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Heavy Metal Tolerance and Antibiotic Sensitivity of Bacterial Strains Isolated From Tannery Effluent

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ABSTRACT

The pollution of the environment with toxic heavy metals is spreading throughout the world along with industrial progress. In the present study, the heavy metal tolerance and antibiotic sensitivity was determined for the bacterial strains isolated from tannery waste water. All the three strains tested were found to exert similar pattern of resistance towards all the heavy metals except chromium where there is very poor growth of all the strains. Pseudomonas aerugunosa and E.coli showed high resistance to cadmium whereas Bacillus subtilis showed high resistance to mercury. Bacillus subtilis and Pseudomonas aerugunosa showed resistance to 50% of the antibiotics tested whereas E.Coli showed resistance to 83% of the antibiotics tested.

Keywords: *Heavy metal tolerance, Bacillus, Pseudomonas, E.Coli, MTC, MIC, Antibiotic sensitivity*

INTRODUCTION

Bioremediation of heavy metal pollution remains a major challenge in environmental Biotechnology. Some industrial processes results in the release of heavy metals into aquatic systems. This has led to increasing concern about the effect of toxic heavy metals as environmental pollutants. This kind of contamination presents a challenge, as the presence of heavy metals in soils and aqueous effluents leads to serious problems because they cannot be biodegraded. Unlike many other pollutants, heavy metals are difficult to remove from the environment [1]. Heavy metals are recognised to be powerful inhibitors of biodegradation activities [2]. These metals cannot be degraded, and are ultimately indestructible. The toxic effects of heavy metals result mainly from the interaction of metals with proteins (enzymes) and inhibition of metabolic processes. These heavy metals such as copper, cadmium, lead, zinc, nickel, mercury and chromium when accumulated in soils, water bodies they can also be present in concentrations toxic to plants, animals, humans and aquatic life [3]. Each heavy metal has unique biofunctions or biotoxicities. For example, copper can enhance microbial growth at low concentrations but repress growth at high concentrations [4] and cadmium has high toxicity at low concentrations [5]. The presences of nonbiodegradable heavy metals in such effluents are 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. Microorganisms have acquired a variety of mechanisms for adaptation to the presence of toxic heavy metals [6]. There is increasing evidence for the evolution of metal resistance in natural populations inhabiting contaminated sites [7, 8, 9].The evaluation of metal resistance 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 [10]. Antibiotic resistance in bacteria is more frequently associated and strongly

correlated with metal resistance [11]. Bacterial species had been isolated from drinking water that was tolerant to metals and antibiotics [12]. The significant increase of Multiple Antibiotic Resistant (MAR) bacteria are observed in various aquatic systems. Human infections caused by such bacteria could be difficult to treat with drugs .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 (R-factor) factor [13]. To survive under metal-stressed conditions, bacteria have evolved several types of mechanisms to tolerate and uptake of heavy metal ions. These mechanisms include the efflux of metal ions outside the cell, and reduction of the heavy metal ions to a less toxic state [14-15]. Therefore this study was performed to determine the antibiotic and heavy metal resistance patterns of bacteria which were isolated from tannery waste water.

MATERIALS AND METHODS

Sampling site and Collection

Effluent samples were collected from the outlet of a tannery industry at Pallavaram, Chennai, Tamilnadu. Samples were collected in sterilized glass bottles aseptically and transported to the laboratory in an ice bucket. Samples were analyzed within 6h of collection.

Processing of Samples for Isolation of Bacterial Strains

Bacterial strains were isolated from effluent samples by serial dilution [16] method. For stock solution 1ml of effluent was diluted to 10ml with distill water. Five test tubes each with 9ml of distill water was prepared and marked as 1-5. One ml from stock solution was aseptically transferred to first test tube making the dilution 10^{-1}. Solution in the test tube was mixed and 1ml from this tube (10^{-1}) was transferred aseptically to next making it as 10^{-2}. Similar transfers were made till 10^{-5} dilution was achieved. From 10^{-2}, 10^{-3}, 10^{-4}, a loop full sample was streaked to sterile M9 Minimal Medium agar plates and spread properly in three different petri plates or in one by making segments, as available. The plates were then kept for incubation at $32 \pm 1^{\circ}C$ for 24 – 48 hrs.

