

Heavy Metals and Antibiotics Resistance Pattern of Bacteria Isolated from Brewery and Plastic Industries Effluent Wastes

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ABSTRACT

In this study, waste water samples from brewery and plastic industrial area were collected and used to determine the antibiotics and heavy metal resistance patterns for bacteria. The preliminary analyses of the samples were taken using atomic absorption spectrophotometer (Varian AA240). Two heavy metal resistant bacteria were isolated and identified as Bacillus sp. and Pseudomonas sp. from oxidation ditch of waste water plants using GSP agar and nutrient agar. The minimum inhibitory concentration of isolates against the heavy metals was determined using spectrophotometric analysis (Astell UV-Vis grating 752W). The identified isolates were resistant to copper, lead, chromium but sensitive to zinc and iron on their culture plates. Forty percent (40%) of Bacillus sp. were resistant to heavy metals while fifty percent (50%) of Pseudomonas sp. were resistant to heavy metal. Iron was found to have the highest percentage of sensitivity while chromium has the lowest percentage. Bacillus sp. was sensitive to all antibiotics used while Pseudomonas sp. was only sensitive to gentamycin, rifampicin and ampiclox. Thus, these heavy metal resistant bacteria could be useful for the bioremediation of heavy metal contaminated ecosystem and antibiotics that are inhibitory can be used for bacterial treatment of Bacillus and Pseudomonas species infections.

Key words: Antibiotic resistance, Bacillus sp., Bioremediation, Metal and Pseudomonas sp.

INTRODUCTION

In the past few decades, uncontrolled urbanization has caused a serious pollution problem due to the disposal of sewage and industrial effluent to waste bodies. Unlike many other pollutants, heavy metals are difficult to remove from the environment (Ren, Geng and Li, 2009). Heavy metals are recognised to be powerful inhibitors of biodegradation activities (Deeb and Altalhi, 2009). These metals cannot be degraded and are ultimately indestructible. The toxic effects of heavy metals result mainly from the interaction of metals with proteins (enzyme) and inhibition of metabolic processes. These heavy metals such as copper, cadmium, lead, zinc, nickel, mercury and chromium when accumulated in soils and water bodies could be toxic to plants, animals, humans and aquatic life (Dowdy and Volk, 1983). Each heavy metal has unique bio function or bio toxicities. The presence of non-biodegradable heavy metals in such effluents is responsible for their persistence in the food chain.

Microbes play massive role in the bio-geochemical cycling of toxic heavy metals and also in cleaning up or remediating metal contaminated environments. Micro-organisms have acquired a variety of mechanisms for adaptation to the presence of toxic heavy metals (Rajbanshi, 2008). There is increasing evidence for the evolution of metal resistance in natural population inhabiting contaminated sites (Klerks, 1989; Sibly, and Shirley, (1999). The evaluation of metal resistance is 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 (Wiener, Müller-Graf, and Barcus, 1998). To survive under metal-stressed conditions, bacteria have evolved several types of adaptation mechanisms to tolerate the uptake of heavy metal ions. These mechanisms include the efflux of metal ions outside the cell, accumulation of the metal ions inside the cell, and the reduction of the heavy metal ions to a less toxic state (Montuelle, Latour, Volat and Gounot, 1994). Antibiotic resistance in bacteria is more frequently associated and strongly correlated with metal resistance (Bell, Elliot, and Smith, 1998). The significant increase of multiple

antibiotic resistant (MAR) bacteria is observed in various aquatic systems. Human infections caused by such bacteria could be difficult to treat with drugs (Chandrasekaran, Venkatesh, and Lalithakumari, 1998). The resistance development may be due to nonspecific mechanism with gene regulation of plasmids and chromosomes, which may be heritable or transferable due to the presence of a resistance factor (R-factor) (Silver and Walderhang, 1992). To survive under metal stressed conditions, bacteria have evolved several types of mechanisms to tolerate the uptake of heavy metals ion. These mechanisms include the efflux of metal ions outside the cell and reduction of the heavy metal ions to a less toxic state (Niles, 1999; Spain, 2003).). The need to isolate and characterize pure cultures of indigenous bacteria from plastic and brewery effluents and establish their resistance to heavy metals and antibiotics led to the present study.

