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An investigation on heavy metal tolerance properties of bacteria isolated from textile effluent

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Abstract

The presence of high concentration of toxic heavy metals in industrial waste directly leads to contamination of receiving soil and water bodies and has deleterious impact on both human health and aquatic life. In the present study samples from textile mill effluent from different areas of Jessore city were analyzed for the identification and characterization of bacteria which shows tolerance to Copper, Mercury and Zinc. The bacterial isolates were characterized on the basis of their morphological and physiological studies including size and shape of the organisms, arrangement of the cells, presence or absence of spores, regular or irregular forms, gram reaction, cultural characteristics, IMViC test, H_2S production, nitrate reduction, deep glucose agar test etc. All the bacterial isolates belonged to 3 genera *Bacillus, Enterobacter* and *Pseudomonas*. All the gram positive isolates used in our study showed highest level of tolerance to Zn and moderate level of tolerance to Cu while gram negative isolates showed higher tolerance to Zn in comparison with Cu in nutrient broth. But all of the isolates showed almost no tolerance to Hg. So, our bacterial isolates have the probability to use in the treatment of industrial effluent containing heavy metals and thus pollution due to heavy metal can be controlled. The goal of this study was to identify heavy metal tolerant bacteria from the textile effluent. This kind of study is very significant for broader investigation to obtain data about metal tolerant bacteria considering their potential use for bioremediation and about the interactions between metals and bacteria.

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Introduction

Various manufacturing processes are carried out for different types of textiles. This process uses a large amount of water. Finishing is the final step in manufacturing and uses chemicals like HS-ULTRAPHIL, ECODESIZEPS- 10 and Amino silicone fluid to treat the cloths for obtaining a better quality (Wang *et al.,* 2002). So the different manufacturing steps, such as mercerization, bleaching, neutralization, dyeing, printing and finishing in Textile industry consumes huge amount of dyes, chemicals and water and also produces large volumes of textile wastewater effluents containing heavy metals Chromium (Cr), Lead (Pb), Copper (Cu), Mercury (Hg) and Zinc (Zn), dyes, organic and inorganic acids and salts, bleaching agent etc., in variable concentration. These untreated industrial effluents not only deteriorate surface water quality, ground water, soil and vegetation, but also cause many diseases like haemorrhage, ulceration of skin, nausea, severe irritation of skin and dermatitis (Nese *et al.,* 2007). The term heavy metal applies to the group of metals and metalloids with atomic density greater than 4000 kg m^{-3}, or 5 times more than water (Garbarino *et al*., 1995). In nature, there are about 50 heavy metals of special concern because of their toxicological effect to human beings and other living organisms although some are necessary for living organisms at certain concentration levels. Heavy metals cannot be degraded or destroyed because they are stable and so persistent environmental contaminants. Bacteria that demonstrate the capacity of surviving in toxic heavy metal concentrations have been isolated from different sources (Basu *et al.*, 1997; Castro-Silva, 2003; Choudhury and Kumar, 1998; Duxbury, 1986; Haefeli *et al.*, 1984; Lima-Bittencourt *et al.*, 2007; Otth *et al.*, 2005). Many bacteria have specific genetic mechanisms of resistance to toxic metals (Mindlin *et al.*, 2001; Silver and Misra, 1988). Aquatic microbes become resistant to antibiotics and metals as a result of contamination with effluents. The term tolerance seems more appropriate to refer to the ability of a bacterial strain to grow in the presence of high concentration of a metal. The presence of those elements in the environment can result in impacts on ecosystems, with alterations in the biomass, diversity of microbial communities and cycling of elements (Roane, 1999; Sobolev, 2008). Microbiology and biotechnological approaches have been used for the removal of metallic ions from industrial waste water 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. Despite the large number of papers describing the action of heavy metals on microorganisms, there are few studies on the effects of toxic metals in the physiology of metal tolerant bacteria, in comparison to those about their inhibitory or deleterious effects on susceptible organisms (Lima *et al*., 2012). So the major goal of this study was identification and characterization of bacteria isolated from the sample of textile effluents possessing tolerance to heavy metals like Cu, Hg and Zn.

Materials and methods

Collection and Preservation of samples

The sample for the research work was taken from textile mill effluent from different sites of Khayertala of Jessore city. Different types of samples were collected in labeled bottles aseptically. After collection, the samples were preserved in the refrigerator.

