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Evaluation of Heavy metal tolerance of bacteria isolated from domestic and paint factory wastewater

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The present study was aimed at isolating potential heavy metal tolerant bacteria which can be applied for bioremediation of heavy metal impacted environments. Waste water samples were collected from both domestic and paint factories. The samples were analyzed using standard microbiological techniques. Bacteria isolates recovered from these samples were screened for tolerance to different heavy metals such as Fe^{2+}, Cu^{2+}, Co^{3+}, Pb^{2+}, Zn^{2+}, Cr^{3+}, and Al^{3+} at 200 and 250 ppm respectively. All bacteria from paint effluents displayed visible growth on all heavy metals while bacteria from household wastewater did not grow on one or more heavy metals. About 42% of the isolates were susceptible to Lead and Chromium while 92% had visible growths on other ions like Zinc and Copper. Three isolates which displayed highest growth and multi metal resistance were selected for heavy metal tolerance evaluation. The 16S rRNA gene sequencing, Basic Local Alignment search tool (BLAST) and phylogenetic profile revealed they had 100%, 97% and 75% homology with *Bacillus subtilis, Serratia liquefaciens* and *Bacillus cereus* respectively. The three isolates exhibited the same growth pattern of gradual decrease in growth with increase in heavy metal concentration however, *Bacillus cereus* had the highest growth compared to the other two bacteria. Paint factory effluents are promising source of heavy metal resistant bacteria. These heavy metal tolerant strains can offer substantial potential for the bioremediation of heavy metals polluted environment.

Keywords: isolation, Screening, heavy metals, wastewater, bacteria, 16S rRNA gene sequencing

INTRODUCTION

Environmental pollution by heavy metals comes from anthropogenic sources such as smelters, mining, power stations and the application of pesticides containing metal, fertilizer and sewage sludge. These heavy metals are accumulated in the environment as they cannot be degraded and pose a threat when they find their way into the food chain. Through their effects on microbial growth, morphology and biochemical activities, heavy metals influence the microbial population, resulting in decreased biomass as well as diversity. In order to survive in heavy metal polluted environments, many micro-organisms have developed resistance to toxic metal ions (Nies and Silver, 1995; Nies, 1999).

Removal of heavy metals from the environment by physiochemical processes are very expensive and generate secondary products, thereby resulting in merely the transfer of the metal from one form into less mobile and available form, but not providing a definitive solution (Kratochvil and Volesky, 1995). Biological processes have long been considered cost effective

environmentally friendly methods for the remediation of heavy metal contaminated soils (Congeevaram *et al.,* 2007). Microbes in this guise must have developed mechanisms to tolerate the metals either by presence of heavy metals through efflux, complexation, or reduction of metal ions or to use them as terminal electron acceptors in anaerobic respiration (Gadd, 1990). Most mechanism reported involves the efflux of metal ions outside the cell, and genes for tolerance mechanisms have been found on both chromosomes and plasmids. Bacteria that are resistant to and grow on metals play an important role in the biogeochemical cycling of those metal ions.

Metal-contaminated environments have been found to usually contain bacteria that exhibit a complex array of biochemical and genetically encoded mechanisms to beat the toxic effects of heavy metals in their surroundings. These may include efflux systems that expel metal ions from the cell by means of transport systems, intracellular sequestration of the metal by specific metal ion–binding proteins, extracellular precipitation into complex compounds, and enzymatic transformation of metal ions to a less toxic species (Malik 2004; Yan and Virarghavan 2000; Lee et al. 2006). The search for heavy metal-resistant bacteria which can be manipulated in a number of ways for remediation of heavy metal–contaminated sites necessitated this study.

MATERIALS AND METHODS

Collection of Samples

Waste water samples were collected aseptically in a sterile Erlenmeyer flask from Bathroom and Kitchen in Ado Ekiti, Ekiti State and waste effluent of paint factory in Akure, Ondo State. The samples were transported aseptically to the laboratory for further microbiological analyses.

