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HEAVY METAL TOLERANCE OF THREE DIFFERENT BACTERIA ISOLATED FROM INDUSTRIAL EFFLUENT

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Abstract

The pollution of the environment with toxic heavy metals is spreading throughout the world along with industrial progress. Microorganisms and Microbial products can be highly efficient bioaccumulations of soluble and particulate forms of metals especially dilute external solutions. Microbes related technologies may provide an alternative or addition to conventional method of metal removal or metal recovery. The present paper deals with the isolation and characterization of heavy metal tolerant bacteria from Industrial effluents and determination of Minimum Inhibitory Concentration (MIC) of the heavy metal resistant bacteria isolates. Among various outcomes of the study, important aspect is the metal accumulating ability of these isolates which can be applied in removal and recovery of metals. Minimum inhibitory concentration (MIC) values for *S. aureus* were 450 µg/ml, *for B. subtilis were* 400 µg/ml *and for E. coli* were 350 µg/ml for the chloride salts of cadmium. Minimum inhibitory concentration (MIC) values for *S. aureus* were resistant to 450 µg/ml, *for B. subtilis were* 300 µg/ml *and for E. coli* were 250 µg/ml for the chloride salts of Nickel. Minimum inhibitory concentration (MIC) values for *S. aureus* were 300 µg/ml *for B. subtilis were* 400 µg/ml *and for E. coli* were 350 µg/ml for the Lead nitrate. These three strains of bacteria that were resistant to heavy metals were chosen for further investigations. Heavy-metal chloride salt of cadmium, Nickel and Lead at varying concentrations (50 µg/ml, 100 µg/ml, 150 µg/ml, 200 µg/ml, 250 µg/ml, 300 µg/ml, 350 µg/ml, 400 µg/ml, 450 µg/ml, 500 µg/ml) were taken for study. The three isolates, all with significant metal up taking ability can be either used as pure isolates or as a mixed consortium to act efficiently in lesser time. The metal accumulation and nanoparticle generation by the cell can be further explored at a molecular level. This can further help in genetic manipulation for more efficiency and practical purpose.

INTRODUCTION

The pollution of the environment with toxic heavy metals is spreading throughout the world along with industrial progress. Microorganisms and Microbial products can be highly efficient bioaccumulations of soluble and particulate forms of metals especially dilute external solutions. Microbes related technologies may provide an alternative or addition to conventional method of metal removal or metal recovery. Heavy metals are recognized to be powerful inhibitors of biodegradation activities. These metals cannot be degraded, and are ultimately indestructible. The toxic effects of heavy metals result mainly from the interaction of metals with proteins (enzymes) and inhibition of metabolic processes. These heavy metals such as copper, cadmium, lead, zinc, nickel, mercury and chromium when accumulated in soils, water bodies they can also be present in concentrations toxic to plants, animals, humans and aquatic life. Microorganisms have acquired a variety of mechanisms for adaptation to the presence of toxic heavy metals. There is increasing evidence for the evolution of metal resistance in natural populations inhabiting contaminated sites. The evaluation of metal resistance a complex process which may involve a variety of mechanisms. Aquatic microbes become resistant to antibiotics and metals as a result of contamination with effluents. Antibiotic resistance in bacteria is more frequently associated and strongly correlated with metal resistance. Microbiological technology used for the removal of metallic ions from industrial waste waters appear to present a low cost application, not an additional factor of environmental pollution and allows recovery of heavy metals from industrial waste waters. Advantages of microbiological method for removing heavy metals led to increased laboratory studies to improve the removal efficiency of metals from industrial waste waters. ***(Cismasiu (2004), Rampelott (2010), Pattanapipitpaisal et.al. (2002)).*** Presence of high concentration of toxic heavy metals in wastewater directly leads to both contamination of receiving water bodies and deleterious impact on aquatic life ***(Moten and Rehman, 1998).*** Use of such polluted water for consumption and other purposes can bring severe problems to human health. At higher concentration,

heavy metals form toxic complex compounds in the cell that are too dangerous for any biological functions. Therefore this study was performed to determine the heavy metal resistance patterns of bacteria which were isolated from tannery waste water. The present study deals with isolation, identification and characterization of heavy metal resistant bacteria, which were isolated from Industrial effluent collected from Agra and Firozabad which involves Isolation and biochemical characterization of bacteria *(Escherichia coli, Staphylococcus aureus and Bacillus subtilis)* isolated from industrial effluents and To determine heavy metals (Cadmium chloride, Nickel chloride and Lead nitrate) resistance of bacteria through Minimum Inhibitory Concentration (MIC) values.

