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ORIGINAL ARTICLE

Isolation and Characterization of Bacteria from Tannery Effluent Treatment Plant and Their Tolerance to Heavy Metals and Antibiotics

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ABSTRACT

The pollution of the environment with toxic heavy metals is spreading throughout the world along with industrial progress. Microorganisms and Microbial products can be highly efficient bioaccumulations of soluble and particulate forms of metals especially dilute external solutions. Microbes related technologies may provide an alternative or addition to conventional method of metal removal or metal recovery. The present study deals with isolation, identification and characterization of heavy metal resistant bacteria was isolated from tannery effluent collected in and around Chennai, South India. Initially, a total of 50 isolates were screened from tannery effluent. The five isolates were selected based on high level of heavy metal and antibiotic resistances. On the basis of morphological, biochemical analysis revealed that, the isolates were authentically identified as Escherichia coli (Isolate-1), Bacillus sps (Isolate-2), Pseudomonas sps (Isolate- 3), Flavobacterium sps (Isolate-4) and Alcaligenes sps (Isolate-5). The identified isolates were resistant to Zinc (Zn), Copper (Cu), Chromium (Cr), Mercury (Hg) and Lead (Pb). The minimum inhibitory Concentration (MIC) of tannery effluent isolates against Pb, Cu, Zn, Cr, and Hg was determined in solid media. All the tannery effluent isolates resistant to Pb (50-90%), Cu (30-85%), Zn (50-80%), Cr (30-70%) and Hg (30-80%). The multiple metal resistances of these isolates were also associated with antibiotics Ampicillin (AM), Ciprofloxacin (C), Cotrimazole (Co), Gentamycin (GM), Kanamycin (K), Nalidixic acid (NA) and Streptomycin (S).The identified heavy metal resistant bacteria could be useful for the bioremediation of heavy metal contaminated tannery effluents.

Key Words: *Leather, Bacteria, Antibiotics, MIC, Bioremediation.*

INTRODUCTION

In the past few decades, uncontrolled urbanization has caused a serious pollution problem due to the disposal of sewage and industrial effluents to water bodies. 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 non-biodegradable 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 [7-9].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 were 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 [13-16].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 [17].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 [18-19]. 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

Isolation of bacteria

The tannery effluent samples were collected from Common Effluent Treatment Plant (CETP), Pallavaram, Chennai during April-May 2008. The samples were collected in sterile plastic container and transported to laboratory for bacteriological analysis. The bacterial isolates were screened on Nutrient Agar (NA) plates supplemented with 5mg/l concentration of each metal one time by the standard pour plate method [20]. Plates were incubated at 37°C /24h and colonies differing in morphological characteristics were selected and used for further studies.

Identification and Characterization of the tannery effluent bacteria

Selected tannery effluent isolates were grown on MacConkey agar (Himedia, India). The shape and colors of the colonies were examined under the microscope after Gram staining. Isolates were biochemically analyzed for the activities of Oxidase, Catalase, MR-VP test, Urease test, Motility, Indole production and Citrate utilization. The tests were used to identify the isolates according to Bergey's Manual of Determinative bacteriology [21-22].

Metal resistance evaluation in solid agar

The isolated bacterial strains were tested for resistance against Lead ($PbCl_2$), Copper ($CuSo_4$), Zinc ($ZnSo_4$), Chromium ($K_2Cr_2O_7$), and Mercury ($HgCl_2$). Brain Heart Infusion Agar (BHIA) was prepared and respective metals were mixed with agar in various (10-80ppm) concentration. The plates were allowed to solidify. New bacterial cultures were streaked on the BHIA surface and the plates were incubated at 37°C/24h. 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 MIC

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]. Eight concentrations (10-80ppm) 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.

Determination of Antibiotic resistance

The antibiotic resistance was done by standard agar disc diffusion method on BHIA using commercial discs (Himedia, Mumbai) [23-24]. 100 µl of fresh bacterial cultures were spread on BHIA. The following antibiotics such as Ampicillin (10µg/ml), Ciprofloxacin (30µg/ml), Co-trimazole (5µg/ml), Gentamycin (10µg/ml), Kanamycin (30µg/ml), Nalidixic acid (30µg/ml) and Streptomycin (10µg/ml) were placed on the plate. The plates were incubated at 37ºC/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.