Isolation and enumeration of Bacteria

Bacterial were isolated from the waste water samples using ten-fold serial dilution and pour plate method as described by Lucious et al. (2013). The inoculated agar plates were incubated at 37ºC for 24 to 48 hours. Colonies developed on the nutrient agar plates after incubation were counted and calculated as CFU/ml. The colonies were streaked on nutrient agar until pure culture was obtained.

Characterization and identification of bacterial isolates

The bacterial isolates were characterized by morphological, cultural, physiological and biochemical characterization. Identities of heavy metal resistant strains were confirmed with 16SrDNA gene sequencing.

Preparation of Heavy metals

Stock solutions of the heavy metals (Chromium, Zinc, Lead, Copper, Iron, Aluminium and Cobalt) were prepared from analytical grade salts of chromium (iii) oxide, Zinc sulphate heptahydrate, Lead nitrate, Copper (ii) chloride dehydrate, Iron ferric sulphate, Aluminium nitrate, Cobaltous chloride. Respective grams of the salts were weighed into 1000 cm^3 volumetric flask, mixed with distilled water to give stock solution (1000 ppm).

Lesser concentrations of 500ppm, 300ppm, 250ppm, 200ppm, 150ppm, 100ppm, 50ppm was prepared from the stock solution using the general formula: (C_1 V_1 =C_2 V_2) where

C_1 = Initial concentration (Stock concentration)

V_1 = Initial volume (Volume to be taken for stock)

C_2= Final concentration (Required concentration)

V_2 = Final volume (Required volume)

Screening of Heavy metal resistant Bacteria

Heavy metal resistant bacteria were screened in nutrient agar (NA) incorporated with different concentrations (200ppm and 250ppm) of heavy metals (Chromium, Zinc, Lead, Copper, Iron, Aluminium and Cobalt) salts.

Heavy metal tolerance of selected bacteria isolates

Heavy metal tolerance of the three selected bacterial isolates was determined using broth dilution method as described by Hossain and Anwar, 2012. Nutrient broth medium sterilized at 121ºC for 15 minutes was supplemented with different concentrations (50, 100, 150, 200, 250, 300, 500 ppm) of filter sterilized heavy metals (Chromium, Zinc, Lead, Copper, Iron, Aluminum and Cobalt). The amended medium was inoculated with the equal amounts of individual selected bacteria isolates from the wastewater samples. Inoculated broth without heavy metal served as the control. The medium was incubated at 37ºC for 48hrs. Growth was determined by reading the absorbance at 600 nm using UV spectrophotometer (Vashishth and Khanna, 2015).

Molecular identification of isolates

DNA Extraction

DNA was extracted using the protocol stated by (Trindade 2007). Single colonies grown on medium were transferred to 1.5 ml of liquid medium and cultures were grown on a shaker for 48 h at 28 ºC. After this period, cultures were centrifuged at 4600g for 5 min. The resulting pellets were resuspended in 520 µl of TE buffer (10 mMTris-HCl, 1mM EDTA, pH 8.0). Fifteen microliters of 20% SDS and 3 µl of Proteinase K (20 mg/ml) were then added. The mixture was incubated for 1 hour at 37 ºC, then 100 µl of 5 M NaCl and 80 µL of a 10% CTAB solution in 0.7 M NaCl were added and votexed. The suspension was incubated for 10 min at 65 ºC and kept on ice for 15 min. An equal volume of chloroform: isoamyl alcohol (24:1) was added, followed by incubation on ice for 5 min and centrifugation at 7200g for 20 min. The aqueous phase was then transferred to a new tube and isopropanol (1: 0.6) was added and DNA precipitated at –20 ºC for 16 h. DNA was collected by centrifugation at 13000g for 10 min, washed with 500 µl of 70% ethanol, air-dried at room temperature for approximately three hours and finally dissolved in 50 µl of TE buffer.