Materials and Methods

The present paper deals with the isolation and characterization of heavy metal tolerant bacteria from Industrial effluents and determination of Minimum Inhibitory Concentration (MIC) of the heavy metal resistant bacteria isolates. Industrial effluent from different sites of Agra and Firozabad were collected for isolation of the microbes. The samples were collected in sterile plastic container and transported to laboratory for bacteriological analysis. 100µl of the effluent water sample was spread on nutrient agar plates. Nutrient agar plates were prepared and the medium was autoclaved at 121^{O}C for 15 min. The growth of the bacterial colonies was observed after 24hrs of incubation at 37^{O}C. The bacteria were isolated and their cultural characteristics and morphological features were confirmed and also subjected to standard biochemical tests (***Krieg and Holt 1984)*** before the experiments. Selected tannery effluent isolates were grown on MacConkey agar (Himedia, India). The shape and colors of the colonies were examined under the microscope after Gram staining. Isolates were biochemically analyzed by Starch hydrolysis test; colonies were grown on MacConkey Agar, Mannitol salt agar. The tests were used to identify the isolates according to Bergey's Manual of Determinative bacteriology ***(Holt et. al., 1994; Manero, A. and Blanch, A. R. (1999).*** To obtain pure culture of *E.coli, B. subtilis* and *Staphylococcus* serial streaking of single colony was done on Nutrient Agar. Pure

cultures of bacteria were maintained in Nutrient broth and were preserved at 4OC until further tested. The bacterial isolates were screened on Nutrient Agar (NA) plates supplemented with various concentration of each metal one time by the standard spread plate method ***(APHA, 1992).*** The spread-plate method involved the addition of a specific heavy-metal-chloride salt added to glycerol-agar medium before autoclaving. Plates were prepared containing one concentration of cadmium chloride, Nickel and Lead. Cadmium, Nickel and Lead plates were made containing 50 µg/ml, 100 µg/ml, 150 µg/ml, 200 µg/ml, 250 µg/ml, 300 µg/ml, 350 µg/ml, 400 µg/ml, 450 µg/ml, 500 µg/ml concentrations. Bacterial cultures were spread onto the surface of all plates to determine the minimum inhibitory concentration (MIC) of each heavy metal. The MIC was defined as the lowest concentration at which no CFU were observed after 24 h incubation at 37^0C. Growth of the bacterial culture was determined visually as positive or negative.Resistance or sensitivity was determined for each strain and each heavy metal. Resistant strains were defined as those having MIC values greater than 5 mM. Sensitive strains were defined as those strains having MIC values at 5 mM.

The broth method of heavy-metal-resistance testing was accomplished using tubes of nutrient broth either alone or containing one heavy metal at one concentration per tube. Concentrations tested were 50 µg/ml, 100 µg/ml, 150 µg/ml, 200 µg/ml, 250 µg/ml, 300 µg/ml, 350 µg/ml, 400 µg/ml, 450 µg/ml, 500 µg/ml of cadmium, Nickel and Lead. The heavy-metal salt was added directly to the broth tube, and these tubes were subsequently autoclaved. Broth tubes were inoculated with 0.1 ml of *S. aureus, B. subtilis and E. coli* grown to an OD 620 of 0.8-1.0, and then incubated on a roller drum for 24h at 37^0C. Following incubation, the OD 620 was measured and recorded. A culture having an OD 620 of greater than 0.1 was considered resistant. The minimum concentration of heavy metals at which no turbidity was observed by spectrophotometer at 620nm was considered as the MIC of bacterial isolates against heavy metals.