Heavy metals used for the study

The chemical characteristics of the heavy metals used were given in Table I.

Table I. Characteristics of heavy metals used

Serial No.	Metal	Salt used	Mol wt.	Atomic wt.
1	Chromium	K2Cr2O7	294	52
2	Cadmium	CdCl2	183	112
4	Copper	CuSO4	160	64
5	Lead	PbCl2	278	207
6	Mercury	HgCl2	130	57

Isolation 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 Cr6+, Cd2+, Co2+, Pb2+, and Hg2+ were prepared separately. The concentration of each heavy metal was maintained at 100 µg/ml of the medium. The bacterial isolates were 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 according to Bergey's manual of systematic bacteriology.

Determination of the effect of heavy metals on bacterial growth:

Growth curves of bacterial isolates were determined with 100µg per mL concentration each of the heavy metals like $CdCl_2$, $PbCl_2$, $CuSO_4$, $K_2Cr_2O_7$ and $HgCl_2$. The inoculated nutrient broth without the addition of heavy metals serves as the control. For bacterial isolates, 50 mL nutrient broth was taken in one set consisting of three flasks, autoclaved and then inoculated with 20 µL of the freshly prepared inoculum. These cultures were incubated at 37 °C in a shaker at 30 rpm. The absorbance of the cultures was measured at 600 nm at an interval of 24 hrs after inoculation upto 48 hrs with 0^{th} hr as the initial absorbance. Growth profiles were determined by using graphs of absorbance versus time. The effects of different concentrations of heavy metal ions on growth were also evaluated.

Metal resistance evaluation in solid agar

The isolated bacterial strains were tested for resistance against Chromium (K2 Cr2O7), Cadmium (CdCl2), Copper (CuSo4), Lead (PbCl2),and Mercury (HgCl2). Brain Heart Infusion Agar (BHIA) was prepared and respective metals were mixed with agar in various (10, 15 and 20 ppm) concentration. The plates were allowed to solidify. New bacterial cultures were streaked on the BHIA surface and the plates were incubated at 37°C for 24hrs. Growth of the bacterial culture was determined visually as positive or negative. Relative growths of the bacterial isolates were expressed as the percentage of those obtained in untreated control which was taken as 100%.

Determination of Minimal Inhibitory Concentration

Metal tolerance was evaluated as the Minimum Inhibitory Concentration (MIC) of metals such as Lead (Pb), Copper (Cu), Zinc (Zn), Chromium (Cr) and Mercury (Hg). The metal tolerance was determined for different bacterial isolates by broth dilution method [12]. Three concentrations (10, 15 and 20 ppm) of each metal were mixed with Brain Heart Infusion Broth. Based on the evaluation, Minimum Inhibitory Concentration (MIC) was determined at 37°C/24h. The minimum concentration of heavy metals at which no turbidity was observed by spectrophotometer (Carry 100, Varian) at 660nm was considered as the MIC of bacterial isolates against heavy metals. The minimal concentration of metal in the Petri plate showing complete inhibition of growth was considered as minimal inhibitory concentration (MIC). The isolated cultures were streaked onto the NA medium containing metal salts using sterile loops and then incubated at 37°C for 24-48 hrs. The plates were checked for bacterial growth. The concentration of metal where there was no growth is observed as the MIC for that salt for that strain [17].

Maximum Tolerance of Heavy Metals

The metals used in the study and detailed procedure to determine the tolerance property, in terms of Maximum Tolerable Concentration (MTC) was carried out [18]. The Maximum Tolerable Concentration (MTC) of heavy metal was designated as the highest concentration of heavy metal that allows growth after 2 days i.e., 48 hrs [19].

Determination of Antibiotic resistance

The antibiotic resistance was done by standard agar disc diffusion method on BHIA using commercial discs (Himedia, Mumbai) [20]. 100 µl of fresh bacterial cultures were spread on BHIA. The antibiotics such as Bacitracin (10µg/ml), Ciprofloxacin (30µg/ml), Norfloxacin (10µg/ml), Gentamycin (10µg/ml), Imipenim (10µg/ml), and Fluconozole (30µg/ml) were placed on the plate. The plates were incubated at 37ºC for 24h. Inhibition zones in diameters were measured in mm using a caliper. Strains were classified as Resistant (R), Intermediate (I) and Susceptible (S) according to the criteria recommended by the National committee for clinical Laboratory Standards, 2001. Control plates were incubated without antibiotic discs. All the experiments were carried out in triplicate.