PCR Amplification and Purification of amplified product

PCR was carried out in a GeneAmp 9700 PCR System Thermalcycler (Applied Biosystem Inc., USA) with a Pcr profile consisting of an initial denaturation at 94°C for 5 min; followed by a 30 cycles consisting of 94°C for 30 s, 50°C for 60s and 72°C for 1 minute 30 seconds ; and a final termination at 72°C for 10 mins. And chill at 4oC.GEL (Wawrik *et al*., 2005; Frank *et al*., 2008). PCR sequencing preparation cocktail consisted of 10 µl of 5x GoTaq colourless reaction, 3 µl of 25mM MgCl2, 1 µl of 10 mM of dNTPs mix, 1 µl of 10 pmol each 27F 5'- AGA GTT TGA TCM TGG CTC AG-3' and - 1525R, 5′-AAGGAGGTGATCCAGCC-3′ primers and 0.3units of Taq DNA polymerase (Promega, USA) made up to 42 µl with sterile distilled water 8µl DNA template. The integrity of the amplified about 1.5Mb gene fragment was checked on a 1% Agarose gel ran to confirm amplification.

After gel integrity, the amplified fragments were ethanol purified in order to remove the PCR reagents. The purified fragment was checked on a 1.5% Agarose gel ran on a voltage of 110V for about 1hr as previous, to confirm the presence of the purified product and quantified using a nanodrop of model 2000 from thermo scientific.

Sequencing

The amplified fragments were sequenced using a Genetic Analyzer 3130xl sequencer from Applied Biosystems using manufacturers' manual while the sequencing kit used was that of BigDye terminator v3.1 cycle sequencing kit. Phylogenetic tree was generated with the MEGA5 software (Tamura et al. 2011) on the basis of evolutionary distances calculated by the neighbour-joining method using Maximum Composite Likelihood model (Tamura et al. 2004).

Physico-chemical Analyses

Standard analytical methods for examination of water and wastewater were used to determine COD, TSS, TS, TDS, DO, BOD (APHA, 2005; Eaton and Clesceri, 2005). Metal analysis was carried out using flame atomic absorption spectrophotometer. Temperature of the samples were recorded at sample collection sites using a simple thermometer (ºC), the pH was measured using a pocket pH meter and electrical conductivity was measured with a conductivity meter.

Statistical analysis

All experiments were performed in duplicate. The statistical calculation was done according to the standard method. The results are given as mean ± SD values.

RESULTS AND DISCUSSION

Total Bacteria Count

Table 1 showed the mean total bacteria counts of wastewater samples from household and paint factory. Higher bacteria count from household compared to that of the factory could be due to the nutritional component of the household wastewater which enhanced the growth of bacteria compared to the presence of toxic compounds in paint effluent which could have eliminated bacteria that are not able to tolerate the toxic effect of the pollutants. It could also reflect the possibility of elimination of some bacteria in the paint effluents as a result of the toxic effect of the paint hydrocarbons and heavy metals. Their growth in this contaminated environment suggests their ability to adapt to, tolerate and grow in the presence of these contaminants.

Table 1: Total bacteria count of wastewater samples

Waste water Samples	Total bacteria count (Log_{10}CFU/ml)
Household	
HD1	6.15±0.03
HD2	5.49±0.04
HD3	3.68±0.02
HD4	4.25±0.01
HD5	4.85±0.05
Mean	4.88
Paint factory	
PF 1	3.95±0.02
PF 2	4.15±0.05
PF 3	3.52±0.04
PF 4	2.25±0.06
PF 5	2.83±0.01
Mean	3.34

Table 2: Qualitative heavy metal resistance screening of Bacteria Isolates from domestic and Paint factory waste water samples

