole, Methyl red, Voges Proskauver, citrate, Urease test, starch hydrolysis, catalase test, mannitol fermentation etc. were performed to identify the organisms (Edmund, 1977).

1. Indole test (tryptone broth)

Inoculate a loopful of bacteria into a tryptone broth. Incubate 48 hours. After incubation: The broth must be turbid. A clear broth indicates that the organism did not grow and cannot be tested. Add a few drops of Indole reagent to the broth culture (tryptone broth). A positive result has a red layer at the top. A negative result has a yellow or brown layer (Fig.1).

2. Methyl Red test (MRVP broth)

Inoculate a loopful of bacteria into MRVP broth. Incubate 3 to 5 days. After incubation: The broth must be turbid. A clear broth indicates that the organism did not grow and cannot be tested. Remove 1 ml of broth and place into a sterile tube. Add 3-4 drops of methyl red to the original broth. A positive result has a distinct red layer at the top of the broth. A negative result has a yellow layer. Many gram-negative intestinal bacteria like *Escherichia, Salmonella*, and *Proteus* can be differentiated (Fig .1).

3. **Voges-Proskauer test**

Inoculate a loopful of bacteria into MRVP broth. Incubate 3 to 5 days. After incubation: A clear broth indicates that the organism did not grow and cannot be tested. Barritt's reagent A (VP A) contains naphthol and Barritt's B (VP B) contains KOH. Test 1 ml of the culture from the MRVP broth. Add the entire contents of the VP A reagent (15 drops) and 5 drops of the VP B reagent to the 1 ml of the broth culture. With a positive reaction the medium will change to pink or red indicating that acetoin is present. With a negative reaction the broth will not change color or will be copper colored. *Enterobacter, Serratia* and some species of *Bacillus* shows positive results (Fig.1).

4. Citrate test (Simmons's Citrate slant)

A loopful of bacteria is streaked onto a citrate agar slant, do *not* stab the butt. Incubate 24 to 48 hours, longer for *Bacillus* species. Incubate with a loose cap. After incubation: A positive reaction is indicated by a slant with a Prussian blue color. A negative slant will have no growth of bacteria and will remain green (Fig.1).

5. Urease test

Inoculate a tube of urea broth with your test organism and incubate at room temperature for 24 to 48 hrs. Urea broth is composed of yeast extract, urea and the pH indicator phenol red. If urease is present, ammonia will be released and the pH will rise. A positive urease test is a change from yellow to cerise (a light cherry color; pH 8.1 or greater). No change in the color of the indicator is a negative test (Fig.1).

Mannitol fermentation

Mannitol Salt Agar contains 7.5% NaCl, which is inhibitory to most bacteria. Bacteria that can grow on this agar can be differentiated based on mannitol fermentation. Fermentation of mannitol results in acidic products which turn phenol red pH indicator from red to yellow. Streak MSA plate and incubate at 37°C for 2 days. Positive test indicates colour change from red to yellow. Negative test shows there is no colour change (Fig 3).

Starch Hydrolysis

Inoculate a starch plate with the organism to be tested. Incubate at optimum temperature for at least 48 hours. Flood plate with iodine, observe results.

Blue color indicates no hydrolyis, while a clear zone indicates hydrolysis. *B.subtilis* is the organism which shows positive results (Fig 4).

Catalase test

Remove a small amount of your environmental unknown from your agar slant, or a loopful of control test organisms from a broth culture and place it on a glass slide.

Mix the organisms with a drop of 3% H2O2 and check for the appearance of gas bubbles (a positive test). No bubbles are a negative test.

Bacillus spp. is used as the positive control and *Streptococcus lactis* is used as the negative control.

Eosin Methylene Blue (EMB) Agar

A selective medium for gram-negative bacteria. Levine's EMB agar contains methylene blue, which inhibits gram-positive bacteria. Differential for enterics: will differentiate lactose fermenters from nonfermenters. After incubation: Lactose nonfermenters will have cream colored colonies. Lactose fermenters will have pinkish colonies, sometimes with dark centers. *E. coli* often has a greenish metallic sheen.

