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Bacterial resistance to chosen heavy metals

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ABSTRACT

The present study is aimed at determining the resistance chosen of bacteria to selected heavy metals. The bacterial isolates were able to tolerate or resist the presence of selected heavy metals like Cd, Cu, Zn, Fe, Mn and Hg. Identification tests for all the bacterial isolates revealed that they to belong to the genera *Aeromonas*, *Escherichia*, *Klebsiella*, *Pseudomonas*, *Proteus* and *Bacillus*. The control experiment showed heavy growth of all the organisms. All the bacterial strains showed resistance against the heavy metals with Minimal Inhibitory Concentration (MIC) values ranging from 50 to 1000 ppm.

KEYWORDS

Heavy metals;
Bacteria;
Resistance;
Minimal inhibitory concentration.

INTRODUCTION

The continuously increasing demand for the commodities produced by chemical industries has triggered heavy metals accumulation in the ecosystem. Mining and metallurgical wastewaters are considered to be the major sources of heavy metal contamination in the environment. Heavy metal contamination exists in aqueous waste streams from diverse industries, such as, metal plating, mining, tanneries, painting, car radiator manufacturing, batteries as well as agricultural sources, where fertilizers and fungicidal sprays are intensively used. Cadmium, copper, iron, lead, nickel, zinc, cobalt, mercury and chromium are harmful heavy metals discharged by industries that pose a risk of contaminating groundwater and other water resources. Heavy metals are not biodegradable and tend to accumulate in living organisms, causing various diseases and disorders[10,34]. Safe and effective disposal of effluents containing heavy metals based on green chemistry is always a challenging task for industrialists and environmentalists as cost-effective treatment alternatives are not available. Conventional technologies for the removal of toxic heavy metals, such as, chemical precipitation, ion exchange or electrochemical processes, are often uneconomical, especially, when used for the removal of heavy metal ions at low concentrations. Biosorption technology based on the ability of biomass to remove metallic ions from aqueous solutions and its potential for industrial effluent treatment has received wide attention[8]. Different types of biomass, such as, algae, bacteria, fungi, yeast and plant based polysaccharides, have been successfully employed to clean-up the industrial effluents[25].

Generally, the higher concentration of heavy metals above threshold levels has deleterious impact on the functional activities of microbial communities in soils. Otherwise, microorganisms exposed to the higher concentrations of toxic heavy metals may develop resistance against the elevated levels of these metals[28]. In addition, microorganisms inhabiting metal polluted soils

have evolved various strategies to resist themselves against metal stress[14]. Such metal resistant microorganisms can be used as successful bioremediation agents[20]. In the present work an attempt has been made to study the resistance of selected bacteria to heavy metals.

MATERIALS AND METHODS

Isolation of bacteria

The soil and water samples were collected from different environmental sources in and around Madurai district, South India. The samples were collected in sterile plastic containers and transported to laboratory for bacteriological analysis. The samples were serially diluted and plated on nutrient agar medium. Plates were incubated at 37°C for 24 hours. The isolated pure colonies of the test organisms were cultivated in nutrient broth for eight hours.

Identification and characterization of the bacteria

Selected isolates were grown on selective media (HiMedia, India). The shape and colours of the colonies were examined under the microscope after Gram staining. Isolates were biochemically analyzed for the activities of lactose fermentation, oxidase, catalase, MR-VP test, urease, TSI-gas production, H_2S production, gelatin hydrolysis, motility, indole production and citrate utilization. The tests were used to identify the isolates according to Bergey's Manual of Systematic Bacteriology[12].

Bacterial isolation to obtain pure culture

Nutrient agar medium was prepared by adding sodium chloride (5.0 g), peptone (5.0 g), beef extract (3.0 g) and agar (15.0 g) in 1000 ml of distilled water at pH 7.0 and autoclaved at 15 psi. Isolates were plated onto nutrient agar and incubated at 37°C for 24 hours. The pure cultures obtained were inoculated into tubes containing five ml of sterile nutrient broth each and incubated overnight at 37°C.