RESULTS AND DISCUSSION

Bacteria isolated from Sample: The number of isolates obtained in M9 minimal medium by serial dilution were given in Table II. The different types of isolates grown in Basal media nutrient agar (NA) incorporated with heavy metals like Cd2+, Pb4+, Cu4+, Cr6+ and Hg2+ and were resistant to fixed concentration of each of heavy metal is given in Table III.

S.No	Dilution	Type of colonies
1	10^{-3}	5
2	10^{-4}	2
3	10^{-5}	2
4	10^{-6}	3

Table III. Different types of Colonies grown in NA media supplemented with Heavy metal

S.No	Dilution	Type of colonies
1	CdCl2	3
2	PbSO4	3
3	CuSO4	3
4	K2Cr2O7	3
5	HgCl2	3

Identification and Characterization of heavy metal resistant bacteria

Their biochemical characteristics have been determined by routine analysis according to Bergey's manual of Systematic Bacteriology. Based on the colony and cellular morphology and their biochemical characteristics, they were identified as *Bacillus subtilis*, *Pseudomonas aerugunosa* and *E.Coli*

Determination of the effect of heavy metals on bacterial growth

Growth Pattern with 10ppm of Heavy metals

The growth pattern of the different strains with the concentration of 10ppm of each of the heavy metal after 24hrs and 48hrs is given in Figure-1 and Figure-2 respectively. The concentration of 10ppm of all the heavy metals showed nearly 50% reduction in growth of all the organisms except Lead and Chromium. All the strains showed varied resistance to chromium. After 48 hrs, tolerance is being developed by all the strains except *Bacillus subtilis* where the growth curve decreases. *Bacillus subtilis* showed high resistant to lead and copper when compared to other heavy metals. *Pseudomonas aeruginosa* showed high resistance lead, copper and mercury. *E.coli* exhibits very high resistance to mercury.