RESULTS AND DISCUSSION

The objective of this study was to identify the microbes which can survive in heavy metal polluted environment. The total number of heavy metals was also detected in the effluent samples. Table1 shows the positive reactions for the biochemical tests and the organism identified is Staphylococcus aureus which is gram positive cocci and shows positive reaction for motility test, coagulase test and mannitol fermentation test .Table 2 showed the positive results for the biochemical tests like vogues proskauer, citrate, urease and catalase tests and the organism also shows positive reactions in the EMB agar. The organism thus identified is Enterobacter species. Table 3 showed the positive results for the biochemical tests like motility test, citrate urease, catalase tests etc. The organism also grows well on Pseudomonas agar. Thus the organism is identified as Pseudomonas species. Table 4 shows the positive results for the biochemical tests like motility test, starch hydrolysis and catalase test. The organism thus identified is Bacillus species. The total number of heavy metals presents in the effluent sample is given in the table 5.

Table 1 shows the presence of the organism Staphylococcus aureus showing the positive results for Mannitol fermentation test and in Motility test.

BIOCHEMICAL TESTS	RESULT
ORGANISMS	Staphylococcus aureus
GRAM STAINING	(+) coccus
MOTILITY	+
EMB AGAR	-
MANNITOL SALT AGAR	+
PSEUDOMONAS AGAR	_

Table 2: shows the positive results for the motility test, vogues proskauer test, citrate, urease test and in EMB Agar

TESTS	RESULT
ORGANISMS	Enterobacter species
GRAM STAINING	(-) rod
MOTILITY	+
INDOLE	_
METHYL RED	_
VOGES	+
PROSKAUER	
CITRATE	+
UREASE	+
STARCH	_
CATALASE	+
EMB AGAR	+
MANNITOL SALT AGAR	_
PSEUDOMONAS AGAR	_

Table 3: shows the presence of the organism subtilis showing positive tests for Motility test, catalase test and starch hydrolysis

TESTS	RESULT
ORGANISMS	Bacillus subtilis
GRAM STAINING	(-) rod
MOTILITY	+
STARCH	+
CATALASE	+
EMB AGAR	_
MANNITOL SALT AGAR	_
PSEUDOMONAS AGAR	_

Table 4: shows the presence of Pseudomonas aeruginosa showing positive results for motility tests, citrate, urease and catalse test and the animal grows on Pseudomonas Agar.

TESTS	RESULT
ORGANISMS	Pseudomonas aeroginosa
GRAM STAINING	(-) rod
MOTILITY	+
INDOLE	_
METHYL RED	_
VOGESPROSKAUER	_
CITRATE	+
UREASE	+
STARCH	_
CATALASE	+
EMB AGAR	_
MANNITOL SALT AGAR	_
PSEUDOMONAS AGAR	+

Table 5 gives the total heavy metals present in the effluent sample

S.No	Name of the Parameter	Sample Details	
		Soil	Water
1.	Total Calcium (%)mg/lit	5.69	432
2.	Total Zinc (ppm)mg/lit	2.56	5.79
3.	Total Copper (ppm)mg/lit	1.29	0.56
4.	Total Chromium (ppm)mg/lit	0.75	9.79
5.	Total Cadmium (ppm)mg/lit	0.26	2.75
6.	Total Nickel (ppm)mg/lit	0.09	1.79
7.	Total Mercury (ppm)mg/lit	0.02	0.12

Heavy metals have been known to disrupt ecosystem structure and functioning for a long time. In the multicellular organisms, heavy metal target specific organs and pathways resulting in disruption of definitive metabolic functions (Fulladosa, *et al*., 2005). The consumption of such contaminated materials may facilitate widespread infections and can ultimately lead to the outbreak of epidemics. The effluent, which is discharged into a manmade lake that is a 'pool of effluent' contaminates nearby streams, food crops on the farm and inadvertently reaches man (Lateef *et al.*, 2005).In high concentrations, heavy metal ions react to form toxic compounds in cells (Nies, 1999).