Preparation of metal stock solutions

Stock solutions of copper, cadmium, mercury, iron, zinc, and manganese were prepared by dissolving specific quantities of their salts separately in double distilled water. Broad range of heavy metal concentrations i.e. 5, 10, 50, 100, 500 and 1000ppm were prepared and sterile paper discs were dipped in respective concentrations.

Minimum inhibitory concentration (MIC) of heavy metals

The isolated bacterial cultures were checked for their respective MICs towards heavy metal ions such as cadmium, copper, zinc, iron, manganese and mercury. A sterile cotton swab was used to collect a swabfull of the pure isolate and directly streaked on the surface of the Muller-Hinton agar plates. Arranged discs containing heavy metal concentrations were impregnated onto the surface of the inoculated plates, in which each disc is made to adhere perfectly to the surface of the agar by gently pressing. The process was repeated for each bacterium on the media incorporated with the selected heavy metals. The plates were incubated at 37°C for 24 hours. After the incubation period, the plates were observed for growth. Identified MIC plates were taken and diameters of inhibition zones were measured from one edge to the other edge with the help of graph sheet. Measured zones were recorded for each heavy metal concentration.

Atomic Absorption Spectrophotometric analysis

Measured MIC plates were taken and discs were removed from the plates. Medium present within the zone (0.5g) was scrapped and digested with concentrated nitric acid. Volume of the digested sample was adjusted by adding deionized water up to 10ml for each sample and the metal ion concentrations were analyzed using Atomic Absorption Spectrophotometer.

RESULTS AND DISCUSSION

The biochemical tests conducted for the identification of six test organisms are shown in TABLE 1. *Aeromonas hydrophila* showed negative reactions for Gram's staining, lactose fermentation, urease activity and H_2S production tests. *Escherichia coli* exhibited negative results for VP, Gram's staining, citrate agar, oxidase, urease activity and H_2S production tests. *Klebsiella sp* gave positive results for motility, VP, citrate agar, catalase and TSI- gas production tests. *Pseudomo-*

nas fluorescens showed negative results for H_2S production, gelatinase, VP, MR, lactose fermentation tests and gram's staining. *Proteus sp* exhibited negative reaction for gelatinase, oxidase, lactose fermentation tests and gram's staining. *Bacillus megaterium* showed negative reaction for indole, MR, VP, oxidase and H_2S production.

TABLE 2 shows the minimum concentrations required to inhibit the growth of the bacterial strains. All the six isolates showed MIC for heavy metals ranging

TABLE 1 : Results of biochemical tests for the identification of test organisms

Name of the test	Test Organisms					
	Aeromonas hydrophila	*E.coli*	*Klebsiella sp.*	*Pseudomonas fluorescens*	*Proteus sp.*	*Bacillus megaterium*
Gram's staining	-	-	-	-	-	+
Lactose fermentation	-	+	-	-	-	+
Motility	+	+	+	+	+	+
Indole	+	+	-	+	-	-
Methyl Red	-	+	-	-	+	-
Voges- proskauer	-	-	+	-	-	-
Simmons Citrate	+	-	+	+	+	+
Oxidase	+	-	-	+	-	-
Catalase	+	+	+	+	+	+
Urease	-	-	+	+	+	+
Gelatinase	+	-	-	-	-	+
TSI – gas production	+	+	+	+	+	+
H_2S production	-	-	-	-	+	-

+ Positive; - Negative

TABLE 2 : Inhibitory zone details for bacteria exposed to copper, mercury, zinc, iron, manganese and cadmium