Isolation, purification and preservation of microorganisms

The success of biotechnological process chiefly depends on the used strain of microorganism. For this reason, the first step is the isolation of concerned microbes from their natural habitats.

a. Media Used for Isolation: For the isolation of microbes nutrient agar media was used.

b. Isolation Procedures: Pour plate technique was applied for enumeration and isolation of bacteria through serial dilution was carried out (Greenberg *et al.*, 1980). One ml of the suspension of effluent was transferred to 9 ml of sterile distilled water for tenfold (1:10) dilution and further diluted up to 10^6 dilutions. Then the media were incubated at 37°C for 24 to 48 hours.

c. Purification: The isolated organisms were then purified through both pour plate and streak plate methods repeatedly on nutrient agar media.

d. Preservation and Maintenance: The purified bacterial and fungal isolates were then transferred to nutrient agar slant in two sets of culture tubes to reduce the possibility of contamination. One set of culture tubes was preserved as stock culture. The second set was used for laboratory work.

Identification of selected isolates

For the identification of selected isolates the following morphological characteristics and physiological behaviors were observed.

A. Morphological studies of the selected isolates

Morphological characters such as size, shape, arrangement, color etc., of selected isolates were observed by microscopic methods.

With a view to identify the selected strains the following morphological characters were studied:

i. Cultural characteristics

Agar colony

Morphological characters such as size, shape, elevation, opacity, surface and color of the colony were studied.

Agar slant

The modes of bacterial growth on slants were studied.

Broth culture

Production of turbidity, sedimentation and surface growth in nutrient broth was observed and noted.

ii. Microscopic characteristics

For the study of size, shapes and sporulation of the vegetative cells microscopic method was used. The length and breadth of cells and spores were measured.

Preparation for microscopic examination

Fixed Stained Smears

The Techniques used to obtain information on shape, anatomy, and taxonomic features of the cells that cannot be easily seen in unstained materials.

Simple staining

For this purpose 5% aqueous solution of basic stains such as methylene blue, crystal violet, mercurochrome, safranin etc. were used. The slides were examined under microscope.

Gram staining

The fixed smear was flooded with ammonium oxalate crystal violet solution for 3 minutes. This was gently rinsed off and an iodine solution was applied for 30 seconds. Followed by gentle washing with water ethyl alcohol (95%) was then applied for 20 seconds to decolorize the strain. Finally safranine was used as a counter stain for 3 minutes. Then the slide was gently rinsed off with water and blotted dry. The result was recorded as gram positive and gram negative.

B. Physiological studies of the selected isolates

Physiological characteristics are more important than morphological characteristics in the identification of bacteria. For this reason, the following studies were done on the physiological activities of the selected bacterial isolates.

i) Catalase test

To observe the activity, nutrient broth tubes were inoculated with 48 to 72 hours old culture and incubated at 37±2°C for 48 hours. After incubation then observation was made closely for the appearance of oxygen bubbles. Production of bubbles indicates

the positive result Fig. 1.

ii) Glucose broth

To observe the growth of organism in liquid culture, glucose broth medium was prepared and inoculated with selected strains. After incubation the appearance of growth were recorded.

iii) Indole test

Indole is nitrogenous compound and a degradation product of amino acid tryptophane by various bacteria. Indole can be detected by the following two methods:

(a) Tryptophane broth tubes were inoculated with 48 to 72 hours nutrient broth cultures. A filter paper strip, which was soaked in a saturated solution of oxalic acid, then dried and kept in the tube by holding them between the cotton plug and the wall of the test tube. After over right incubation the paper becomes pink. This indicates the formation of indole.

(b) Kovac's Method

Tryptophane broth tubes were inoculated with the 48 to 72 hours nutrient broth cultures. After incubation at 37±2°C for 24 to 48 hours, few drops of kovac's reagent was added to it, shaken and allowed the reagent to float. Indole formation is detected by the appearance of a pink colour at the top. Controlled tube will produce bottle green ring at the top of the medium.

iv) Test for nitrate

Test reduction of nitrate is brought about by several bacteria. The end products of nitrate reduction are nitrite ammonia, nitrous oxide, nitrogen gas etc. The organisms contain the enzyme nitrate reductase is able to reduce the nitrate to nitrite.

The following reagents were required for this test

Reagent A

Sulfanilic acid was dissolved in one liter of 5N acetic acid and stored in brown glass bottle.

Reagent B

6.0 ml of dimethyl napthalamine was added to one liter of 5N acetic acid and stored in brown bottle.

Reagent C

Small amount of zinc dust.