Result and Discussion

Bacterial strains were isolated from tannery effluent. Industrial effluent (water samples) were collected from Agra and Firozabad cultured on nutrient agar media and then tested through various biochemical tests for identification of *S. aureus, B. subtilis* and *E. coli.*

In this study, *Staphylococus aureus* was a gram positive cocci and found positive Mannitol salt agar test and negative for starch hydrolysis test and MacConkey Agar test whereas *and Bacillus subtilis* was gram positive rods and found positive for starch hydrolysis test and negative for mannitol salt agar test and MacConkey Agar test (**Table 1**). *E. coli* was found gram negative rods and gave positive tests for MacConkey Agar test and negative for starch hydrolysis test and Mannitol salt agar test.

Determination of Minimum Inhibitory Concentration (MIC)

MIC of the heavy metal resistant bacterial isolates grown on heavy metals incorporated media, against respective heavy metal was determined by gradually increasing the concentration of the heavy metal, 50 µg/ml each time on NA plate until the strains are unable to grow on the plate. The starting concentration used was 50 µg/ml. The culture growing on the last concentration was transferred to the higher concentration by streaking on the plate. MIC was noted when the isolates were unable to grow on plates even after 10 days of incubation.

HEAVY METAL RESISTANCE TESTING

Bacterial strains of *S. aureus, B. subtilis* and *E. coli* were taken for resistance to Cadmium, Nickel and Lead using a disc-sensitivity method, and preliminary results indicated the presence of cadmium resistance in one of these strains. Heavy metal resistance was tested using a disc sensitivity method, and growth in either broth or on a solid media that contained varying concentrations of heavy metals.

BROTH METHOD

S. aureus, B. subtilis and *E. coli* was inoculated into Nutrient broth containing heavy-metal salt (cadmium, Nickel and Lead) at varying concentrations (50 µg/ml, 100 µg/ml, 150 µg/ml, 200 µg/ml, 250 µg/ml, 300 µg/ml, 350 µg/ml, 400 µg/ml, 450 µg/ml, 500 µg/ml). A control tube was also inoculated which lacked heavy-metal

salt. It was clear, even prior to inoculation, that a precipitate was formed in broths containing these heavy-metal salts. Following inoculation and 24 hours of incubation at 37^0C, there were obvious, visible clumps in the broths, likely consisting of the heavy-metal salt as well as cell mass. These clumps were not dispersible by vortexing or agitation, and therefore the OD readings were very low and did not accurately measure the extent of culture growth.

SENSITIVITY METHOD

In the present study, it was evident that different bacterial strains such as *S. aureus, B. subtilis and E. coli*, showing tolerance to heavy metals When *S. aureus, B. subtilis and E. coli* was grown on nutrient Agar plates containing varying concentrations of heavy-metal salts, minimum inhibitory concentration (MIC) values for *S. aureus* were 450 µg/ml, *for B. subtilis were* 400 µg/ml *and for E. coli* were 350 µg/ml for the chloride salts of cadmium. Minimum inhibitory concentration (MIC) values for *S. aureus* were resistant to 450 µg/ml, *for B. subtilis were* 300 µg/ml *and for E. coli* were 250 µg/ml for the chloride salts of Nickel. Minimum inhibitory concentration (MIC) values for *S. aureus* were 300 µg/ml *for B. subtilis were* 400 µg/ml *and for E. coli* were 350 µg/ml for the Lead nitrate. The resistance of the isolates to heavy metals was neither lost nor altered when isolates were stored in nutrient agar at refrigerated temperature. The MIC determined for the metals were between 250 µg/ml and 450 µg/ml, varied depending on the metal and bacterial isolate. This study showed a high incidence of metal resistance for the bacterial isolates. Many bacterial species isolated from industrial zones had been shown to develop resistance to heavy metals ***(Osborn et. al., 1997; Ansari and Malik 2007).*** In this study it is clearly seen that the bacterial isolates also show non vulnerability to different antibiotics Earlier bacterial strains resistant to Gentamycin and Penicillin were also resistant against to heavy metals ***(Dhakepalker and Chopade, 1994; Basu, et. al., 1997).*** The spread of multiple antibiotic resistant bacteria has been the most serious threat to the successful treatment of disease ***Kumar et. al., 2005; Ramteke, 1997).*** The bacterial isolates used in the study were resistant to the heavy metals. This increase in the MIC