Isolates' Codes	Heavy metals (ppm)													
	Fe		Pb		Cr		Co		Zn		Al		Cu	
	200	250	200	250	200	250	200	250	200	250	200	250	200	250
HD01	+	-	-	-	+	-	+	-	+	+	-	-	-	-
HD02	++	-	-	-	-	-	+	-	+	+	-	-	-	-
HD03	+	-	+	-	+	+	++	-	+	+	++	+	+	-
HD04	+	++	-	-	-	-	++	+	++	++	+	++	+	+
HD05	+	+	+	+	-	-	+	+	+	+	+	++	+	++
HD06	+	+	+	-	+	-	-	-	+	+	+	-	+	-
HD07	-	-	-	-	+	-	+	+	+	-	-	+	+	+
HD08	+	-	+	+	-	-	+	-	+	++	++	+	+	+
HD09	++	+	-	-	+	-	+	-	++	+	++	+	++	+
HD10	+	-	+	-	+	-	+	-	+	+	++	+	++	+
HD11	+	-	-	-	-	-	+	-	+	+	++	+	++	+
HD12	+	++	-	-	-	+	+	+	+	+	+	+	+	+
HD13	+	-	+	+	-	+	+	+	+	+	+	+	+	+
HD14	++	+	++	+	-	+	+	-	+	-	+	++	+	++
HD15	++	++	+	-	-	++	+	-	++	+	++	+	+	-
PF01	++	+	++	+	++	++	+	++	++	++	++	++	++	+
PF02	++	++	++	+	+	++	++	+	++	++	+	++	+++	+
PF03	+++	++	++	+	+	++	+	++	++	+++	++	++	++	++
PF04	++	++	+	++	+	++	++	++	+	++	++	+	++	++
*PF05	+++	++	+++	++	++	++	++	++	+++	+++	+++	+++	+++	++
PF06	+	++	++	++	+	++	+	++	++	+	+	++	++	++
PF07	++	+	++	+	+	++	+	++	+	+	++	+++	++	+
PF08	+	++	+++	++	+	+	+	++	++	++	++	+	+	++
*PF09	++	++	+++	+++	+++	+++	++	++	+++	++	+++	++	+++	++
*PF010	+++	++	+++	++	++	+	+++	++	++	+++	++	+++	++	+++
PF011	+	++	++	++	++	+	+	++	++	++	++	++	++	++

Table 3: Physico-chemical properties and Mineral analysis of wastewater samples

PARAMETER	Household Sample	Paint factory Sample
Temperature (^{0} C)	34.20±0.02	28.20±0.01
Turbidity (NTU)	9.43 ±0.05	128±0.04
Conductivity (mS/m)	115±0.08	102±0.07
pH	9.40±0.02	6.30±0.02
Total dissolved solid (mg/L)	378.1±0.05	278.03±0.10
Total solid (mg/L)	418.36 ±1.20	319.65±0.08
Total suspended solid (mg/L)	28.7±0.05	29.62±0.03
Total alkalinity (mg/L)	20.25±0.02	15.4±0.07
Acidity as $CaCO_3$ (mg/L)	4.60±0.01	4.40±0.02
Total hardness (mg/L)	82.00±1.04	112.50±1.21
DO (mg/L)	5.3±0.01	4.5±0.03
BOD (mg/L)	134.3 ±0.08	112±0.04
COD (mg/L)	265.30 ±1.34	256±1.03
Na (ppm)	7.70±0.01	9.70±0.02
K (ppm)	8.50±0.03	10.10±1.23
Ca (ppm)	9.50±0.07	10.50±0.05
Mg (ppm)	10.20±0.05	18.00±0.01
Zn (ppm)	2.20±0.03	5.40±0.05
Fe (ppm)	5.50±0.06	9.20±0.02
Cu (ppm)	1.64±0.05	3.25±1.16
Pb (ppm)	0.35±1.35	1.08±1.24
Cd (ppm)	ND	3.38±1.15
Cr (ppm)	0.31±0.04	3.67±0.07