After incubation a few drops of solution A and equal volume of solution B was added and shaken well. Formation of red to pink color indicates the reduction of nitrate to nitrite. A small piece of Zn dust was added to the tubes, where solution A and B was already added. Any remaining nitrate (in case) would be reduced to nitrite resulting red to pink color.

v) Citrate utilization

The ability to utilize citrate as sole source of carbon and energy can be used to distinguish certain gram negative rods. For this test citrate medium was inoculated with at 37±2°C for 48 hours. Appearance of turbidity or growth in the medium indicated the utilization of citrate.

vi) Production of hydrogen sulphide

Peptone iron agar medium was inoculated and lead acetate paper was introduced in each of the tubes containing peptone iron agar medium by holding them between the plug and the wall of the test tube. After incubation the production of hydrogen sulphide was indicated by blackening of the lead acetate paper.

vii) Starch hydrolysis

After the microbial growth iodine solution was added to each of the starch agar plates. Development of clear white to bluish brown color indicated the complete and partial hydrolysis of the starch respectively, hence the presence of enzyme amylase. Development of deep blue color indicated that the starch had not been hydrolyzed.

viii) Voges-Proskaur (V.P.) test

The acidic product produced by some bacteria converted to metabolic intermediate, pyruvic acid and then to neutral products and CO_2. Voges-Proskaur medium was inoculated and incubated. After the

growth 3 ml napthol solution was added to each of the test tubes followed by one ml of potassium hydroxide-creatine solution. The tubes were then shaken vigorously for 1-2 minutes. Appearance of a crimson ruby color in the tube indicated the positive result.

ix) Methyl red reaction

After incubation of the prepared V.P. medium a few drops of methyl red solution were added in each tube. A distinct red color indicated methyl red positive and yellow color indicated methyl red negative.

x) Casein hydrolysis

Nutrient skimmed milk medium was prepared by mixing sterilized skimmed milk (1-2 ml) and sterilized nutrient agar (10-15 ml) in petri plates. After inoculation and incubation, the plates were observed as the organisms produced caseinase were surrounded by clear zone of hydrolysis of casein. But the controlled plate was found opaque due to the presence of casein Fig. 2.

xi) Motility test

Deep tube of nutrient agar medium was prepared with indicator terazolium chloride and sterilized. At 45°C the tubes were inoculated by the selected isolated microorganisms and incubated for 2 to 5 days. The organisms which were motile changed the color of the medium form colorless to pink to red.

Evaluation of metal tolerance

The bacterial isolates were tested for their tolerance to Cu, Hg and Zn by broth dilution method. For this test, different concentration of heavy metal containing nutrient broth media was prepared and dispensed into the test tubes (10 ml/tubes) and sterilized in an autoclave. These modified broths were inoculated with the equal amounts of individual selected organism isolated from the industrial effluent by preparing suspension. For comparing the growth response at different ppm, one set of nutrient broth (without the heavy metal) containing test tubes were inoculated with the selected organisms and used as control. Then incubated at 37°C for 2 to 4 days and observed periodically.

Results

During the period of study, a total number of 10 bacterial colonies were isolated. Out of these isolates, 5 isolates were selected for further study on the basis of their morphological and cultural characteristics and better growth on nutrient agar media. The selected isolates were designated as NS1, NS2, NS3, ES1 and CS1.

On nutrient agar plate bacterial isolates showed different type of colonies. They were differed from each other in color, form, margin and elevation. Bacterial isolates were characterized on the basis of their morphological characteristics including size and shape of the organisms, arrangement of the cells, presence or absence of spores, regular or irregular forms, gram reaction, cultural characteristics, IMViC test, H_2S production, nitrate reduction, deep glucose agar test etc.,(Table.1). All these characteristics were then compared with the standard description of "Bergey's Manual of Determinative Bacteriology" 8thed. (Buchanan and Gibbons, 1974). All the isolates were found to belong to 3 genera, such as, *Bacillus, Enterobacter* and *Pseudomonas*. An attempt was made to identify them up to species and provisionally identified as *Bacillus circulans* (NS3), *Bacillus pulvifaciens* (NS2), *Bacillus sphaericus* (NS1), *Enterobacter cloacae* (ES1) *and Pseudomonas putida* (CS1).

All the three gram positive bacteria NS1, NS2 and NS3 shown similar kind of characteristics specially spore formation and turbid and sediment formation in both Nutrient and Glucose broth. On the other hand positive gram negative bacteria ES1 and CS1shown similar characteristics especially no spore formation. But differences were noticed for belonging to different genus. Microbial tolerance to Copper (Cu), Mercury (Hg) and Zinc (Zn) was studied visually. Selected five isolates were allowed to grow in nutrient broth modified with Cu, Hg and Zn concentrations range from 10 ppm to 150 ppm.