MATERIALS AND METHODS

Sample Collection

The sample area was the plastic effluent treatment plant at Chikason Group of Companies Limited, Nnewi, Anambra State and Brewery effluent treatment plant at Awo-mma, Imo State, Nigeria. The sampling site was the aeration tank where biological treatment of waste water takes place. Samples were collected in sterile plastic container and transported to laboratory for bacteriological analysis. A total number of four samples were collected, two from each of the sample areas.

Heavy Metal Analysis

Heavy metal analysis was conducted using Varian AA240 Atomic Absorption Spectrophotometer according to the method of American Public Health Association (APHA, 1998). The sample was thoroughly mixed by shaking and 100mls of it was transferred into a glass beaker of 250mls, to which 5mls of concentrated nitric acid was added and heated to boil till the volume was reduced to about 15.20mls. The mixture was cooled, transferred and made up to 100mls using metal free distilled water. The sample was aspirated and analyzed under the oxidizing air-acetylene flame.

Isolation, Identification and Characterization of the Bacteria

The isolation of bacteria was done using Nutrient Agar and GSP agar (a selective agar for *Pseudomonas* sp.). GSP agar has the following composition: soluble starch, 20g/l; sodium -L- glutamate, 10g/l; MgSO₄, 0.5g/l; KH₂PO₄, 2g/l; phenol red, 0.6g/l; agar 12g/l. Ten fold dilutions of the waste water samples were done before streaking on the GSP agar for the isolation of *Pseudomonas*. For the isolation of *Bacillus* sp., the waste water sample was pasteurized at 80°C for 10mins in a water bath before inoculating into the Nutrient agar. The plates were incubated at 37°C for 24 hours. The isolates were purified by streaking on Nutrient Agar. The morphological characteristics of the colonies were observed and examined under the microscope after Gram and endospore staining. Isolates were biochemically analyzed for the activities of oxidase, catalase, urease, motility, indole, TSI (H₂S) and fermentation (Glucose, Sucrose, Maltose, Galatose, Mannose, Fructose and Cellulose) tests. The tests were used to identify the isolates according to Bergey's Manual of determinative bacteriology (Cheesbrough, 2000; Holt *et al.* 1994; Manero, and Blanch, 1999).

Evaluation of Metal Resistance in Solid Agar

The isolated bacteria strains were tested for resistance. Brain heart infusion agar was prepared and respective metals (Fe, Cu, Pb, Cr, and Zn) were mixed each with agar in various concentration: 25µg/ml, 50µg/ml, 75µg/ml and 100µg/ml. The medium was poured and allowed to solidify. The isolates were streaked on Brain heart infusion agar surfaces and the plates were incubated at 37°C for 24 hours. Growth of the bacteria was determined visually as positive or negative. Relative growth of the bacterial isolates was expressed as the percentage of those obtained in untreated control which was taken as 100% (Schmidt and Schlegel, 1994).

Determination of Minimum Inhibitory Concentration

Metal tolerance was evaluated as the minimum inhibitory concentration (MIC) of the metals. The metal tolerance was determined for *Bacillus* sp. and *Pseudomonas* sp. by Broth dilution method. The bacterial organisms were grown on peptone water for 24 hours at 37°C. The concentration of each metal was mixed with brain heart infusion broth that is 0.05g of metal in 100ml of medium. The mixture was done separately for each metal. Then 3ml suspension of grown organism and 10ml of medium were pipetted on test tubes for each metal and incubated. Based on the evaluation, MIC was determined at 37°C for 24 hours and was observed by spectrophotometer model Astell

UV- Spectrophotometer (UV- Vis grating 752W) at 660nm. It was considered as the MIC of bacterial against heavy metals (Schmidt and Schlegel, 1994).

Determination of Antibiotics Resistance

The antibiotics resistance was done by standard agar disc diffusion method on Muller Hinton agar using commercial discs. After solidification of agar, 0.1ml of fresh bacterial cultures was spreaded on Muller Hinton agar and the following antibiotics were placed: Ciprofloxacin, Norfloxacin, Gentamycin, Amoxil, Streptomycin, Rifampicin, Erythromycin, Chloramphenicol, Ampiclox and Levofloxacin. The plates were incubated at 37°C for 24 hours. Inhibition of the zones in diameter were measured in millimetre using a pair of divider and meter rule and also classified based on resistance or sensitive according to National Committee for Clinical Laboratory Standard (NCCLS, 2002).