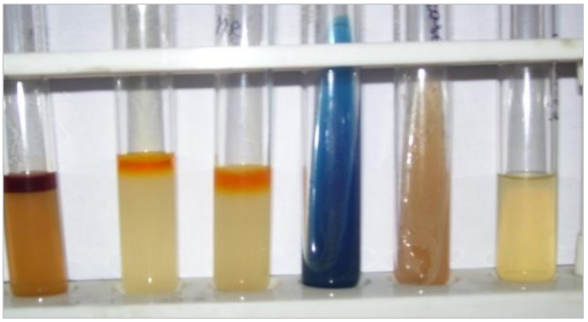

Fig. 1: shows the presence of *Enterobacter* species

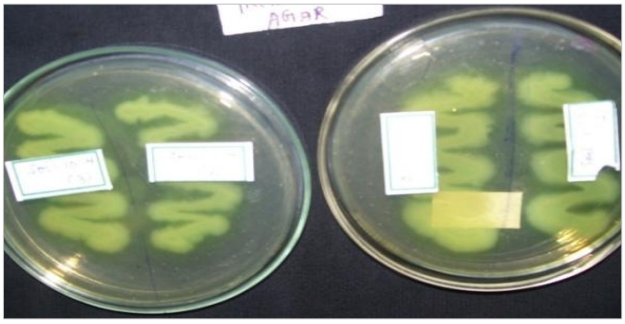

Fig. 2: shows the presence of *Pseudomonas* species which grows on a P*seudomonas* agar.

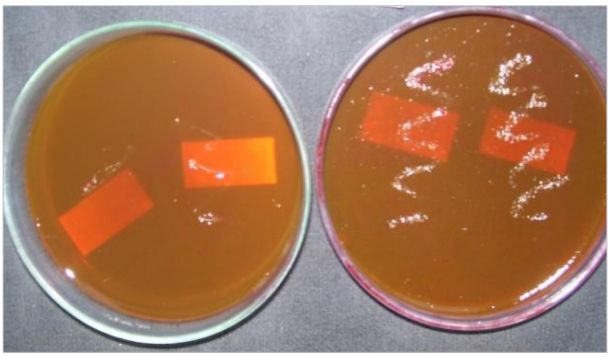

Fig. 3: shows the presence of *Staphylococcus aureus* which b grows well on a Mannitol salt agar.

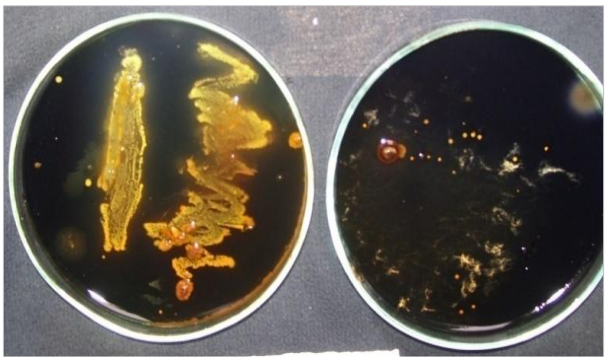

FIG 4 shows the presence of Bacillus subtilis which hydrolysis the starch

To have a toxic effect, however, heavy metal ions must first enter the cell. B ecause some heavy metals are necessary for enzymatic functions and bacterial growth, uptake mechanisms exist that allow for the entrance of metal ions into the cell. There are two general uptake systems — one is quick and unspecific, driven by a chemiosmotic gradient across the cell membrane and thus requiring no ATP, and the other is slower and more substrate-specific, driven by energy from ATP hydrolysis. While the first mechanism is more energy efficient, it results in an influx of a wider variety of heavy metals, and when these metals are present in high concentrations, they are more likely to have toxic effects once inside the cell (Nies and Silver, 1995). To survive under metal-stressed conditions, bacteria have evolved several types of mechanisms to tolerate the uptake of heavy metal ions. These mechanisms include the efflux of metal ions outside the cell, accumulation and complexation of the metal ions inside the cell, and reduction of the heavy metal ions to a less toxic state (Nies, 1999). Thus the organisms isolated and identified from a heavy metal polluted environment developed the mechanisms to survive n a highly toxic environment. This study thus highlights the presence of bacteria which survives in a highly heavy metal polluted environment.