Table 1. Morphological, cultural and biochemical characteristics of different isolates.

	ES1	CS1	NS1	NS2	NS3
Vegetative cell	Short rod	Short rod	Rod	Rod	Rod
Cell arrangement	Single	Single	Single or in pair	Single or in pair	Single or in pair
Gram staining	Gram Negative	Gram Negative	Gram positive	Gram Positive	Gram Positive
Spore staining	Non spore former	Non spore former	Spore former	Spore former	Spore former
Nutrient broth	Turbid, Sediment	Sediment	Turbid, Sediment	Turbid, Sediment	Turbid, Sediment
Glucose broth	Turbid, Sediment	Turbid, Ring	Turbid, Sediment	Turbid, Sediment	Turbid, Sediment
Motility test	-	-	-	-	-
Indole test	-	-	-	-	-
MR test	-	-	-	-	-
VP test	+	-	-	+	-
Catalase test	Slightly +	+	+	+	+
Citrate test	+	+	+	+	+
Nitrate reduction test	-	-	-	-	-
H_2S production	-	-	-	-	-
Casein hydrolysis	+	+	+	+	+
Starch hydrolysis	-	-	-	-	-
	Enterobacter cloacae	*Pseudomonas putida*	*Bacillus sphaericus*	*Bacillus pulvifaciens*	*Bacillus circulans*

+: Positive reaction, -: Negative reaction.

In the present study only the NS1 isolates were able to grow with copper ($CuSO_4$. $5H_2O$) upto the concentration of 130 ppm. The gram positive bacteria have shown more tolerance than the gram negatives (Table 2).

Table 2. Copper ($CuSO_4$. $5H_2O$) tolerance of selected isolates.

Isolate No.	Growth activity (turbidity) in nutrient broth	Tolerance activity at various ppm														
		10	20	30	40	50	60	70	80	90	100	110	120	130	140	150
ES1	+++	+++	+++	++	++	++	+	+	+	+	-	-	-	-	-	-
CS1	+++	+++	+++	+++	+++	++	++	++	++	+	+	-	-	-	-	-
NS1	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+	+	+	-	-
NS2	+++	+++	+++	+++	+++	+++	++	++	++	++	+	+	-	-	-	-
NS3	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++	+	+	-	-	-

Note: + = Positive (+= scanty, ++=moderate, +++= heavy), - = Negative.

All the isolates were sensitive to mercury being able to tolerate only 10 ppm mercury ($HgCl_2$). This states that the textile effluent discharges no or very small amount of Hg (Table 3).

Table 3. Mercury ($HgCl_2$) tolerance of selected isolates.

Isolate No.	Growth activity (turbidity) in nutrient broth	Tolerance activity at various ppm														
		10	20	30	40	50	60	70	80	90	100	110	120	130	140	150
ES1	+++	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CS1	+++	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NS1	+++	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NS2	+++	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NS3	+++	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Note: + = Positive (+= scanty, ++=moderate, +++= heavy), - = Negative.

All the isolates of this study were more tolerant to zinc ($ZnCl_2$) than copper and mercury. The result also indicates the higher Zn tolerance of gram positives than gram negatives. The highest (140 ppm) tolerance was observed in NS1 while the isolate ES1 was able to grow up to the concentration of 110 ppm Zn. It result also tells that the textile effluent releases Zn at very high concentration (Table 4).

Table 4. Zinc ($ZnCl_2$) tolerance of selected isolates.

Isolate No.	Growth activity (turbidity) in nutrient broth	Tolerance activity at various ppm														
		10	20	30	40	50	60	70	80	90	100	110	120	130	140	150
ES1	+++	+++	+++	+++	+++	+++	++	++	++	+	+	+	-	-	-	-
CS1	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++	+	+	-	-
NS1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+	+	-
NS2	+++	+++	+++	+++	+++	+++	+++	++	+++	++	++	++	+	+	-	-
NS3	+++	+++	+++	+++	+++	+++	+++	++	++	++	+	+	+	-	-	-

Note: + = Positive (+ = scanty, ++ = moderate, +++ = heavy), - = Negative.

Discussion

Industrial effluents discharged into river or in land without any treatment can cause severe pollution with carcinogenic substances. Microbes have wide spread capacity to remove, transform and precipitate these chemical pollutants from the surroundings (Faisal and Hasnain, 2004; Kumar *et al.* 2005; Raghukumar *et al.* 2006).