Metals	MIC details	Bacterial Strains					
		E.coli	*P.fluorescens*	*A.hydrophila*	*Proteus sp.*	*Klebsiella sp.*	*B.megaterium*
Copper	MIC concentration (ppm)	100	90	200	100	85	60
	Zone diameter (mm)	19	15	17	18	20	14
	Copper concentration in the zone (ppm)	85	32	140	30	48	18
Mercury	MIC concentration (ppm)	250	220	60	110	70	70
	Zone diameter (mm)	22	28	21	24	18	15
	Mercury concentration in the zone (ppm)	200	180	48	88	31	15
Zinc	MIC concentration (ppm)	50	225	125	75	125	50
	Zone diameter (mm)	14	16	16	18	18	16
	Zinc concentration in the zone (ppm)	8	125	75	33	86	27
Iron	MIC concentration (ppm)	75	160	250	80	150	125
	Zone diameter (mm)	20	22	15	15	17	20
	Iron concentration in the zone (ppm)	35	48	110	36	41	55
Manganese	MIC concentration (ppm)	150	60	150	125	250	175
	Zone diameter (mm)	18	15	17	17	22	18
	Manganese concentration in the zone (ppm)	75	15	80	78	125	98
Cadmium	MIC concentration (ppm)	250	150	225	400	125	70
	Zone diameter (mm)	20	15	19	21	28	16
	Cadmium concentration in the zone (ppm)	110	81	121	186	62	28

from 50 – 400ppm. The highest MIC of copper to *A.hydrophila* was observed to be 200ppm. *B.megaterium* showed the lowest MIC at 60ppm. For mercury, *E.coli* showed the highest MIC at 250ppm and the least was noted for *A.hydrophila* at 60ppm. The maximum MIC for zinc to *P.fluorescens* was observed as 225ppm for *B.megaterium* and *E.coli* showed the minimum MIC at 50ppm. The highest MIC for iron to *A.hydrophila* was noticed as 250ppm and *E.coli* exhibited the lowest MIC at 75ppm. For manganese, *Klebsiella sp.*showed the highest MIC at 250ppm while the least was observed in *P.fluorescens* at 60ppm. The maximum MIC for cadmium to *Proteus sp.* was observed as 400ppm and *B.megaterium* showed the minimum MIC at 70ppm of cadmium.

E.coli exhibited the highest MIC for mercury and cadmium and the lowest at 50ppm of zinc. In *P.fluorescens* the maximum MIC for zinc was found and the minimum was observed for manganese. The highest MIC for *A.hydrophila* was observed for iron while the lowest was noted for mercury. *B.megaterium* exhibited the maximum MIC for iron and the minimum for zinc. *Proteus sp.* showed the highest MIC for cadmium and lowest for zinc whereas, *Klebsiella sp.* exhibited the highest MIC for manganese and the least for mercury.

The microbial level of resistance or tolerance of each concentration of heavy metal was depicted by the level of growth on the agar. The microbial load decreased with an increase in the concentration (0.25 mg/mL) of heavy metal indicating the toxic effect of the heavy metals on the growth of microorganisms as stated by Badar *et al*.[29]. However, no observable growth of microorganisms at high concentrations explains the theory earlier stated by Konopka *et al*.[2] that resistance mechanisms do not offer protection at extremely high levels of free metal ions and a lethal toxic effect is observed. Badar *et al*.[30] stated that bacterial resistance or tolerance can be used to minimize the effect of heavy metals on total biological activity of the ecosystem.

In the present study, *Bacillus megaterium* exhibits the highest resistance to copper, mercury and cadmium. *P.fluorescens* exhibits the second highest resistance to cadmium, when compared to the other organisms. Cooksey[13] reported that resistance against copper in the plant pathogen *Pseudomonas syringae* was mainly due to copper accumulation and compartmentalization in the cell's periplasm and the outer membrane and concluded that the protective mechanism against copper in *P. syringae* was due to four types of proteins (CopA, CopB, CopC and CopD). *E.coli* exhibits the highest resistance to zinc and iron, when compared to the other organisms. For manganese more resistance was identified in *P. fluorescens* than the other organisms. In the present study, based on the results, it can be concluded that *B. megaterium*, *P.fluorescens* and *E.coli* exhibit the highest resistance for all the metals.

