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RESEARCH ARTICLE

MULTIPLE METAL RESISTANCES AND ITS CORRELATION TO ANTIBIOTIC RESISTANCE IN BACTERIA ISOLATED FROM INDUSTRIAL EFFLUENT

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ABSTRACT

The different microorganisms such as bacteria, fungus has ability to treat water contaminated with heavy metal. The study carried out for heavy metal, removed by bacteria isolated from infected area to treat contaminated water collected from effluent of industries. Characterization of isolated bacteria showed resistance to heavy metal. The total 73 bacterial isolate were screened, using LB agar. Three resistant bacteria were selected using different agar media containing different concentration of Ar, Cu, and Zn. These bacteria showed optimum growth when incubated for 24 hour at 30℃. The isolates were further examined using different heavy metal concentration.The selected organisms which show higher resistance to metal was identified as *Bacillus, Staphylococcus* and *Klebsiella* .antibiotic resistance of heavy metal tolerance was analyzed in detail showing resistance against Ampicillin and ciprofloxacin and minimum inhibitory concentration at 100µg/ml and 120µg/ml for *Klebsiella* and *Staphylococcus* respectively.This result reveals that, the bacterial strain shoes remarkable tolerance against heavy metal could be potent agent for the development of soil inoculum applicable in bio-augmentation of heavy metal sites.

Key words: Bio-augmentation, industrial effluent, *Staphylococcus,* heavy metal degradation, growth factor.

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INTRODUCTION

The related anthropogenic activities lead to substantial release of toxic metals into the environment purposely. Heavy metal pollution is generating hype in recent years. With the rapid expansion of many industries (mining, surface finishing, energy and fuel producing, fertilizer, pesticide, metallurgy, iron and steel, electroplating, electrolysis, etc.), wastes comprising heavy metals are unswervingly discharged into the environment causing severe environmental pollution and threatening human life. The danger of heavy metals is intensified by their almost indefinite persistence in the environment due to their absolute nature. Heavy metals such as As, Cr, Pb, Hg, Cd and U, etc. are persistent components of industrial effluents. Even the aquatic ecosystem is triggered by the heavy metals pollution from industrial and domestic foundations, due to which there has been an increased bioaccumulation and exaggeration of toxicants in the food chain. The occurrence of these heavy metals in the environment has been a topic of great worry due to their toxicity, non-biodegradable nature and the long biological half-lives for their removal from biological tissues Aiking *et al.* (1984).

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The wastewater, discharged from various industries, contain different kinds of pollutants and these toxic pollutants contaminate the fresh water bodies posing a stern risk to the environment and living organisms Schwarzenbach *et al.* (2010). Among different types of aquatic pollutants, heavy metals are the most significant ones, because they are very toxic even atvery low concentrations and are persistent in the environment thereby, threatening the environment and biota Seiler et al (2009), Yadanaparthi et al (2009). Electroplating industry, tanneries, electronics manufacturing industry, coal-fired power plants and mining operationare key sources of heavy metal pollution in water. Thus, it is essential to treat the industrial effluents containing metals before their discharge to prevent heavy metal pollution of water (Wang and Chen,2010), An. et al (2002), Li et al (2002), Sanders et al (2009). Agrawal et al (2005).Chromium, another toxic heavy metal pollutant, is mainly present in the effluents of leather tanning, electroplating, metal finishing, textile industries ,and chromate preparations (Mohan and Pittman, 2006). World Health Organization (WHO) guideline for Cr(VI) in drinking water is 50 µg L^{-1}.In Geneva, (2011)exposure to elevated levels of Cr(VI) might lead. Metallic elements are intrinsic components of the environment. Their presence is measured unique in the sense that it is problematic to eliminate them totally from the environment.

Metal taken as a significant class of toxic material which is encountered in frequent occupational and environmental situations. The effect of these toxic element on human health is currently an region of intense interest due to the ubiquity of exposure. The increasing use of these wide verity of metals in industry and in our everyday life, problematic from the environment have serious dimensions. Technical advancements by humans which resulted in the pollution of water bodies resulting in the presence of toxic contaminants at concentrations well above the limits fixed by Environmental protection agency (EPA) and (WHO) World Health Organization (Celik and Demirbas 2005)The harms imposed to human being and aquatic life related with exposure to metals like chromium, cadmium, lead, mercury and arsenic have been well established in the literature Gonzalez et al (2005), Boening (2000), Hetherington (2007), Forray FL, Hallbauer (2000), (Kotas and Stasicka, 2000). The toxic nature of such metal ions raise due to their non degradable nature they accumulate in the living cells and damaging the normal functions of many organs of living organisms. Technologies like electrochemical separation, chemical precipitation, membrane separation, ion exchange, reverse osmosis and adsorption resins however effective for remediation of metal, yet are not economical in manufacturing application Juttner *et al.* (2000), Yang *et al.* (2001), Bose et al (2002),Wingen felder *et al.* (2005), Matis *et al.* (2004), Chakravarti *et al.* (1995).