RESULTS

In this study, two genera of bacteria namely *Bacillus* sp. and *Pseudomonas* sp. were isolated from brewery and plastic waste water samples. Morphological characteristics and physiological characteristics were carried out for the isolates. Morphologically, *Pseudomonas* sp. was Gram positive, endospore negative, shape is rod and size is 4.2mm (Table 1). On the other hand, *Bacillus* sp. was Gram negative, endospore positive, shape is rod and size is 5.25mm (Table 1). Physiologically, *Pseudomonas* sp. was catalase, oxidase and H₂S positive, while indole, urease and fermentable sugars were negative (Table 2). On the other hand, *Bacillus* sp. was catalase, oxidase, indole, Glucose, Sucrose, Galatose, Mannose, Fructose and Cellulose positive, while H₂S, urease and maltose were negative (Table 2). The preliminary analyses for the samples (brewery and plastic effluent) are presented in Table 3. The ones with remark of pass shows that they are in line with the standard according to American Public Health Association while ones with fail were not in line. Table 4 showed the isolate growth on plates, which indicated their resistance to heavy metals. Both organisms are resistant to Lead, Copper and Chromium but sensitive to Zinc and Iron as against the control. The isolates were later subjected to Minimum Inhibitory Concentration (MIC) and the results were represented in graph (Figures 1-5). The maximum percentage is 70% of iron in *Pseudomonas* sp. and 50% in *Bacillus* sp. The MIC of Chromium is found to be the same with the two isolates. The result of the Antibiotic sensitivity test was shown in Table 5. *Bacillus* sp. was highly resistant to Streptomycin but susceptible to Gentamycin while *Pseudomonas* sp. was highly resistant to Rifampicin but susceptible to Ampiclox. Antibiotic resistance of isolate against Gentamycin, Rifampicin and Ampiclox was shown in Figure 6.

TABLE 1: Morphological characteristics of isolates

Morphology	<i>Bacillus</i> sp.	<i>Pseudomonas</i> sp.
Shape and size	Rod, 5.25mm	Rod with rounded end, 4.2mm
Motility	Motile	Motile
Gram reaction	Positive	Negative
Endospore	Positive	Negative
Growth	Aerobic	Aerobic
Slant culture	Abundant, spreading	Abundant, spreading
Stab culture	Rhizoid	Filiform
Nutrient agar colonies	Flat, translucent, smooth	Raised, translucent, mucoid

TABLE 2: Physiological characteristics

Biochemical reaction	<i>Bacillus</i> sp.	<i>Pseudomonas</i> sp
Indole test	+	-
Urease test	-	-
Catalase test	+	+
Oxidase test	+	+
TSI test (Triple Sugar Iron)	-	+
Hydrogen sulphide production		+
Hydrogen sulphide production	+	+
Glucose	+	-
Sucrose	-	-
Maltose	+	-
Galatose	+	-
Mannose	+	-
Fructose	+	-
Cellulose	+	-

Key: + = Positive; - = Negative

TABLE 3: Values of the physical and chemical properties of samples

Parameter	Plstic effluent	Brewery effluent	Reference Value	Remark
pH	5.15	5.31	< 6.5 – 8.5	Pass
Turbidity (NTU)	056	855	< 200ppm	fail
Chlorine (mg/l)	1.5	1.8	< 200ppm	pass
Total suspended Solid (mg/l)	0	0	<250ppm	pass
Chemical oxygen	18.66	85.33	–	–
Biological oxygen Demand (BOD)	7.45	45	–	–
Colour	clear	pale white	–	–
Lead (ppm)	0.00	0.01	<0.05ppm	Pass
Chromium (ppm)	0.00	0.00	< 0.005ppm	Pass
Iron (ppm)	0.363	1.052	< 1.00ppm	fail
Copper (ppm)	0.012	0.068	< 1ppm	Pass
Calcium (mg/l)	2.350	5.825	< 10.00ppm	Pass

Key: - = Not detected

TABLE 4: Growth of isolates on different metals

Metal	<i>Bacillus</i> sp.	<i>Pseudomonas</i> sp.
Zinc	-	-
Lead	+	+
Copper	+	+
Iron	-	-
Chromium	+	+
Control	+	+

Key: + sign indicates “resistance”, which implies that there was growth of the organism on the solid agar plate.
 – sign indicates “sensitivity” which implies that there was no growth on the solid agar plate.