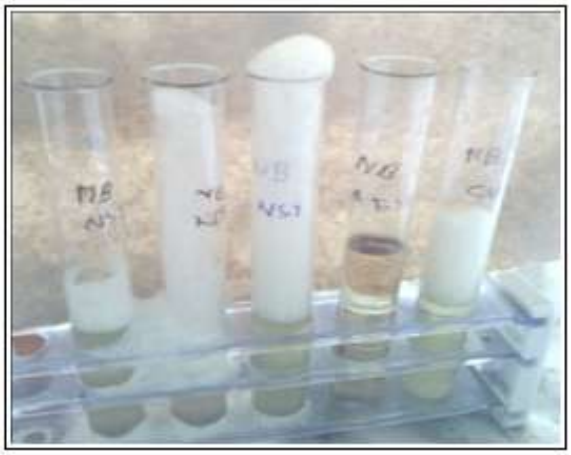

Fig. 1. Catalase Test: Production of oxygen bubbles *by Bacillus, Enterobacter* and *Pseudomonas.*

In the present study, five bacterial species were isolated from the textile mill effluent belonging to 3 genera, such as, *Bacillus, Enterobacter* and *Pseudomonas.* The isolates were putatively indentified as *Bacillus circulans*, *Bacillus pulvifaciens*, *Bacillus sphaericus*, *Enterobacter cloacae and Pseudomonas putida* on the basis of their cultural, morphological and biochemical characteristics. Their heavy metal tolerance was studied by growing them in nutrient broth modified with Cu, Hg and Zn concentrations ranging from 10 ppm to 150 ppm. Similar findings of occurrence of heavy metal resistant bacteria in contaminated soil samples have been reported (Olukoya *et al.*, 1997; Fagade and Adetutu, 1999; Oyetibo *et al.*, 2010;).

Fig. 2. Casein hydrolysis by *Enterobacter cloacae*.

The study says that *Bacillus sphaericus* and *Bacillus circulans* shows highest tolerance to Cu of 130 ppm and 120ppm respectively in comparison with the rests showing tolerance to 100 ppm. This result states that the textile effluent discharges considerable amout of

Co. In case Hg, all the isolates were sensitive to mercury being able to tolerate only 10 ppm whereas De *et al.,* (2003) isolated two strains from an area with intense shipping traffic, which grew on seawater nutrient agar solid medium with 75 ppm mercury. According to another study of Durve *et al.,* (2012) the actual heavy metal concentration was calculated and it was seen that Pseudomonas aeruginosa could tolerate 294.60 ppm of Mercury resulting that the effluent of the textile do not release considerable amount of Hg.

The study shows that all the isolates were more tolerant to Zn than Cu and Hg where both *Pseudomonas putida* and *Bacillus pulvifaciens* were moderately tolerant to 110 ppm and able to grow up to the concentration of 110 ppm on the other hand *Enterobacter cloacae* could tolerate up to 140 ppm concentration of Zn. The result states that the effluent releases Zn more than Cu and Hg and in considerable amount.

Presence of metal tolerant bacteria in a given environment may be an indication that such area is affected by heavy metals. Isolation of bacteria from metal polluted environment would represent an appropriate practice to select metal resistant strains that could be used for heavy metal removal and bioremediation purposes (Malik, 2004). Microbial metal bioremediation is an efficient strategy due to its low cost, high efficiency and eco-friendly nature. Microbiological detoxification of polluted water is economical, safe and sustainable (Eccles, 1995).

Under conditions of high levels of heavy metals in their environment, metal resistance in bacteria most likely help them to adapt faster by the spread of resistant factors. The organisms with heavy-metal resistance isolated and identified in this study have potential application in bioremediation of environments polluted with metals and may also help to overcome the inhibition that heavy metals exert on the biodegradation of organic pollutants. The studies assessing the potential ability of the selected isolates to remove heavy metals from contaminated industrial waste water and their plasmid profiles are currently underway.

Conclusion

The present study demonstrates that all the isolates used in our study showed high level of resistance to zinc and moderate level of resistance to copper and mercury in nutrient broth. More investigation can be done to obtain data about metal tolerant bacteria considering their potential use for bioremediation, as well as about the impact resulting from the interactions between metals and metal tolerant bacteria. Pollution by heavy metal can be prevented or reduced in a large through the implication of biotechnology in huge textile effluent management.

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