DO= Dissolved Oxygen, BOD= Biochemical Oxygen Demand, ND = Not Detected

**Mean and standard error of five samples*

Screening of Heavy metal resistant Bacteria

Twenty-six bacteria isolates recovered from the waste water samples were screened for heavy metal resistance as shown in Table 2. The bacteria isolates exhibited varying growth responses to high heavy metal concentrations (200 ppm, 250 ppm) they were exposed to. Bacteria isolates from paint effluents displayed higher resistance to heavy metal as shown by moderate to heavy growth on heavy metal amended medium compared to their counterpart from household waste water samples. All bacteria from paint effluents also displayed visible growth on all heavy metals while bacteria from household wastewater did not grow on one or more heavy metals. Elimination of growth of 42% of the bacteria in Lead and Chromium medium reflect their susceptibility to these metal ions compared to other ions like Zinc and Copper where 92% of the isolates had visible growths. Variation in the response of these bacteria could be as a result of their pre exposure to some of these metals in the environment which they must have developed mechanisms to tolerate. The physicochemical properties of the samples analyzed also revealed the presence of some of these metal ions. Ability of some of these bacteria to grow on high concentrations of these heavy metals is a reflection of their ability to adapt and tolerate the toxic effect of these heavy metals. Isolates PF05, PF 09 and PF10 which had moderate to heavy growth in all the heavy metals were selected for heavy metal tolerance evaluation.

Molecular identification of isolates

Fig. 1 showed the amplification of the DNA of the three bacteria. It was shown that each of the isolates amplified the 16SrDNA with 1500 base pair.

Molecular identification of the three bacteria revealed they are related to *Bacillus subtilis, Serratia liquefaciens* and *Bacillus cereus* with 100%, 97% and 75% similarities respectively. The phylogenetic position of the species is shown in Fig. 2.

Physico-chemical parameters of wastewater samples

The Physico-chemical parameters of the wastewater samples presented in Table 3 showed that the parameters such as pH, Turbidity, TSS, TDS, COD, BOD exceeded the permissible limit (FEPA 1991). The result also revealed the samples contained different heavy metals in varying concentrations higher than the permissible limit however, wastewater samples from paint factory contained higher concentrations of heavy metals. Cadmium was not found in household wastewater but paint factory wastewater had 3.38 ppm value for Cd which is higher than FEPA (1991) recommended concentration level (< 1ppm).

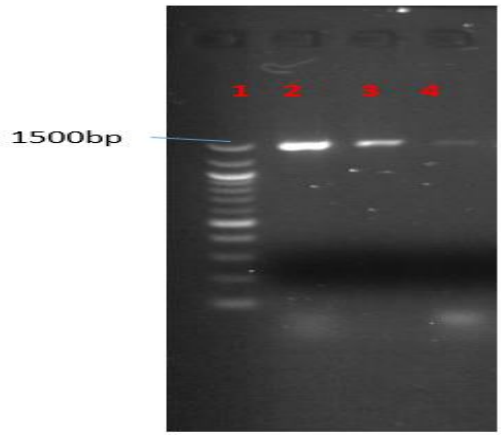

Fig. 1: Agarose gel electrophoresis indicating the positive amplification of the bacteria 16S region loading arrangement

Key: 1- molecular marker,

2- Rukayat 10-6,

3- Rukayat 2nd B 10-5 A.C

4.-Rukayat 10-6 P.C

Table 3: Molecular identification of the heavy metal resistant isolates using the 16SrRNA sequences

Isolates	Molecular identification
RUKAYAT 10-6,	100% identical to *Bacillus subtilis*
Rukayat 2nd B 10-5 A.C	99% identical to *Serratia liquefaciens*
Rukayat 10-6 P.C	75% identical to *Bacillus cereus*