TABLE 5: Zone of inhibition (mm)

Antibiotics	<i>Bacillus</i> sp.	<i>Pseudomonas</i> sp.
Ciprofloxacin	11	ND
Norfloxacin	13.75	ND
Gentamycin	14	8
Amoxil	10	ND
Streptomycin	8	ND
Rifampicin	11	7.75
Chloramphenico	11	ND
Ampiclox	9	9
Levofloxacin	13.5	ND

Key: ND= Not determined

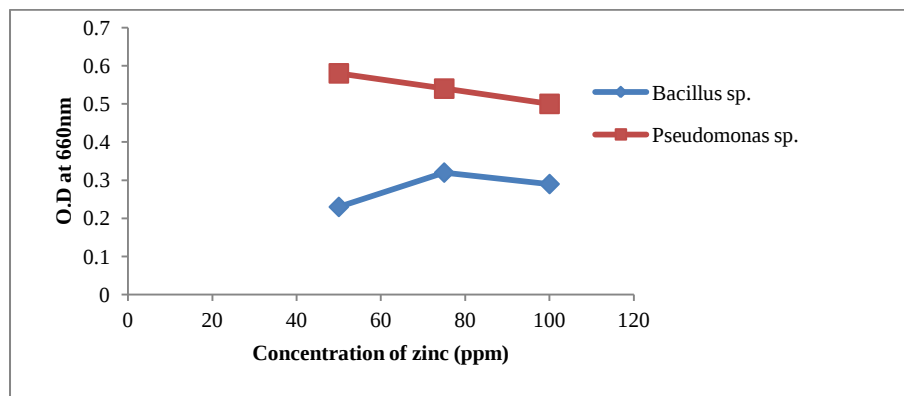


Figure 1: Resistance of the isolates against zinc

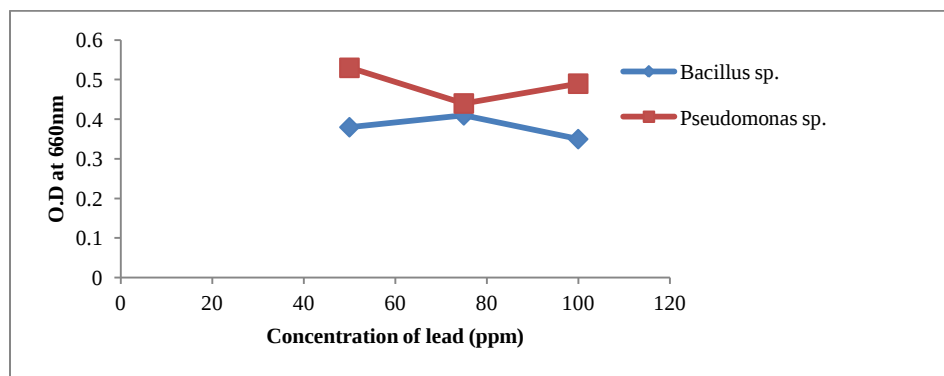


Figure 2: Resistance of the isolates against lead

