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Study on Heavy Metal Resistant Bacteria in Guheswori Sewage Treatment Plant

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Abstract
Removal of heavy metals from wastewater needs advance chemical technology and is more expensive too. The cheaper alternative for this is the bioremediation using heavy metals resistant microorganisms. In this study, 10 heavy metal resistant bacteria were isolated from oxidation ditch of wastewater treatment plant of Bagmati Area Sewerage Project. These include chromium resistant *Staphylococcus* spp, *Escherichia coli, Klebsiella* spp; cadmium resistant *Acinetobacter* spp*, Flavobacterium* spp, *Citrobacter* spp; nickel resistant *Staphylococcus* spp, *Bacillus* spp; copper resistant *Pseudomonas* spp; and cobalt resistant *Methylobacterium* spp. All the isolates showed high resistance to heavy metals with Minimum Inhibitor Concentration (MIC) for heavy metals ranging from 150 µg/ml to 500 µg/ml. Six resistant isolates showed multiple tolerance to heavy metals. All the 10 isolates also showed antibiotic resistance of which 10% were resistant to single antibiotic and 90% were multi-antibiotic resistant. Heavy metal tolerance test showed maximum microbial tolerance to chromium and minimum tolerance to nickel in mixed liquor sample of oxidation ditch.

Key words**:** Heavy metal resistant bacteria, multiple tolerance, antibiotic resistance, Guheswori Sewage Treatment Plant

Introduction

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 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.

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, extracellular precipitation and enzymatic oxidation or reduction to a less toxic form, and efflux of heavy metals from the cell has been reported (Mergeay, 1991; Hughes and Poole, 1991; Nies, 1992; Urrutia and

Beveridge, 1993; Joshi-Tope and Francis, 1995).

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. This study aimed to isolate and identify heavy metal resistant bacteria from Guheshwori Wastewater Treatment Plant situated at the bank of Bagmati River near Guheshwori temple.

Materials and methods

Sample collection

The sampling area was the wastewater treatment plant at Guheshwori constructed by high-powered committee for implementation and monitoring of the Bagmati Area Sewerage Construction / Rehabilitation Project (BASP). The sampling site was the oxidation ditch of the treatment plant. It is the aeration tank where biological treatment of wastewater takes place.

Samples were collected in sterile plastic bottles from the surface of oxidation ditch. A total of eight samples were taken for the study.

Isolation and identification of heavy metal resistant bacteria

For the selective isolation of heavy metals resistant bacteria, heavy metals incorporated media were used. Basal media nutrient agar (NA) incorporated with heavy metals like Cd^{2+}, Ni^{2+}, Co^{2+}, Cu^{2+}, and Cr^{6+} were prepared separately. The concentration of each heavy metal was maintained at 100 μg/ml of the medium. The wastewater sample from oxidation ditch was directly streaked on these media and incubated at 25°C for 24-48 h. After the incubation period the plates were observed for any kind of growth on the media. The isolated and distinct colonies on these selective media were subcultured repeatedly on the same media for purification. The pure culture was identified on the basis of their morphology and biochemical characters.

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, 10 μg/ml each time on NA plate until the strains failed to give colonies on the plate. The starting concentration used was 100 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 failed to grow on plates even after 10 days of incubation (Shakoori *et al*., 1998).

Determination of co-resistance to other heavy metals

Various bacterial isolates resistant to one heavy metal were tested for their resistance to the rest of the heavy metals chosen for this study. The starting concentration of heavy metals in this test was 50 μg/ml, which was gradually increased 10 μg/ml each time until MIC was achieved.

Determination of antibiotic sensitivity and resistance pattern

Antibiotic sensitivity and resistance of the isolated heavy metal resistant isolates were assayed according to the Kirby-Bauer disc diffusion method given by Bauer *et al*. (1996) (Mackey and McCartney, 1996). After incubation, the organisms were classified as sensitive or resistant to an antibiotic according to the diameter of inhibition zone given in standard antibiotic disc chart.

Determination of microbial tolerance of mixed liquor of the oxidation ditch to different heavy metals

Tolerance of microorganisms of oxidation ditch to heavy metals was determined by pour plating the oxidation ditch sample into PCA (Plate count agar) media containing increasing concentration of heavy metals. The initial concentration of heavy metals in this test was taken as 50 μg/ml of the media. After each pour plating procedure, the plates were incubated at 30°C for 24-48 h and the total numbers of microbial colonies were counted in each plate. The concentration of heavy metals in PCA media was increased until the complete inhibition in the growth.