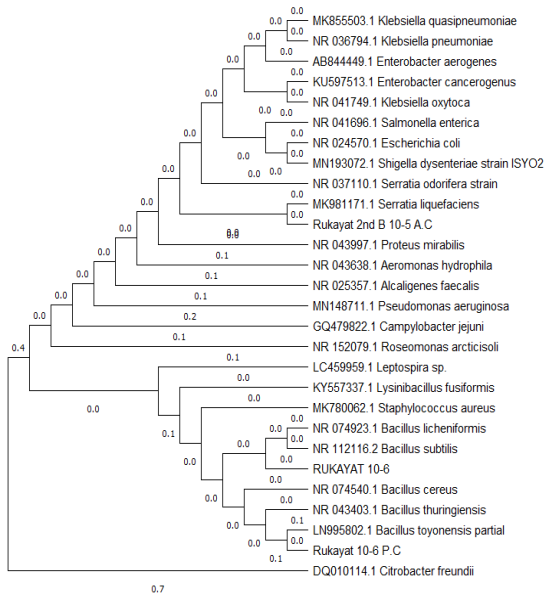

Fig. 2: Phylogenetic tree based on 16S rDNA sequences of DNA extracted from three selected heavy-metal resistant bacteria isolates

Heavy metal tolerance of selected bacteria isolates

The growth profile of the three selected heavy metal resistant bacteria in Lead at varying concentrations is shown in fig 3. The bacteria showed substantial growth in all Lead concentrations examined. There was a slight decline in their growth at 50 ppm compared to the control which had no heavy metal prior to increase in their growth at 100ppm. Their growth gradually declined as the concentration increased but was not totally inhibited at 500ppm. The decline in their growth indicates the toxic effect of the heavy metals on the growth of microorganisms as earlier stated by Badar *et al.,* 2000. Many heavy metals, like Zn, Cu, Co, Ni, Mn, and Fe have been reported to have nutritional characteristics known as essential "trace elements" necessary for living organisms because at a certain concentration levels, they participate in some enzyme activities (Rathnayake *et al.,* 2009) but when in excess concentrations, toxicity can occur as a result of alterations in enzymes specificity and in the conformational structure of the nucleic acids and proteins and interference with oxidative phosphorylation and osmotic balance (Bruins *et al.,* 2000; Hussein *et al.,* 2005; Pereira *et al.,* 2012).

The bacteria exhibited the same growth patterns in other heavy metals (fig 4-9). *Bacillus cereus* had the highest growth compared to the other two bacteria. This agrees with the findings Mgbemena *et al*. (2012) and also similar to a report of Rathnayake *et al.* (2010) which investigated the tolerance of trace metals such as Cd^{2+}, Cu^{2+} and Zn^{2+} by *Paenibacillus* sp. and *Bacillus thuringeinsis* isolated from a pristine soil.

Varying microbial tolerance to heavy metals has been attributed to a variety of resistance mechanisms such as differences in uptake and/or transport of the metal, the efflux of metal ions outside the cell, enzymatic transformation of the metals by oxidation, reduction, methylation or demethylation into chemical species which may be less toxic or more volatile than the parent compound (Issazadeh et al., 2013; Lucious et al., 2013).

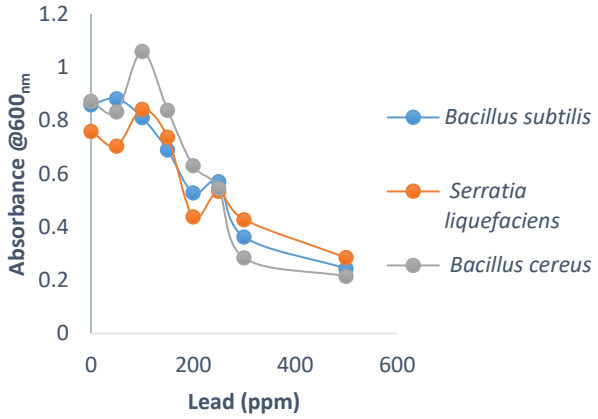

Fig. 3: Growth Pattern of heavy metal resistant bacteria at different concentrations of lead