of metals as well as the antibiotic resistance among bacterial population in any system may be an indication of risk to the safety. Association between resistance to antibiotics and heavy metals has been reported ***(Dhakepalker and Chopade, 1994)***. Among 90% of bacteria were tolerant to Co-trimazole, Gentamycin and Streptomycin.

Conclusion

During the present investigation efforts were made to isolate strains of bacteria which tolerate and accumulate heavy-metal salt (cadmium, Nickel and Lead). Heavy-metal chloride salt of cadmium, Nickel and Lead at varying concentrations (50 µg/ml, 100 µg/ml, 150 µg/ml, 200 µg/ml, 250 µg/ml, 300 µg/ml, 350 µg/ml, 400 µg/ml, 450 µg/ml, 500 µg/ml) were taken for study. Minimum inhibitory concentration (MIC) values for *S. aureus* were 450 µg/ml, *for B. subtilis were* 400 µg/ml *and for E. coli* were 350 µg/ml for the chloride salts of cadmium. Minimum inhibitory concentration (MIC) values for *S. aureus* were resistant to 450 µg/ml, *for B. subtilis were* 300 µg/ml *and for E. coli* were 250 µg/ml for the chloride salts of Nickel. Minimum inhibitory concentration (MIC) values for *S. aureus* were 300 µg/ml *for B. subtilis were* 400 µg/ml *and for E. coli* were 350 µg/ml for the Lead nitrate. Biological metal removal (biosorption) has distinct advantages over conventional methods: it is non-polluting and it can be highly selective, more efficient, easy to operate, and hence cost-effective for treatment of large volumes of wastewaters containing low metal concentrations ***(Puranik and Pakniker, 1999).*** The industrial effluents are enriched media to grow and spread microbial population, which are resistant to different metals. The identification of resistance against different metals may provide a useful tool for the simultaneous monitoring of several toxic pollutants in the environment. It is clearly indicated that domestic waste and industrial waste are responsible for the development of bacterial resistance along with the risk of human health and environment. Among all the isolates showed resourceful tolerance against all the heavy metals used. Hence, this species can be used as a bioremediation tool for the treatment of effluent from leather and other industries handling heavy metals.

Table 1 Characteristics of bacterial isolates from tannery effluents

S.No	Sample code	Simple staining	Negative staining	Gram's staining	Amylase test	Mackonkey Agar	Mannitol Salt Agar
1	Isolate 1	+	-	Gram negative	-	+	-
2	Isolate 2	-	+	Gram positive	-	-	+
3	Isolate 3	+	-	Gram positive	+	-	-

- **Negative; + Positive**

Starch Hydrolysis Test **Mannitol Salt Agar**

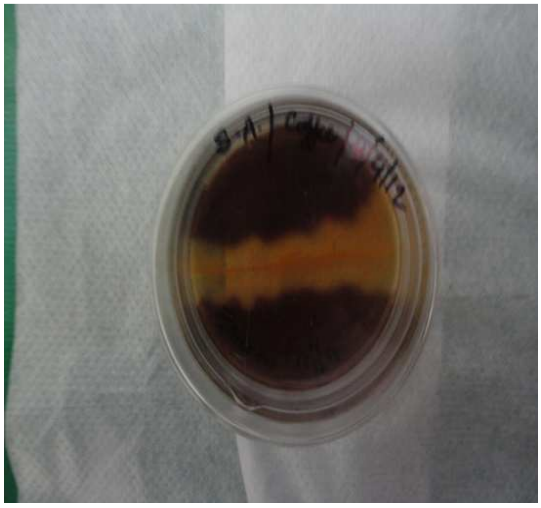

Fig 1: Starch hydrolysis test showing growth of *Bacillus subtilis*

Fig 2: *S. aureus* colonies have yellow zones at 48 h when grown on Mannitol Salt Agar