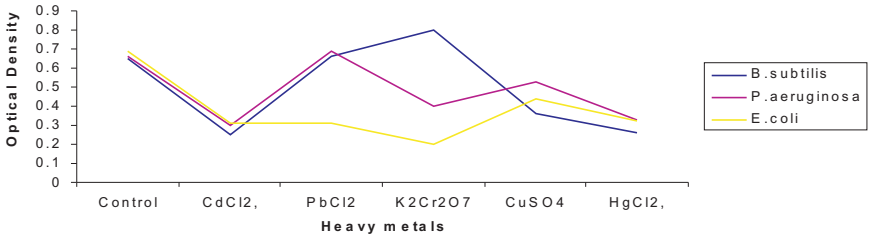


Figure 1. Growth Pattern with 10ppm of Heavy metals after 24 Hrs

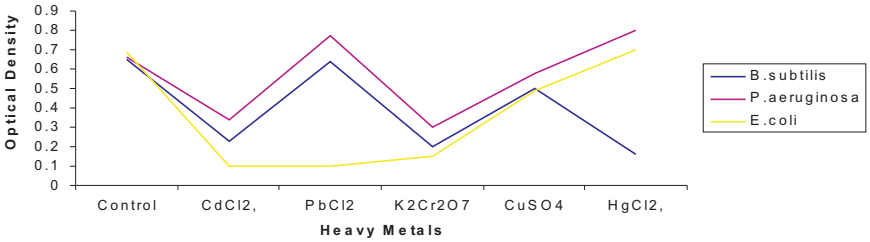


Figure 2: Growth Pattern with 10ppm of Heavy metals after 48 Hrs

Growth Pattern with 15ppm of Heavy metals

Growth profiles were determined by using graphs of absorbance versus time. The effects of different concentrations of heavy metal ions on growth were also evaluated. These observations suggest that the toxicity of the metal ions to the bacterial isolates were dependent on the bacterial strains. Some media support better growth of microbes than the others [21]. The growth pattern of the different strains with the concentration of 15ppm of each of the heavy metal after 24Hrs and 48 Hrs is given in Figure 3 and, Figure 4 respectively. All the isolated showed high resistance to lead and copper whereas there is poor growth of all the 3 strains in all the other heavy metals tested. This indicates their inability to grow in increased concentration of the respective heavy metals. The growth pattern of the different strains with 15 ppm of heavy metals after 48hrs, showed similar resistance to lead, copper and chromium. However, it also proved the inability of the strains to grow upon prolong exposure to cadmium and mercury.

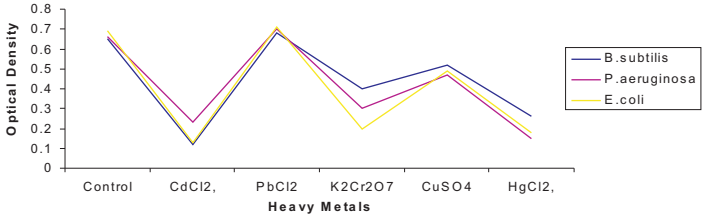


Figure 3. Growth Pattern with 15ppm of Heavy metals after 24 Hrs

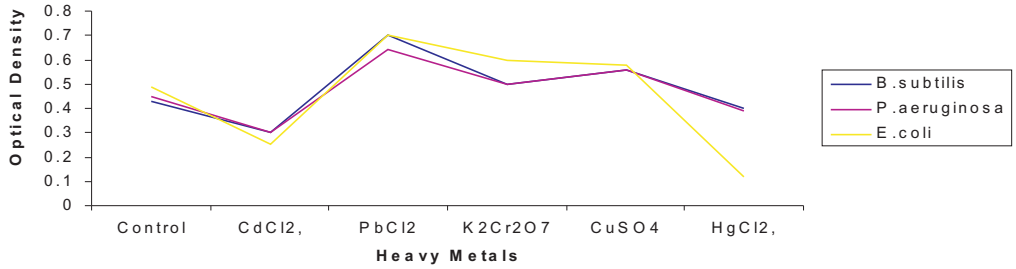


Figure 04. Growth Pattern with 15ppm of Heavy metals after 48 Hrs

Growth Pattern with 20ppm of Heavy metals

The growth pattern of the different strains with the concentration of 20mg/ml of each of the heavy metal after 24Hrs and 48 Hrs is given in Figure 5 and Figure 6 respectively. Though all the strains showed similar pattern of resistance to all the heavy metals, the poor growth curve was noted in this high concentration after both 24 and 48hrs which proved the intolerance of growth with high concentration of heavy metals.