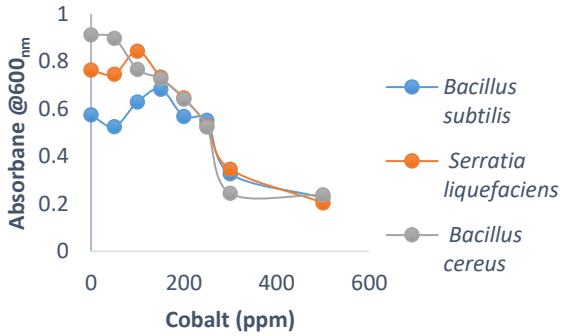

Fig. 4: Growth Pattern of heavy metal resistant bacteria at different concentrations of Cobalt

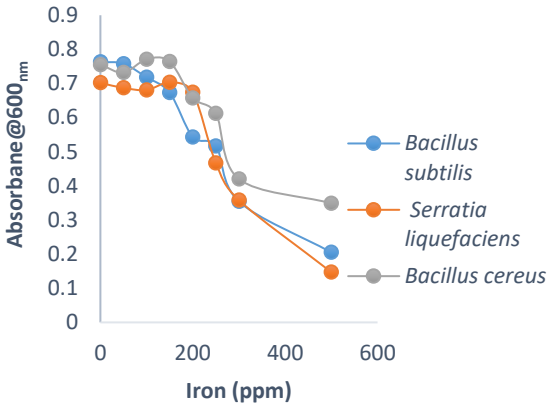

Fig. 5: Growth Pattern of heavy metal resistant bacteria at different concentrations of Iron

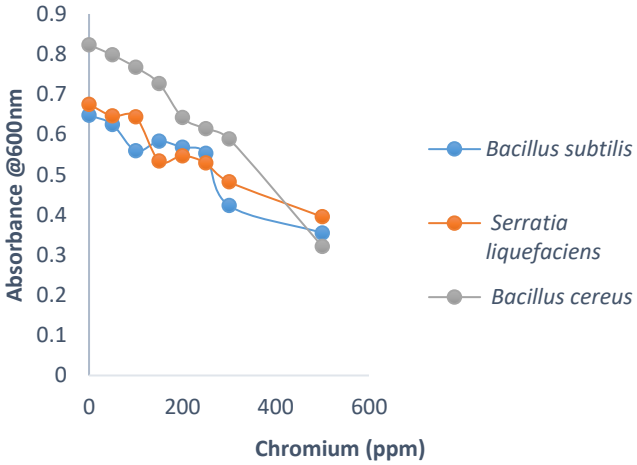

Fig. 6: Growth Pattern of heavy metal resistant bacteria at different concentrations of Chromium

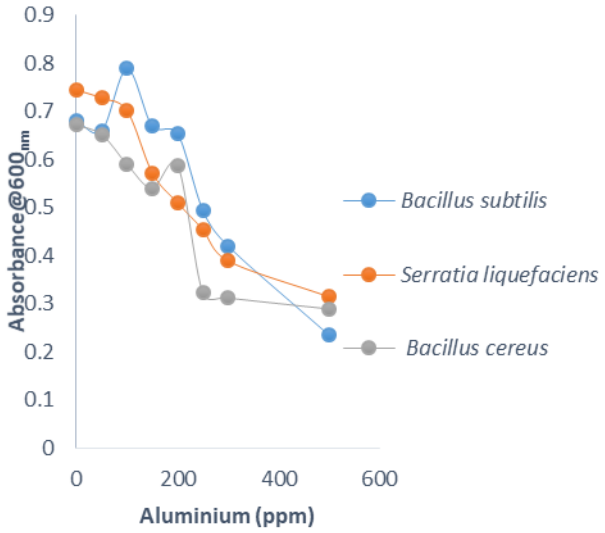

Fig. 7: Growth Pattern of heavy metal resistant bacteria at different concentrations of Aluminum