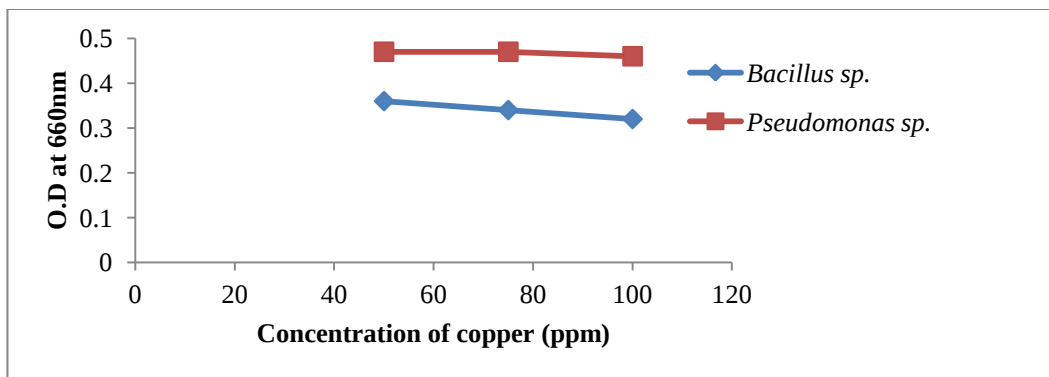


Figure 3: Resistance of the isolates against copper

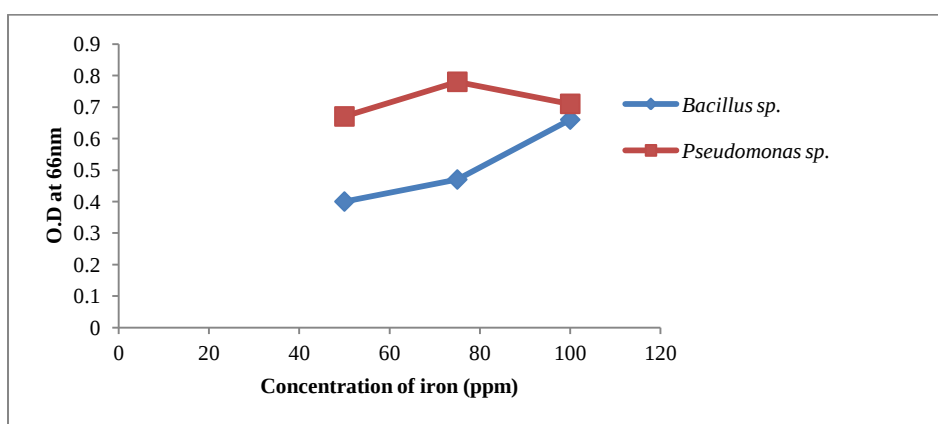


Figure 4: Resistance of the isolates against iron

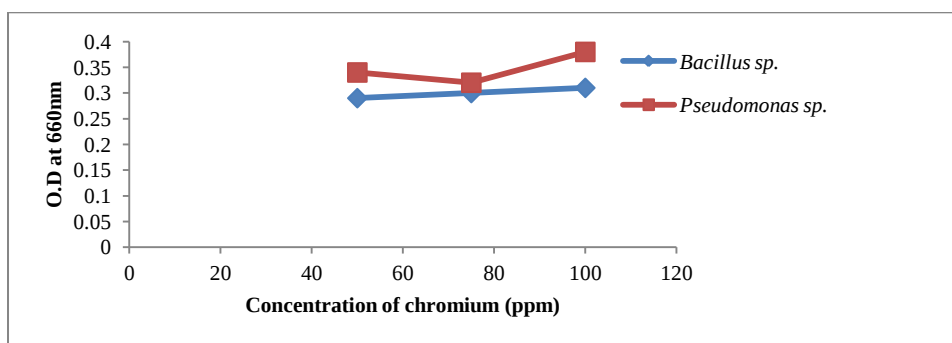


Figure 5: Resistance of the isolates against chromium

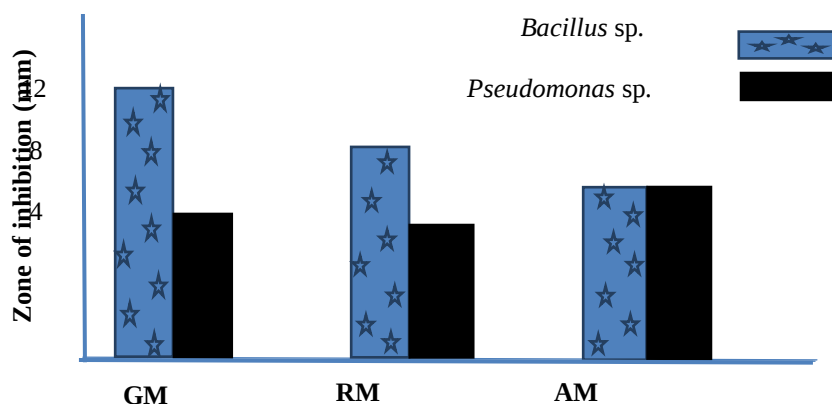


Figure 6: Antibiotic resistance of isolates against gentamycin, rifampicin and ampiclox

Key: GM – Gentamycin; RM – Rifampicin; AM – Ampiclox.