Biosorption is the features exhibited by inactive, non-living matters of biological origin to bind and to gather metal ions from aqueous solution Dabrowski (2001), Volesky (2001)In other words the biomass interaction with metal ions is physicochemical, metabolism independent process with the underlying mechanism being surface complexation, absorption,, ion exchange, adsorption, and precipitation Aksu (2005), (Stasinakis and Thomaidis, 2010), Tsezos (2001). The another technology using living biomass for heavy metal removal is bioaccumulation, It is an active process reliant on the living organism (Ahluwalia and Goyal, 2007, Ahemad and Malik, 2011). But the toxic nature of metal ions interrupt metal uptake by living organisms and that's why bioaccumulation has lesser efficiency and lower kinetics as compared to biosorption. Bioremediation by microbes such as bacteria, fungi, algae and some agricultural wastes that emerged as an eco-friendly, effective and low cost material option could offer potential inexpensive alternatives to the conventional adsorbents (Vallsand Lorenzo, 2002) Different species of *Aspergillus, Pseudomonas, Sporophyticus*, *Bacillus, Phanerochaete*, etc., have been reported as efficient chromium and nickel reducers. The response of microorganisms towards toxic heavy metals is very important for reclamation of polluted sites Congeevaram *et al.* (2007). Thus, there is the need to degrade this heavy metal from waste water using microorganism before it mix with river water. For this point of few, this study was undertaken to study heavy metal degradation.

MATERIALS AND METHODS

Sampling and pre-treatment: Effluent from Iron- steel industry and plastic industry Richai (industrial area in Jabalpur), were obtained from the sedimentation tank of the the industry. Before collection water was allowed to settled at room temperature followed by decanting the supernatant sample were collected in pre-sterilized amber bottles pH, temperature, color was observed and B.O.D was locked and sample was kept at 4 degree in ice jacket and was taken to microbiology lab St. Aloysius college For further study Sample was processed within 24 hours .All these experiments was performed in replicates.

Heavy metal determination: Three heavy metals (Cu, Zn, Ar) in the waste water effluent were determined Spectrophotometry. (Following the standard method of APHA 1981)

Isolation of heavy metal-tolerant bacteria

Metal Tolerant bacterial isolates were collected from different effluent with elevated levels of Cu, Ar, Co, Cr (prepared as 10 % stock solutions of CuSO4, Arcl2, ZnSo4 salts) and metal mixture Bestawy *et al.* (2012) Tolerant bacteria were isolated from the final stage of the metal enrichment experiment .One-milliliter liquor from each reactor was used to inoculate liquid Luria–Bertani (LB) medium containing the investigated heavy metal (sterilized by filtration) in that reactor. Heavy metal concentrations used during bacterial isolation were 15, 100, 40 and 40 mg/l for the individual Cu, Ar, and Zn respectively. While a mixture of Cu, Ar, Zn and Co at 2, 10, 10 and 10 mg/l respectively was used for isolation of strains with multiple accumulation ability. After 24 h incubation at 30 _C, 100 ll of the grown culture was transferred into 10-ml fresh LB medium containing the same metal concentration and left for another24 h under the same conditions. The highest metal concentration reduced the activated sludge activity by 50 %was amended in Petri dish containing 20 ml sterile LB agar and mixed well. 100 ll of the grown culture was spread on the surface of the plate and incubated for 24 h at 30 _C. Large identical colonies from each plate were picked out and cultured for purification and identification.

Screening for heavy metal resistance pattern

Effect of the individual metals on the growth (measured as optical density, OD at 600 nm) An overnight culture of each strain was inoculated in 20-ml LB medium supplemented with definite concentrations of the corresponding heavy metals and incubated for 24 h under the previously mentioned conditions (Gupta and Kumar, 2012).

Determination of Antibiotic resistance

The antibiotic discs contains antibiotics, Penicillin101U),tetracycline(30µg),ampicillin(10µg),gentamycin(10µg), cephalexin (30µg),erythromycin(5µg), streptomycin (10µg), ciprofloxacin (5µg). The strain was tested for its sensitivity to the 8 antibiotic. The 0.1 ml culture was spread on LB agar plate .the antibiotic disks were placed on plate and incubated at 37˚c for 1-2 days. Ampicillin and ciprofloxacin was used for MIC determination Ayten *et al.* (2015) .