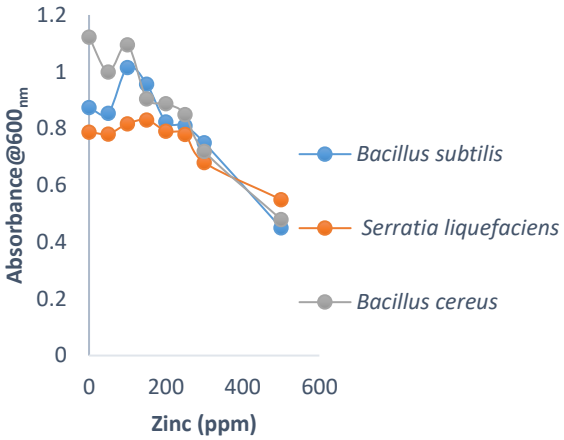

Fig. 8: Growth Pattern of heavy metal resistant bacteria at different concentrations of Zinc

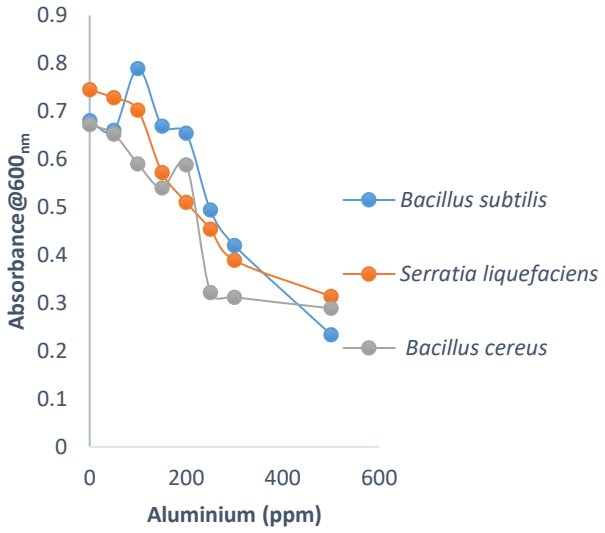

Fig. 8: Growth Pattern of heavy metal resistant bacteria at different concentrations of Aluminum

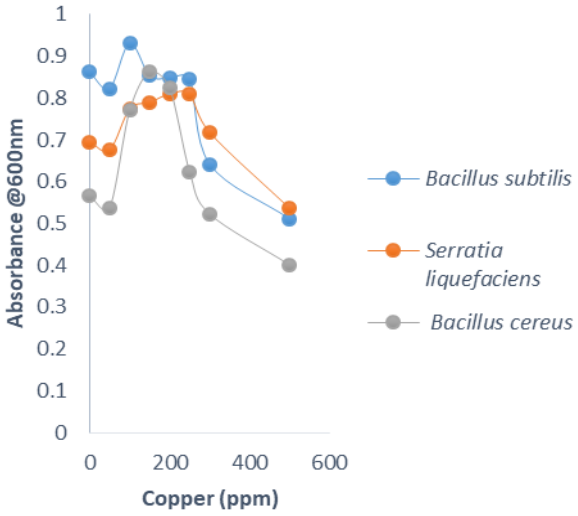

Fig. 9: Growth pattern of heavy metal resistantbacteria at different concentrations of Copper

CONCLUSION

The findings of this study revealed that wastewater is an excellent source of heavy metal resistant bacteria. There was variation among the bacteria isolates with respect to their tolerance towards different heavy metals, however higher tolerance and multi resistance potentials of bacteria from paint factory to high concentrations of heavy metals compared to bacteria from household wastewater confirmed the role pre exposure to pollutants play in building resistance mechanisms in microorganisms. These heavy metal resistant bacteria can serve as useful tools in the remediation of metal contaminated environments.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

ACKNOWLEGEMENT

Add acknowledgements here

AUTHOR CONTRIBUTIONS

TOO, TRO and PIO designed the experiments, TOO, TRO, PIO and RK carried out the experiment and also wrote the manuscript. AOO and OOO prepared the heavy metals, performed the data analysis and reviewed the manuscript. All authors read and approved the final version.

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