Results

***Isolation and identification of heavy metals resistant bacteri*a**

Antibiotic sensitivity of heavy metals resistant isolates

All the isolates were resistant to antibiotics of which cobalt resistant *Methylobacterium* sp. was found to be single antibiotic resistant while the rest of the isolates were found to be multi-antibiotic resistant while the rest of the isolates were found to be multi-antibiotic resistant (Table 3, Figure 2).

Altogether ten heavy metal resistant bacteria were isolated from the oxidation ditch of Guheswori Treatment Plant. All the isolates showed high resistance to heavy metals with MIC for heavy metals ranging from 150 μg/ml to 500 μg/ml (Table 1). All the heavy metal resistant bacteria were tested for their co-resistance to other heavy metals. Among the tested organisms, copper resistant *Pseudomonas* sp., nickel resistant *Staphylococcus* sp. and *Bacillus* sp., cadmium resistant *Acinetobacter* sp. and *Flavobacterium* sp. and chromium resistant *Staphylococcus* sp. showed multiple-tolerance to heavy metals (Table 2).

Microbial tolerance to heavy metals

The tolerance test indicated that among five experimented heavy metals, maximum tolerance was shown to chromium showing the growth of microorganisms up to 500 μg/ml and minimum tolerance to nickel showing no growth above 200 μg/ml. The microbial tolerance at each concentration of heavy metal was depicted by the microbial load on PCA plate expressed as C.F.U/ml. The microbial load decreased with the increase in concentration of heavy metals indicating toxic effect of the heavy metals on the growth of microorganisms (Figure 1).

The Guheswori Treatment Plant of BASP collects all the domestic as well as industrial wastewater from Gokarna, Bouddha, and Jorpati areas. The wastewater coming from domestic and industrial sources is the appropriate environment where the microorganisms can develop resistance to heavy metals and antibiotics. The presence of small amount of antibiotics and heavy metals in the wastewater induce the emergence of

Table 1. Heavy metals resistant bacteria.

Bacteria	Resistant to	MIC
Staphylococcus sp.	Chromium	500µg/ml
Escherichia coli	Chromium	200µg/ml
Klebsiella sp.	Chromium	150µg/ml
Citrobacter sp.	Cadmium	220µg/ml
Acinetobacter sp.	Cadmium	150µg/ml
Flavobacterium sp.	Cadmium	300µg/ml
Pseudomonas sp.	Copper	300µg/ml
Staphylococcus sp.	Nickel	150µg/ml
Bacillus sp.	Nickel	200µg/ml
Methylobacterium sp.	Cobalt	250µg/ml

Table 2. Co-resistance to other heavy metals

Organisms	**Co-resistance to**	**MIC**
Cu^{2+}-resistant *Pseudomonas* sp.	Cobalt	150µg/ml
	Cadmium	120µg/ml
Ni^{2+}-resistant *Staphylococcus* sp.	Cobalt	150µg/ml
Ni^{2+}-resistant *Bacillus* sp.	Cobalt	110µg/ml
Cd^{2+}-resistant *Acinetobacter* sp.	Copper	200µg/ml
	Cobalt	180µg/ml
Cd^{+2}-resistant *Flavobacterium* sp.	Copper	220µg/ml
	Cobalt	200µg/ml
Cr^{6+}resistant *Staphylococcus* sp.	Cobalt	150µg/ml
	Cadmium	140µg/ml
	Nickel	150µg/ml

Table:3 Antibiotic sensitivity of heavy metals resistant bacteria

SN	**Organisms**	**Sensitive to**	**Resistant to**
1	Cr^{6+}resistant *Staphylococcus* sp.	Ciprofloxacin, Vancomycin, Methicillin	Penicillin, Tetracycline, Erythromycin,
2	Cr^{6+}resistant *E. coli*	Gentamycin, Choramphenicol.	Ampicillin, Cotrimoxazole, Ciprofloxacin, tetracycline.
3	Cr^{6+}resistant *Klebsiella* sp.	Gentamycin, Cotrimoxazole	Tetracycline, Ciprofloxacin, Chloramphenicol, Ampicillin.
4	Ni^{2+} resistant *Staphylococcus* sp.	Tetracycline, Ciprofloxacin, Vancomycin.	Penicillin, Erythromycin.
5	Cd^{+2} resistant *Citrobacter* sp.	Ampicillin, Gentamycin, Ciprofloxacin.	Tetracycline, Choramphenicol.
6	Cd^{+2}resistant *Acinobacter* sp.	Chloramphenicol, Cotrimoxazole, Gentamycin, Ciprofloxacin	Tetracycline, Ampicillin
7	Cd^{+2}resistant *Flavobacterium* sp.	Chloramphenicol, Gentamycin, Ciprofloxacin.	Tetracycline, Ampicillin, Co-trimoxazole.
8	Co^{+2} resistant *Methylobacterium* sp.	Tetracycline, Ciprofloxacin, Cotrimoxazol, Ampicillin, Gentamycin.	Chloramphenicol