Treatability studies

The most efficient strains for metal removal were selected based on the resistance screening bioassays and manipulated in effluent waste treatment contaminated with heavy metals and organic matter. The acclimatized sludge was prepared by centrifugation to remove the supernatant which was replaced by wastewater. The resistant strain for each investigated metal was seeded individually in LB-medium and incubated at 30˚C and 200 rpm for 24 hour. Cultures were centrifuged and the supernatants were replaced with wastewater and used as

inoculation for batch reactors for the treatability study with moderate concentrations of heavy metals. Three sets of 500-ml reactors were used to compare the removal efficiencies of the effluent, bacterial strains and mixture of both.

Statistical analysis

Triplicate measurement was done in all the case during the observation and assessment of bacterial growth incorporated with different level of heavy metal. Data were captured into Microsoft Excel Software, version 2010 which was used to calculate mean standard deviation .Student's t-test was applied to confirm that the observed changes were statistically significant.

RESULTS

Physicochemical characteristics of chemical waste: sample was collected from Richai area of Jabalpur with latitude 23.2296067 and longitude 79.9766125 .the temperature and pH was noted at the site and was found to be 32.5°c and 4.2 respectively and other physiochemical parameter were also above BIS limit (Słomczynska and Słomczynski, 2004) (table 1).

Table 1. Physiochemical parameter of collected water sample

Temperature	32.5°C
pH	4.2
TDS	19.4mg/lit.
TSS	21.6mg/lit.
DO	7.6mg/lit.
COD	73.43mg/lit.
BOD	23.76mg/lit.

Identification of bacterial isolates: sample was analyzed for the presence of heavy metal tolerant bacteria. Potent bacterial isolates which are resistant to antibiotics were identified using biochemical test and staining .Isolated strains were preliminary identified according to Bergey's manual of study and result were analyzed using PIB win. The result are presented in (Table 2).

Table 2. Biochemical identification of isolated bacterial strain

Characteristics	*Bacillus*	*Staphylococcus*	*Klebsiella*
Catalase	+ve	+ve	+ve
Oxidase	Variable	-ve	-ve
Urease	-ve	+ve	+ve
Gas production	-ve	-ve	+ve
Indole	-ve	-ve	-ve
Methyl red	-ve	+ve	-ve
Nitrate reduction	+ve	+ve	+ve
Gelatin hydrolysis	+ve	+ve	-ve
Citrate utilization	+ve	+ve	+ve
Glucose utilization	+ve	+ve	+ve
Lactose utilization	Variable	+ve	+ve
Maltose utilization	+ve	+ve	+ve
Xylose utilization	+ve	-ve	+ve
Pigment	-ve	+ve	-ve

Metal process ability: eavy metal processing capacity of all the potent bacterial isolates was checked by adding Cu, Ar, and Zn in culture media. Minimum metal tolerance for *klebsiella* was 900µg/ml, *Staphylococcus* 700µg/ml, and *Bacillus* for copper 500µg/ml. These activity was analyzed both quantitatively and qualitatively Acheampong *et al.* (2010). (fig.1.)

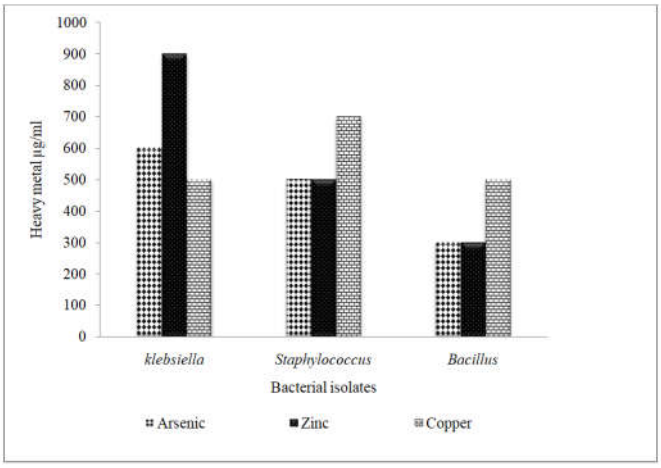

Figure 1. Metal tolerance concentration of Heavy metals against bacterial isolates