DISCUSSION AND CONCLUSION

Pollution of the environment with toxic heavy metals is spreading throughout the world and bacteria resistance to antibiotics has become a worldwide problem. Over the years, researches have been carried out to find the lasting solution to these menaces. In the present study, heavy metal resistant bacteria were isolated from brewery and plastic effluents. Aquatic organisms are sensitive to pH changes of biological treatment require pH control or monitoring the toxicity of heavy metals also gets enhanced at particular pH. Thus, pH is having primary important in deciding the quality of waste water effluent. The range of desirable pH of water prescribed for drinking purpose by ISI and WHO is 6.5 to 8.5. The average pH value of the two effluent samples collected from different industrial units vary between minimum of 5.15 and 5.31. In this case, pH values were slightly acidic which lie slightly below the permissible unit. The biological conditions such as biological oxygen demand (7.45mg/l), chemical oxygen demand (18.66mg/l) were also suitable for the microbial activities in the contaminants sample that makes the microbial consortium to bioaccumulate the metals and remediate the contaminants. Metals concentration studies in waste disposal samples have been presented in Table 3.

The heavy metal analysis also shows that brewery sample contains more of iron above its standard according to American Public Health Association (1998). The organism isolated were *Bacillus* sp. and *Pseudomonas* sp., which were characterised both physiologically and morphologically. *Bacillus* sp. is Gram positive, spore formers, produces more of carbon (IV) oxide and measured 5.25mm in diameter. *Pseudomonas* sp. is Gram negative, non-spore formers, produces less gas and measured 4.2mm in diameter. It was evident that *Bacillus* and *Pseudomonas* species were showing tolerance to five heavy metals zinc, copper, lead, chromium and iron. This difference in resistance could be attributed to metalophilic nature of *Pseudomonas* to heavy metals than *Bacillus*. The report is similar to the work done by Rajbanshi, (2008) and Tamil, Anjugam, Madhan, and Archana, (2012). The resistance of the bacterial to heavy metals was neither lost nor altered when isolates were stored in nutrient agar. The minimum inhibitory concentration determined for the five heavy metals (Zinc, Lead, Chromium, Iron and Copper) at 660nm wavelength showed that 40% of *Bacillus* sp. was resistant while 50% of *Pseudomonas* sp. showed resistant. This study showed a high incidence of metal resistance for the bacterial isolates. Bacterial species isolated from industrial zone has been shown to develop resistance to heavy metals (Osborn, Bruce, Strike, and Ritchie, 1997; Ansari and Malik, 2007).

It is also clearly seen that the *Bacillus* sp. shown sensitive to different antibiotics and that of *Pseudomonas* sp. shown resistance to ciprofloxacin, norfloxacin, amoxil, streptomycin, levofloxacin and some vulnerability to gentamycin, rifampicin and ampiclox antibiotics. The histogram chart in figure 6 shows that *Bacillus* sp. has the least resistance to those antibiotics (gentamycin, rifampicin and ampiclox) which shows that gentamycin can be used in treatment of *Bacillus* infections. The spread of multiple antibiotic resistant bacteria has been the most serious threat to the successful treatment of disease (Basu, Bhattacharya, and Paul, 2005; Ramteke, 1997). This increase in the minimum inhibitory concentration of metals as well as the antibiotic resistant 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 (Dhakepalkar and Chopade, 1994). Comparatively, the whole analyses revealed that *Pseudomonas* sp. is highly resistant to heavy metals especially iron as well as to some antibiotics while *Bacillus* sp. is less resistant to heavy metals and sensitive to most antibiotics.

CONCLUSION

The identification of resistance against different metals may provide useful tool for the simultaneous monitoring of several pollutants in the environment. It is clearly indicated that industrial waste are responsible for the development of bacterial resistance along with the risk of human health and environment. These and future studies should provide some insight into heavy metals resistance in bacteria. Therefore, it appeared that *Bacillus* sp. and *Pseudomonas* sp. can be used as bioremediation tool for the treatment of effluents from plastic, brewery and other industries handling heavy metals.