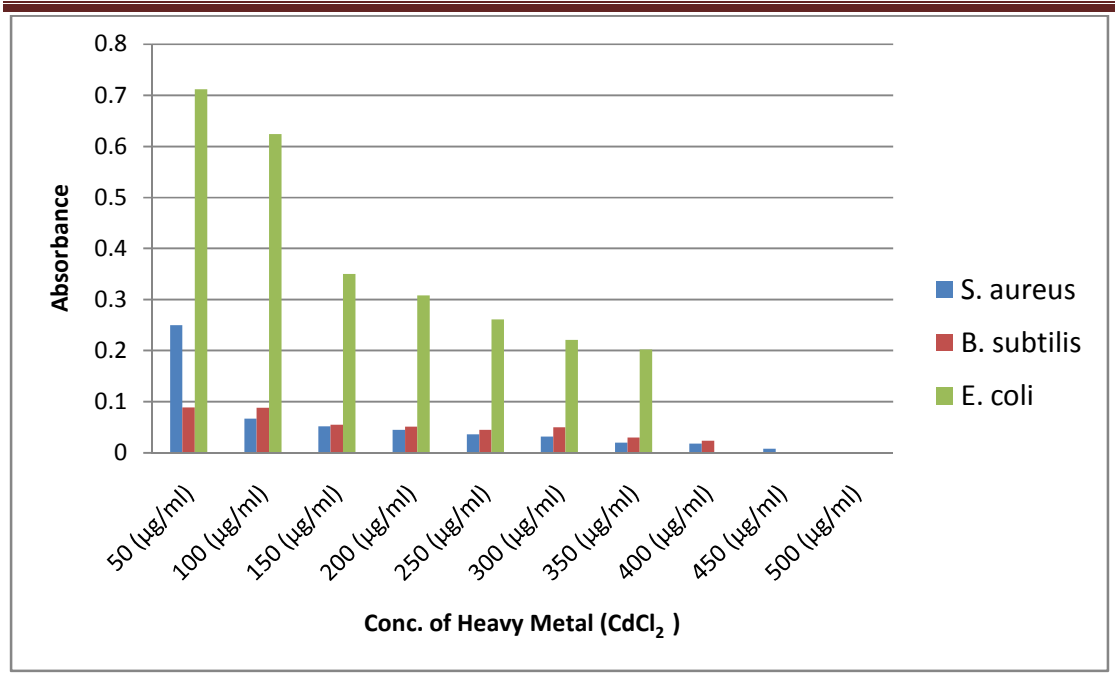

Fig: 3 Graphical representation of Heavy Metal Resistance Testing of Cadmium Chloride against Microorganisms by Broth Method