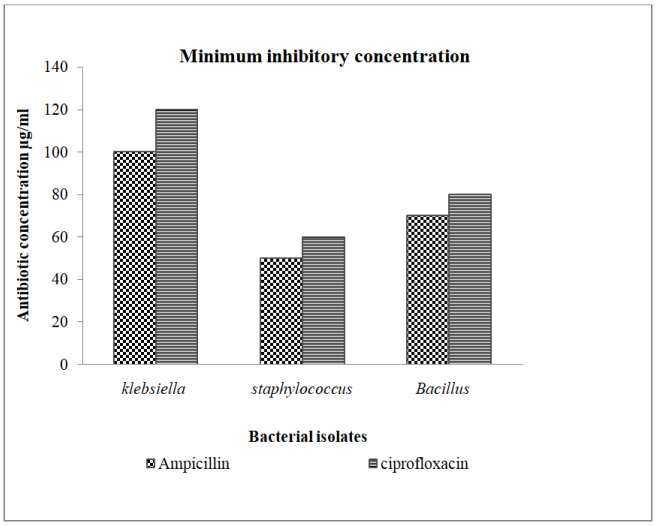

Figure 2. Antibiotic sensitivity profiling of selected isolates

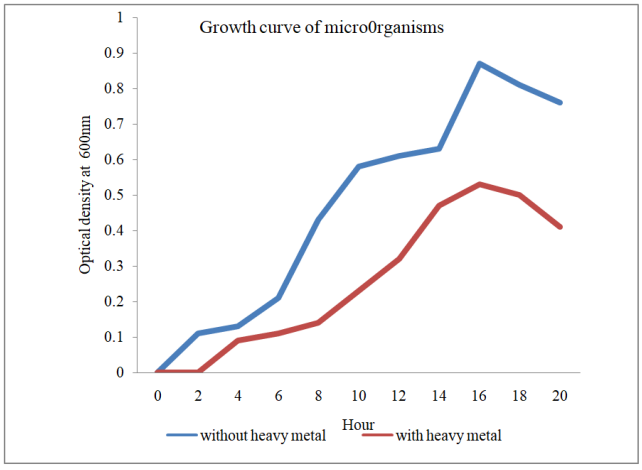

Figure 3. Growth of bacterial isolates with and without heavy metal

Antibiotic profile*:* IC for antibiotic for all selected bacterial isolates was determined among all the bacterial isolates *Klebsiella .and Staphylococcus* and *Bacillus* showed high degree of resistant against ampicillin and ciprofloxacin. susceptibility test of isolated bacteria strain against different antibiotic is shown in: *Klebsiella* shown highest resistant against Ciproflaxacin but was sensitive to penicillin and streptomycin similarly *Staphylococcus* and *Bacillus* showed

resistivity against ampicillin and ciprofloxacin. Ability of microorganisms to resist of antibiotic and heavy metal seems to be result of exposure to heavy metal contaminated environment that cause co-incidental selection of resistant factor for antibiotic and heavymetal (fig.3.)

DISCUSSION

Even metal exert their toxic effect on microorganisms through various mechanisms ,and metal-tolerant bacteria could survive in these habitats and could be isolated and selected for their potential application in the bioremediation of contaminated sites Piotrowska- seget *et al.* (2005) .In recent study ,we have isolated bacteria which could be resistant to heavy metal. Based on the relative ability of Cu, Ar, Zn solubilization of the tested strains (table-1),bacterial strain *Staphylococcus, Bacillus* and *Klebsiella*was selected as the most active strain for the experiments. Preliminary screening of collected samples for heavy metal resistant ability showed that all samples were positively grown utilizing heavy metal (Cu, Ar, and Zn) in their culture media .Serial dilution of all sample yield distinct isolates from the heavy metals resistant bacteria population based on their morphology. The bacterial isolates were than characterized by morphologically, biochemical tests, multiple heavy metal resistance capacity, MIC and comparative heavy metal degradation capacity .Identification of bacterial isolates were done according to Bergey's Manual of determinative bacteriology Borrow et al (1974), Bergey *et al.* (1974).