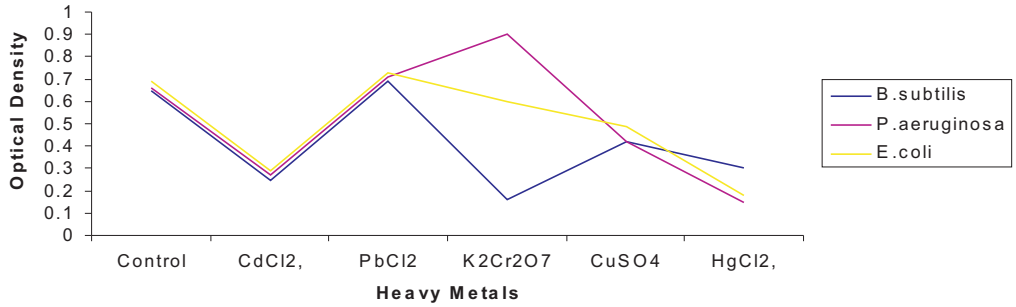


Figure 05. Growth Pattern with 20ppm of Heavy metals after 24 Hrs

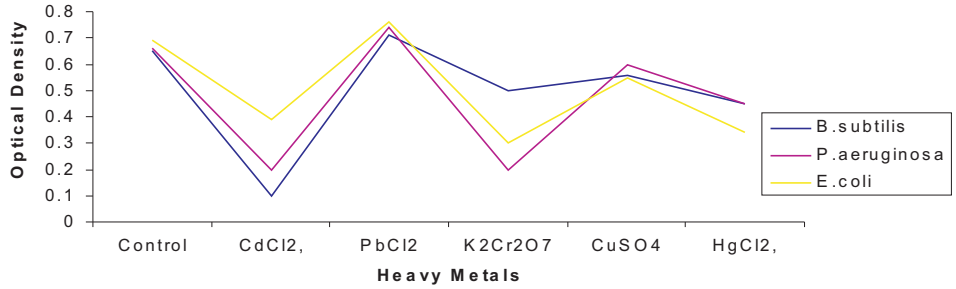


Figure 06. Growth Pattern with 20ppm of Heavy metals after 48Hrs

Minimal Inhibitory Concentration of Heavy Metals

The minimal inhibitory concentrations of heavy metal ions were also determined in liquid media for the bacterial strains in order to establish baseline levels of resistance against which to compare values determined with the other bacterial species. The minimal inhibitory concentration of various heavy metals towards all the isolated strains was shown in Figure 7 to Figure 11. Different strains showed difference in resistance towards all the heavy metals tested. All the heavy metals tested were found to exert similar pattern of resistance among all the three strains except chromium where there is very poor growth of all the strains. This implicates the inability of all the strains to tolerate chromium even in less concentration. Further an increase in concentration of the heavy metals resulted in decrease in growth of the bacterial strains with an exception of cadmium. This implicates the tolerance developed by the strains towards cadmium.

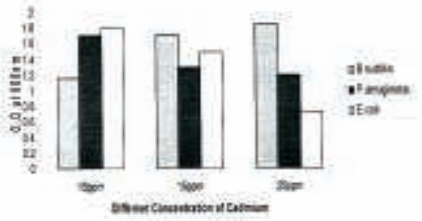


Figure 07. MIC of Cadmium

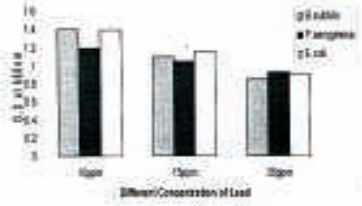
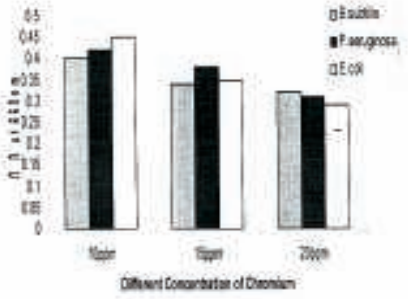


Figure 08. MIC of Lead



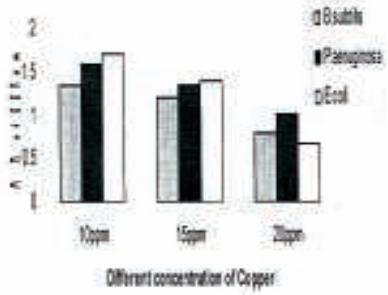


Figure 09. MIC of Chromium

Figure 10. MIC of Copper

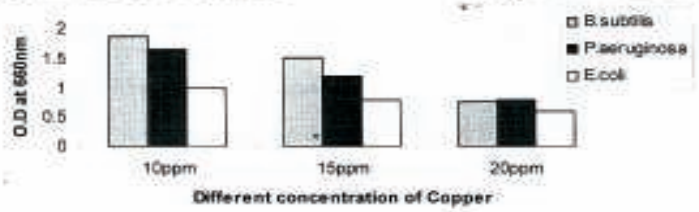


Figure 11. MIC of Mercury at Different concentration

In gram-positive bacteria, accumulation of Cd2+ leads to the expression of the *CadA* resistance system, which is located on plasmid p1258 and related plasmids [22- 23]. *CadA* mediates resistance by active ion efflux [23-24]. The *CadA* resistance determinant is inducible and the *CadC* gene product is known to be trans-acting DNA-binding regulatory protein of this system [25, 23]. The microbial growth decreased with the increase in concentration of heavy metals indicating toxic effect of the heavy metals on the growth of microorganisms. Previous study by reported that copper and chromium were tolerated heavy metals whilst mercury was the most toxic heavy metal for the bacteria [21]. Responses of bacterial isolates to heavy metals were very heterogeneous. All isolates were Gram-negative bacteria. However, Foster has reported the resistance to heavy metals in both Gram-positive and Gram-negative bacteria. All isolates tolerant for cadmium and mercury were Gram-positive aerobic spore-formers, and very few of these strains were antibiotic-resistant [26]. Generally Gram-negative soil species appeared to be more tolerant than Gram-positive [27]. This difference in results could be explained by the conditions of each bacterial isolation and the