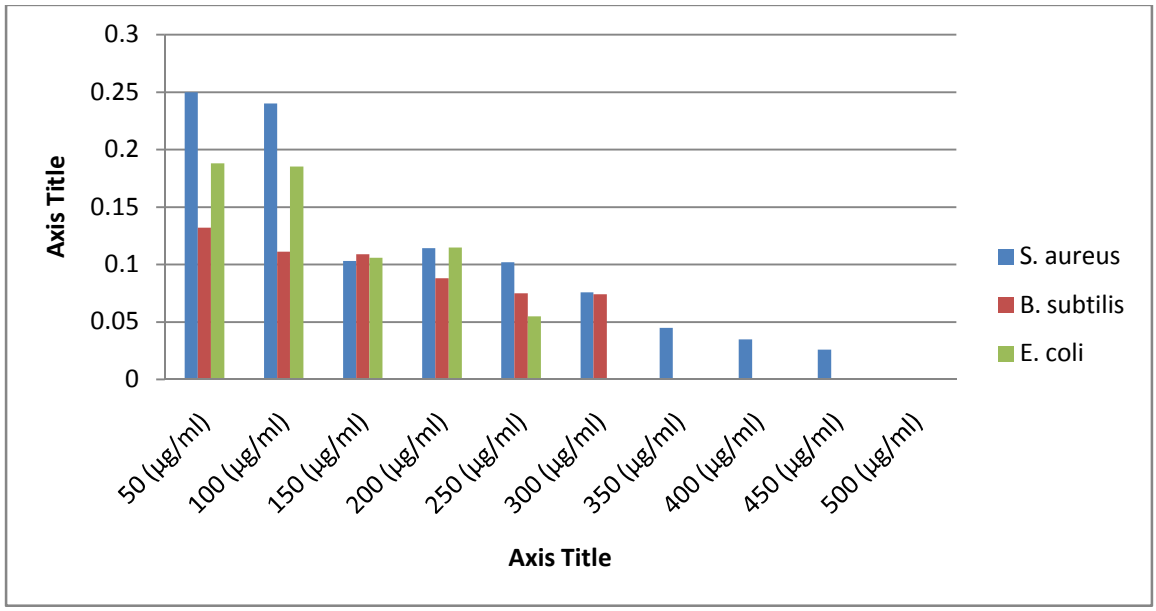

Fig: 3 Graphical representation of Heavy Metal Resistance Testing of Nickel Chloride against Microorganisms by Broth Method

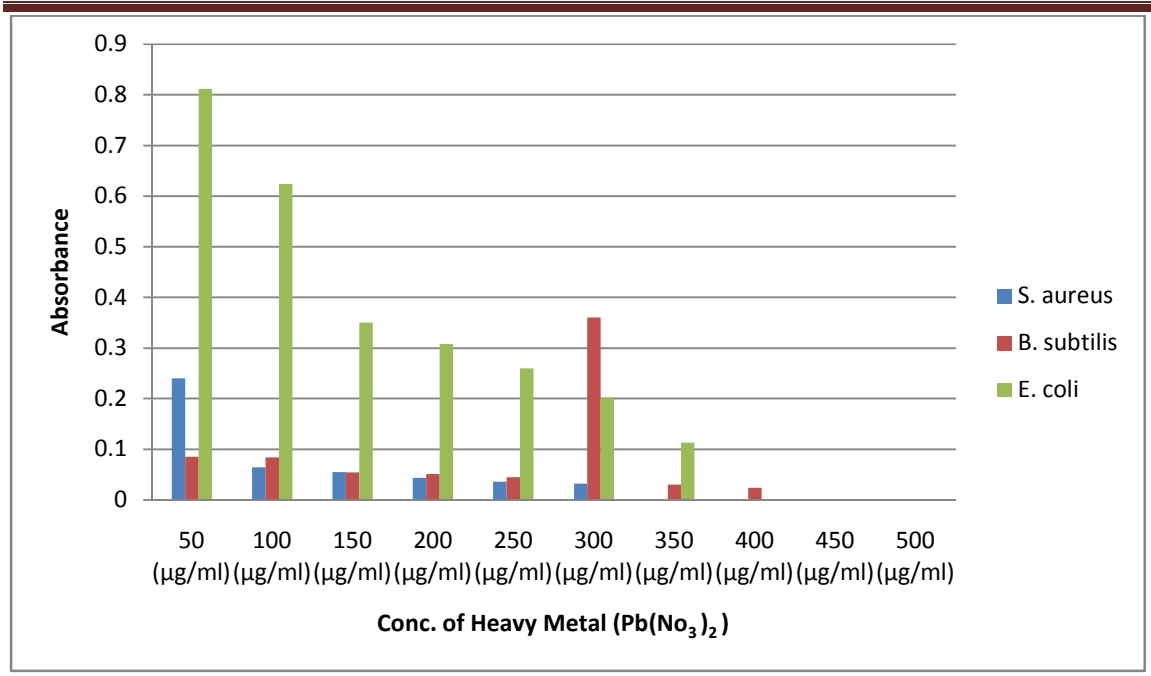

Fig: 4 Graphical representation of Heavy Metal Resistance Testing of Lead Nitrate against Microorganisms by Broth Method

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