LITERATURE CITED

Afshin Qishlaqi and Farid Moore, 2007. Statistical Analysis of Accumulation and Sources of Heavy Metals Occurrence in Agricultural Soils of Khoshk River Banks, Shiraz, Iran. *American-Eurasian J. Agric. & Environ. Sci.,* **2**(5): 565-573

Aksu Z and Kutsal T, 1990. A comparative study for biosorption characteristics of heavy metal ions with C.vulgaris. *Environ. Technol.,* **11**: 979-987

Aksu Z, 1998. Biosorption of heavy metals by microalgae in batch and continuous systems. In: Algae for waste water treatment.eds Y.-S. Wong and N. F. Y. Tam, 99.37-53. Springer,Germany.

Avery SV and Tobin JM, 1993. Mechanism of adsorption of hard and soft metal ions to Saccaromyces cerevisiae and influence of hard and soft anions. *Appl. Environ. Microbiol,* 59: 2851-2856.

Brady D., Stoll A.D., Starke L and Duncan JR, 1994. Bioaccumulation of metal cations by Saccaromyces cerevisiae. *Appl. Microbiol. Biotechnol*., **41**: 149-154.

Doenmez G and Aksu Z, 1999. The effect of copper(II) ions on the growth and bioaccumulation properties of some yeasts.Process *Biochem*., **35**: 135-142.

Doenmez G and Aksu Z, 2001.Bioaccumulation of copper(II) and Nickel (II) by the non adapted and adapted growing Candida sp. *Wat. Res*., **35**: 1425-1434

Facchinelli A, E Sacchi and L Mallen, 2001.Multivariate statistical and GIS-based approach to identify heavy metals sources in soils. *Environ.Pollu*.,**114**:13-324

Huang C, Huang C and Morehart AL, 1990. The removal of copper from dilute aqueous solutions by Saccharomyces cerevisiae. *Wat. Res*., **24**: 433-439

Lateef JK Oloke and EB Gueguimkana, 2005.The prevalence of bacterial resistance in clinical, food, water, and some environmental samples in Southwest Nigeria. *Environmental Monitoring and Assessment.,* 100: 59–69

Macaskie L and Dean ACR, 1989. Microbial metabolism, desolubilisation and deposition of heavy metals: Metal uptake by immobilised cells and application to the detoxification of liquid wastes. *Adv. Biotechnol. Proc*., **12**: 159-172

Nies AD Nies and S Silver, 1990. Nucleotide sequence and expression of a plasmid-encoded chromate resistance determinant from Alcaligenes eutrophus. *J. Biol. Chem*., **265**: 5648-5653

Nies DH and Silver S, 1995. Ion efflux systems involved in bacterial metal resistances. *Journal of Industrial Microbiology.*, **14**: 186-199.

Nies DH, 1999. Microbial heavy metal resistance. *Appl. Microbial. Biotechnol*., 51: 730-750.

Rehman A Shakoori FR , 2007. Multiple metal resistance and uptake by a ciliate, Stylonchia mytilus isolated from an industrial effluent and its possible use in wastewater treatment. *Bull. Environ. Contam. Toxicol*., **79**. 410-414

Summers AO, 1992. Untwist and shout: A heavy metal - responsive transcriptional regulator. *J. Bacteriol*., **174**:3097-3101.

Volesky B, May H and Holan ZR, 1992. Cadmium biosorption by Saccharomyces cerevisiae. *Biotechnol. Bioeng.*, **41**: 826-829.