selectivity of microbial culture techniques adopted in each study, particularly with respect to the nature and specificity of growth media. Due to the presence of highly concentrated areas of heavy metals in the environment, heavy metal resistance mechanisms are commonly found in bacterial genomes. There is no general mechanism for resistance to all heavy metal ions. However, the mechanisms of resistance usually involve either enzymatic detoxification or efflux pumping which pump the toxic ions out of the cell prohibiting them from accumulating to levels high enough to inhibit growth or cause cell death.

In order to assess the bioremediation potentials of the isolates, it was also necessary to evaluate their resistance profiles and the minimal inhibitory concentrations (MICs). Resistance of the isolates was determined in both TYG and MH media. The resistance patterns shown by the three isolates to the heavy metal ions and the degree of resistance differed among them.

Copper ions are required as co-factors by many enzymes, such as oxidases and hydroxylases, but are highly toxic when present in excess [28]. They are possibly accumulated by the CorA-Mg2+ transporter and additionally by P-type ATPases under copper starvation. Mechanisms for copper metabolism occur naturally in all living organisms, and they are generally chromosomally encoded [29]. In contrast, mechanisms that specify resistance to copper in bacteria are often plasmidencoded [30]. The plasmid and chromosomal systems may interact with each other to maintain copper homeostasis in bacteria [31-34]. Inside the cells, copper may be bound by various compounds to form copper complexes [35]. P-type ATPases seem to detoxify copper via efflux in some species, however, the copper resistance systems of the *Pseudomonas* type usually encode four proteins which bind copper in the periplasm or close to the outer membrane [35]. Copper toxicity is based on the production of hydroperoxide radicals [36] and its radical character makes Cu2+ very toxic [35], and many organisms are reported to be sensitive to copper [37]. Also, the nature of growth media, pH and temperature of incubation could affect the metal tolerance [38]. These observations suggest that the toxicity of the metal ions to the bacterial isolates were dependent on the bacterial strains. Also, the bacterial isolates were more tolerant to the metal ions in MHA than TMA which suggests that the bacterial tolerance to the metal ions was dependent on the type of medium used. Mercury-resistance determinants have been found in a wide range of Gram-negative and Gram-positive bacteria isolated from different environments. These resistance determinants vary in the number and identity of genes involved and is encoded by the *mer* operon located on plasmids [39-41].

Maximum Tolerance of Heavy Metals

The isolated strain showed different levels of tolerance to the metals under investigation and MTC were used to determine metal tolerance of bacteria as given in Table IV. The heavy metal tolerance pattern among all the three strains isolated was shown in Table V.

Table IV. Maximum Tolerance Concentration

Isolated Strain	Heavy Metals				
	CdCl2,	PbCl2	K2Cr2O7	CuSO4	HgCl2
Bacillus subtilis	20	15	20	20	15
Pseudomonas aeruginosa	20	15	15	20	15
E.coli	20	20	15	20	20

Table V. Heavy Metal Resistance Pattern

S.No	Isolates Identified	Resistance Pattern
1	*Bacillus subtilis*	Hg>Pb>Cu>Cd>Cr
2	*Pseudomonas aeruginosa*	Cd >Hg> Cu >Pb>Cr
3	*E.coli*	Cd >Cu >Pb>Hg>Cr

Pseudomonas aeruginosa and *E.coli* showed high resistance to cadmium whereas *Bacillus subtilis* showed high resistance to mercury. However, all organisms showed least resistance towards chromium. This proves the adaptation of the isolates to chromium toxicity in the environment. Toxicity testing in liquid medium allows a good evaluation of metal toxicity in polluted environments, such as industrial effluents and sewage sludge leachates [21]. Liquid medium toxicity testing is different from toxicity testing on solid medium, where the conditions of diffusion, complexation and availability of metals are different from those in solid medium. The levels of tolerance of environmental bacteria to the different divalent metal ions including Cu2+, Co2+, Cd2+ and Zn2+ in nutrient broth was tested and reported that the

test in liquid media was sensitive at concentrations 10 to 1000 times lower than those obtained in solid media [21].