REFERENCES

- American Public Health Association (1998). Direct air-acetylene flame method standard methods for the examination of water and waste water, 20th ed. *Journal of Health*, **7**: 28-32.
- Ansari, M.I. and Malik, A. (2007). Biosorption of nickel and cadmium by metal resistant bacterial isolates from agricultural soil irrigated with industrial waste water. *Biological Review of Technology*, **98**: 3149- 3153.
- Basu, M., Bhattacharya, S. and Paul, A.K. (1997). Isolation and characterization of chromium resistant bacteria from tannery effluents. *Bulletin of Environment and Contaminant Toxicology*, **58**: 535-542.
- Bell, J.B., Elliot, G.E. and Smith, D.E. (1998). Influence of sewage treatment and urbanization on selection of multiple faecal coliform populations. *Applied Environmental Microbiology*, **46**: 227-232.
- Chandrasekaran, S., Venkatesh, B. and Lalithakumari, D. (1998). Transfers and expressions of a multiple antibiotic resistances plasmid in marine bacteria. *Current Microbiology*, **37**: 347-351.
- Cheesbrough, M. (2000). District Laboratory Practice in Tropical Countries Pt.2, Cambridge UK. Pp.18- 402.
- Deeb, B.E. and Altalhi, A.D. (2009). Degradative plasmid and heavy metal resistance plasmid naturally coexist in phenol and cyanide assimilation bacteria. *American Journal of Biochemical and Biotechnology*, **5**(2): 84-93.
- Dhakepalkar, P.K. and Chopade, B.A. (1994). High levels of multiple metals resistance and its correlation to antibiotics resistance in environment isolates of *Acinetobacter*. *Journal of Biometals*, **7**: 67-74.
- Dowdy, R.H. and Volk, V.V. (1983). Movement of heavy metals in soils. In: D.W. Nelsen et al, (Ed.) Chemical Mobility and Reactivity in Soil Systems, SSSA Spec. Publ. 11, SSSA, Madison, WI. Pp.229-240.
- Holt, J. G., Kreig, N. R., Sneath, P. H. A., Staley, J. T. and Williams, S. T. (1994). Bergey's Manual of Determinative Bacteriology. 9th edn. Williams and Wilkins: A waverly Company, Baltimore Maryland, USA. Pp.73 - 589.
- Klerks, P.L. (1989). Adaptation of metals in animals. In: heavy metal tolerance in plants. *Evolutionary Journal of Plant and Animal*, **4**: 313-321.
- Manero, A. and Blanch, A. R. (1999). Identification of *Enterococcus* spp. with a biochemical key. *Applied Environmental Microbiology*, **65**: 4425- 4430.
- Montuelle, B., Latour, X., Volat, B. and Gounot, A. M. (1994). Toxicity of heavy metals to bacteria in sediments. *Bulletin of Environment and Contaminant Toxicology*, **53**: 753- 758.
- Niles, D.H. (1999). Microbial heavy metal resistance. *Microbial Biotechnology*, **51**:730-750.
- National Committee for Clinical Laboratory Standards (2002). Performance standards for antimicrobial disc and dilution susceptibility tests for bacteria isolated from animal *Clinical and Laboratory Standards Institute*, **22**: 13 – 14.
- Osborn, A.M., Bruce, K., Strike, P. and Ritchie, D.A. (1997). Distribution, diversity and evolution of the bacterial mercury resistance *operon*. *Microbiology Revised*, **19**: 239-262.
- Rajbanshi, A. (2008). Study on metal resistant bacteria in Guheswori sewage Treatment plant. *Our Nature*, **6**:52-57.
- Ramteke, P.W. (1997). Plasmid mediated co-transfer of antibiotic resistance and heavy metal tolerance in coliform. *Industrial Journal of Microbiology*, **37**: 177- 181.
- Ren, W.X., Li, P.J., Geng, Y. and Li, X.J. (2009). Biological leaching of heavy metals from a contaminated soil by *Aspergillus niger*. *Journal of Hazardous Material*, **167**(1-3): 164-169.
- Schmidt, T. and Schlegel, H.G. (1994). Combined nickel-cobalt-cadmium resistance encoded by the ncc locus of *Alcaligenes xylosoxidans* 31A. *Journal of Bacteriology*, **176**: 7045-7054.
- Sibly, R. M. and Shirley, M.D. (1999). Genetic basis of a between environment trade off involving resistance trait in *Azotobacter chroococcum* isolated from rhizospheric soil. *Biosphere Technology*, **86**:7-13.
- Silver, S. and Walderhang, M. (1992). Gene regulation of plasmid and chromosomes determined inorganic ion transport in bacteria. *Microbiology Review*, **5**:195-228.
- Spain, A. (2003). Implications of microbial heavy metal tolerance in the environment Reviews in Undergraduate Research, **2**: 1-6.
- Tamil, A., Anjugam, E., Madhan, B.N. and Archana, R. (2012). Isolation and characterization of bacteria from tannery effluent treatment plant. *Asian Journal Expert of Biological Science*, **3**(1): 34-41.
- Wiener, P., Müller-Graf, C. and Barcus, V. (1998). Bacterial evolution in modern times: trends and implications. *Integrative Biology*, **1**: 149- 160.