In recent years, heavy metal pollution has become one of the most serious environmental problems in both developed and developing countries of the world. Heavy metal contamination of soil is widespread due to metal processing industries, tannery, combustion of wood, coal and mineral oil, traffic, and plant protection. The toxic effects of heavy metals result mainly from the interaction of metals with proteins (enzymes) and inhibition of metabolic processes. In contrast to organic pollutants, metals are not mineralized by microorganisms but can be oxidized or reduced, transformed to different redox stages, or complexed by organic metabolites[6]. Some metals are subjected to bioaccumulation and may pose a risk to human health when transferred to the food chain[18]. The presence of heavy metals even in traces is toxic and detrimental to both flora and fauna[17].

Arsenic and cadmium, for instance, can cause cancer. Mercury can cause mutations and genetic damage, while copper, lead, and mercury can cause brain and bone damage. Iron exists in two forms, soluble ferrous iron (Fe^{2+}) and insoluble ferric particulate iron (Fe^{3+}). The presence of iron in natural water may be attributed to the dissolution of rocks and minerals, acid mine drainage, landfill leachate, sewage or engineering industries. Iron in water is generally present in the ferric state. The concentration of iron in well aerated water is seldom high but under reducing conditions, it may exist in groundwater, lakes or reservoirs and in the absence of sulphate and carbonate, high concentrations of soluble ferrous iron may be found. The presence of iron at concentrations above 0.1mg/l will damage the gills of the fish. The free radicals are extremely reactive and short lived. The free radicals formed by iron on the surface of the gills will cause oxidation of the surrounding tissue

and lead to massive destruction of gill tissue and anaemia. Iron is an essential element in human nutrition and it is contained in a number of biologically significant proteins, but ingestion in large quantities results in haemochromatosis where in tissue damage results from iron accumulation[29].

Mercury is generally considered to be one of the most toxic metals found in the environment[26]. Once mercury enters the food chain, progressively larger accumulation of mercury compounds takes place in humans and animals. The major sources of mercury pollution in environment are industries like chlor-alkali, paints, pulp and paper, oil refining, rubber processing and fertilizer[9], batteries, thermometers, fluorescent light tubes and high intensity street lamps, pesticides, cosmetics and pharmaceuticals[1]. Methyl mercury causes deformities in the offspring, mainly affecting the nervous system (teratogenic effects). Children suffer from mental retardation, cerebral palsy and convulsions. Mercury also brings about genetic defects causing chromosome breaking and interference in cell division, resulting in abnormal distribution of chromosome. Mercury causes impairment of pulmonary function and kidney, chest pain and dyspnoea. The harmful effect of methyl mercury on aquatic life and humans was amply brought out by the Minamata episode in Japan[32].

The heavy metal copper is utilized by bacterial cells in small quantities in biosynthesis of metabolic enzymes like, cytochrome C oxidase. However, bacteria in different ecosystems including soil and water, are exposed to very high concentration of this metal as high levels of copper exist in soil ecosystem due to its wide application in mining, industrial processes, and agricultural practices[31]. Consequently, bacteria have evolved several types of mechanisms to defend against the high copper concentration and copper induced biotoxicity[4].

Bacillus, *Micrococcus*, *Arthrobacter*, *Sphingomonas*, and *Microbacterium* are common metal-tolerant Gram negative and Gram-positive bacteria[19]. A gene cluster, czr, involved in cadmium and zinc resistance was identified in *P. aeruginosa* CMG103[27]. Bioaccumulation is an active process dependent upon metabolic energy of microorganisms. In other words, bioaccumulation is an energy-dependent heavy metal transport system. Heavy metal transport through bioaccumulation has been reported in many genera like, *Citrobacter sp*. (lead and cadmium), *Thiobacillus ferrooxidans* (silver), *Bacillus cereus* (cadmium), *Bacillus subtilis* (chromium), *Pseudomonas aeruginosa* (uranium) *Micrococcus luteus* (strontium), *Rhizopus arrhizus* (mercury), *Aspergillus niger* (thorium) and *Saccharomyces cerevisiae* (uranium)[3,21].

CONCLUSION

Among the six bacterial strains tested for resistance against the selected heavy metals, *Bacillus megaterium*, *Pseudomonas fuorescens* and *Escherichia coli* exhibited the highest resistance for all the six metals.

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