Antibiotic Resistance Test

The sensitivity of the isolated strains towards various antibiotics and their zone of clearance is shown in Table VI. All the three strains showed resistance towards Gentamycin, Fluconazole and Norfloxacin. *Bacillus subtilis* showed high resistance to gentamycin whereas the other two strains show high resistance towards Fluconazole. All the strains were sensitive to Imipenem. Only *E.coli* is resistant to Bacitracin and Ciprofloxin whereas the other two strains were sensitive as there is no zone formation. *Bacillus subtilis* and *Pseudomonas aeruginosa* showed resistance to 50% of the antibiotics tested whereas *E.Coli* showed resistance to 83% of the antibiotics tested Figure 12).

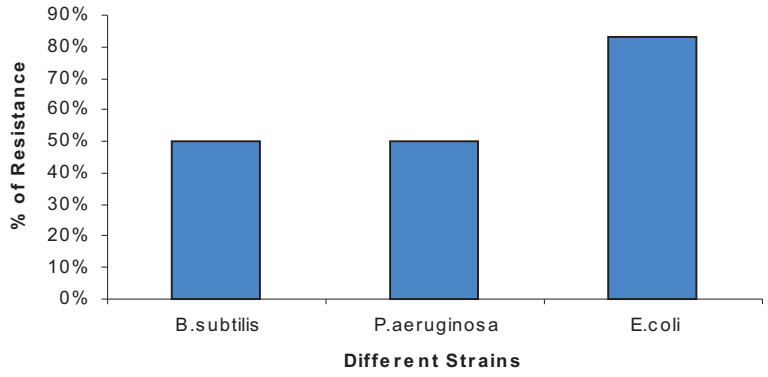


Figure 12: % Antibiotic resistance

Table VI. Resistance towards Antibiotics

S.NO	ANTIBIOTIC USED	DISC CONTENT/ml	*Bacillus sp* mm	*Pseudomonas sp* mm	*E.coli* mm
1	Bacitracin	10 µg	**S**	**S**	**R** 0.2
2	Gentamycin	10 µg	**R** 1.4	**R** 0.5	**R** 0.7
3	Norfloxacin	10 µg	**R** 0.3	**R** 0.6	**R** 0.6
4	Imipenem	10 µg	**S**	**S**	**S**
5	Ciprofloxin	30 µg	**S**	**S**	**R** 1.3
6	Fluconazole	30 µg	**R** 0.6	**R** 1.0	**R** 1.0

A correlation exists between metal tolerance and antibiotic resistance in bacteria because of the likelihood that resistance genes to both (antibiotics and heavy metals) may be located closely together on the same plasmid in bacteria and are thus more likely to be transferred together in the environment. Multiple resistance isolates exhibits resistance towards group of antibiotics but increase in heavy metal concentration leads to decrease in antibiotic resistance [42-43]. 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[44]. It is therefore, likely that selective pressure by one such compound indirectly selects for the whole set of resistances.

CONCLUSION

Bacterial resistance to antibiotics and heavy metals is an increasing problem in today's society. Resistance to antibiotics is acquired by a change in the gene makeup of bacterium, which can occur by either a gene mutation or by transfer of antibiotic resistance genes between bacteria in the environment. The increasingly use of antibiotics in

health care, in agriculture and animal husbandry is in turn contributing to the growing problem of antibiotic resistant bacteria. Heavy metals used in industry and in household products are, along with antibiotics creating a selective pressure in the environment that leads to the mutations in microorganisms that will allowed them better survive and multiply. Microbes have adapted to tolerate the presence of metals or can even use them to grow. Thus, a number of interactions between microbes and metals have important environmental and health implications.

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