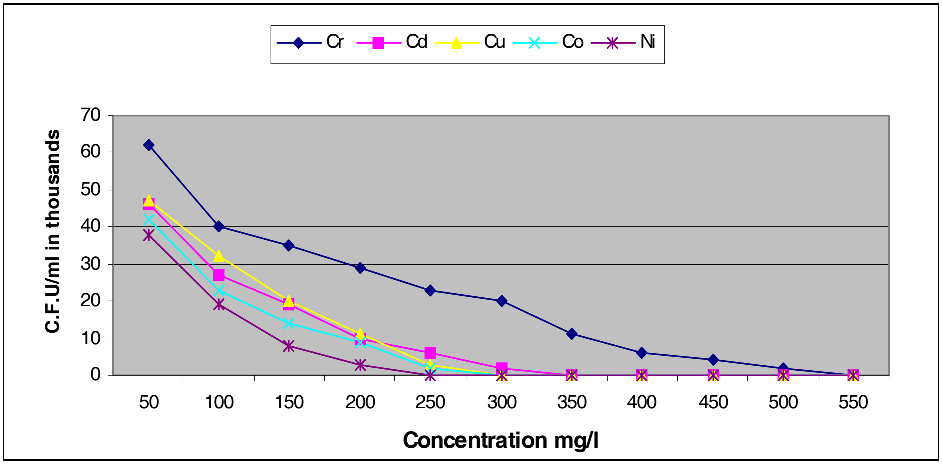

Figure 1. Microbial tolerance to heavy metals

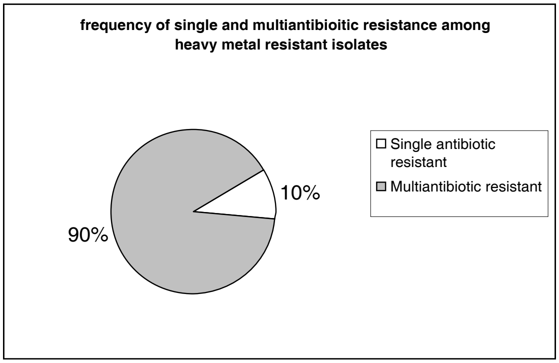

Figure 2. Antibiotic resistance of heavy metal resistant isolates

antibiotic resistant and heavy metal resistant microorganisms. Most of the isolates in the present study showed multiple tolerances to both heavy metals and antibiotics. The microbial resistance to heavy metal is attributed to a variety of detoxifying mechanism developed by resistant microorganisms such as complexation by exopolysaccharides, binding with bacterial cell envelopes, metal reduction, metal efflux etc. These mechanisms are sometime encoded in plasmid genes facilitating the transfer of toxic metal resistance from one cell to another (Silver, 1996). Filali et al. (1999) studied wastewater bacteria isolates

Psuedomonas aeroginosa, *Klebsiella pneumoniae*, *Proteus mirabilis* and *Staphylococcus* resistant to heavy metals and antibiotics. Similarly, Sharma *et al*. (2000) isolated highly cadmium resistant *Klebsiella* that was found to precipitate significant amount of CdS. The heavy metal resistant organism could be a potential agent for bioremediation of heavy metals pollution. Multiple tolerances occur only to toxic compounds that have similar mechanisms underlying their toxicity. Since heavy metals are all similar in their toxic mechanism, multiple tolerances are common phenomena among heavy metal resistant bacteria. In wastewater, there are some substances that have the potential to select for antibiotic resistance even though they are not antibiotics themselves. Heavy metals and biocides are two of them. The exposure to heavy metals or biocides results in the selection of bacterial strain also able to resist antibiotics. This happens because genes encoding heavy metals and biocides are located together with antibiotic resistance genes or alternatively because bacteria can have unspecific mechanism of resistance common to different substances including heavy metals, biocides and antibiotics (Dalsgarrd and Guardbassi, 2002). It is therefore, likely that selective pressure by one such compound indirectly selects for the whole set of resistances.

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