Bioremediation is considered to be most safe and secure technique as it depend on micro organisms that found naturally in soil and cause no harm to environment and the people who living in that region. Bioremediation is the method which can be simply carried out on area without making major disturbance of normal actions and threats to environment and human throughout transportation. Bioremediation is less effective than other technique that is used for removal of hazardous waste. Yet still few sources of bioremediation for example bacteria, fungi, algae, yeasts, and plants are available, but, the biological management by itself is not sufficiental one to treat the pollutants. Different forms of biological organisms has a different requirements for their growth (temperature, nutrients, pH etc.) so we have to find and isolate those types, which can cultured without difficulty in the laboratories, with minimum requirement and can be helpful in treatment of different forms of pollutants. A comparative study of area wise and pollutant type data is required to decide the priority area and the need for the effective removal of the pollutant from the contaminated area. Our bacterial isolates were identified as *Staphylococcus, Bacillus* and *Klebsiella* which was resistant to ampicillin and ciprofloxacin. As regular use of resources are main assets to humans their adulteration for causing long time effects of pollution (radiation and noise), global warming, greenhouse gases and ozone layer depletion. The sanitization of these natural assets is essential for the conservation of environment and nature by using bioremediation method (Figure 3). The bioremediation method may be conducted by the microorganisms, which naturally found the water and soil environment undergoing cleansing, or by other microorganisms, that obtain from differenten vironments. There are many microbes that can be used to eliminate metal from environment, such fungi, yeast, bacteria, and algae (Davis and. Vieira Wat, 2000). Solid and liquid wastes have many type of heavy metals may be effectively treated by different methods of biotechnology Many metals can be oxidized and reduced by definite. enzymes of microorganisms. Microbial metabolismproduce products such as oxygen, hydrogen, that can be use for metal oxidation/reduction. The reduction oroxidation of metals is typically accompanied by metalprecipitation or solubilization. Metals solubilization and precipitation may also be mediated by microbial metabolites. Microbial production of CO and phosphate will increase precipitation of non-solubilized phosphates, sulfides and carbonates of heavymetals like arsenic, cadmium, copper, nickel, lead, mercury, chromium, ;production of H2S by bacteria of sulfate-reducing particularly important for removal of heavy metals and radionuclides from liquid waste of nuclear facilities, wood straw or saw dust, sulfate-containing mining drainage waters, tailing pond of hydrometallurgical plants. Production of organic acid from cellulose by anaerobic fermentation, may taken as source of reduced carbon for reduction of sulfate and advance precipitation of metals (EPA2006).

The microbial cells surface is covered by negatively charged phosphate and carboxylic groups, and amino groups which are positively charged. So, depending onpH, there may be significant adsorption of heavy metals to the microbial surface Irobi, et al(1996). Biosorption, by example of fungal fermentation residues, isused to collect uranium and other radionuclide efrom waste water. Minerals having metals such as sulfides can be oxidized and metals can be solubilized. This process issued for the bioleaching of heavy metals from sewagesludge before biotransformation or land filling Xiang et al (2003) For example, metal such as arsenic and mercury may be volatilized by methylation due to theaction of anaerobic microbes. Methylation of arsenic by methanogenicbacteria and fungi to volatile toxic trimethylarsineanddimethylarsineor can be transformed to less toxic non-volatile dimethylarsinic and methane arsenic acids through algae. Thus, there is a fundamental need to study the effect of various microorganisms in amalgamation against a variety of pollutants for the conservation of natural resources and environment protection. Bacteria is one of vital microbial applicant which needs to be broadly explored for their ability of bioremediation and though, studies have been proceed out in the said region, more broad and comprehensive studies need to be approved out for extracting bacteria systems as "heavy-metal contamination alleviators".

Conclusion

Of the all the isolates recovered at different levels metal concentration, total of 73 bacterial was isolated. Toxicity to metals at different concentration were checked both on solid and liquid media. Result of solid media substantiates liquid medium growth profile. The availability of metal compound is higher in liquid than in solid medium. Thus, a particular concentration becomes more toxic for organisms while grown in liquid broth. Therefore, the tolerance level for metal was greater in solid medium than liquid medium. Biodiversity of heavy metal resistant bacteria was assist from 2-20 hours. Nutrient broth without heavy metal was used as positive control. After 8 hours optical density found to be 0.14 which increased to 0.53 in 16^{th} hour indicating increased in bacterial growth even in presence of heavy metal .this study shows that bacteria are capable to degrade multiple heavy metals. The use of bacteria as a tool for bioremediation is a very useful, convenient and cost-effective method. There have always been needs for the identification and isolation of new species of

bacteria, which are showing resistance to pollution such as heavy metal, ammonia, hydrocarbons etc. The presence of bacteria capable of tolerating heavy metal from soil samples from heavily contaminated petroleum sites was investigated. Bacteria that resist high level of heavy metal were isolated in pure culture. In summary our result that isolate strain characterized with remarkable tolerance against heavy metal ,could be potential agents for the development of a soil inoculants applicable in bio augmentation of heavy metal polluted sites. The genetic modification of bacteria can be opened new horizon of bioremediation in heavy metal pollution .genetic upgrading may help to expand the field of accessible methodologies to refining process. Regardless the genome sequencing of microorganisms and use of proteomics, genomic, metabolites that could be involved in heavy metal tolerance.

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Conflict of interest: Author declares that they do not have any conflict of interest.

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