RESULTS

Isolation of heavy metal resistance bacteria

Bacterial strains were isolated from tannery effluent. Fifty colonies were screened from initial level of heavy metal

supplemented NA medium. Out of fifty bacterial strains, ten bacterial isolates were enteric of which six were *Escherichia coli* (Isolate 1) and four were *Bacillus species* (Isolate2). Twelve were *Pseudomonas species* (Isolate 3), nine belongs to the *Achromobacter* of which five were *Flavobacterium species* (Isolate 4) and four were *Alcaligenes species* (Isolate 5). The rest of the isolates remained unidentified. The different isolates showed different optimum pH and optimum temperature.

Table -1. Characteristics of bacterial isolates from tannery effluents

Morphological/ Physiological/Biochemical characteristics.	Isolate1	Isolate2	Isolate3	Isolate4	Isolate5
Gram reaction	-	+	-	-	-
Endospore	-	+(central)	-	-	-
Motility	+	+	+	+	+
Fluorescence(UV)	-	-	+	-	-
Growth under	Ae	Ae	Ae	Ae	Ae
On MacConkey agar	+	-	-	+	+
Growth at P^H	5.7-8.0	4.5-8.5	4.0-8.0	5.0-7.5	5.5-10.8
Growth at temperature (°C)	37	35	32	32	35
Indole test	+	-	-	+	+
Methyl red test	+	-	-	-	-
Voges-Proskauer test	-	-	-	-	-
Citrate test	+	+	+	+	+
Urease test	-	+	+	+	+
Catalase test	+	+	+	+	+
Oxidase test	-	+	-	+	-
Oxidation/fermentation (O/F)	F	F	NR	ND	F
H_2S production	-	-	+	-	+
Acid production from carbohydrates:					
Lactose	AG	A	-	A	Al
Dextrose	AG	A	-	A	Al
Sucrose	AG	A	-	A	A
Mannitol	G	A	A	A	Al
Type strain	*E.coli*	*Bacillus spp.*	*Pseudomonas spp.*	*Flavobacterium spp.*	*Alcaligenes spp*

- **Negative;** + **Positive;** Ae: **Aerobic;** F: **Fermentative;** O: **Oxidative;** AG: **Acid/Gas;** G: **Gas;** A: **Acid,** Al: **Alkaline,** NR: **Noreaction;** ND**Not determined.**

Effect of heavy metals on the growth of identified isolates

The growth of bacterial isolates in the presence of different heavy metals is mentioned in **Table 2.** More than 80% of *Pseudomonas sps* isolated were found to be highly resistant to all heavy metals. *E. coli* isolates (70%) were resistant against Pb and Cr and only 30% against Cu. *Bacillus sps* (50%) were resistant against Pb, Cu, Zn and 30% against Cr and Hg respectively. *Flavobacterium sps* (90%) were resistant against Pb and 55% to Cr. *Alcaligenes sps* (90%) were

found to be resistant against Pb and 30% to Cr.

Table - 2. Metal resistance incidence of isolates in percentage (%).

Metals	*Pseudomonas*	*E. coli*	*Bacillus*	*Flavobacterium*	*Alcaligenes* spp.
Pb	90±0.2	70± 0.2	50 ± 0.1	90 ± 0.1	90 ± 0.1
Cu	85 ± 0.1	30 ± 0.1	55± 0.1	70± 0.3	65 ± 0.2
Zn	80± 0.2	60± 0.2	50± 0.2	75 ± 0.2	70± 0.3
Cr	70 ± 0.1	70± 0.1	30± 0.1	55 ± 0.1	30 ± 0.1
Hg	80 ± 0.2	55 ± 0.1	30± 0.1	70 ± 0.2	65 ± 0.2

Values are average of three determinations

Evaluation of heavy metal resistance

The minimum inhibitory concentrations (MIC) of different metals for various bacteria were shown in **Fig. 1** to **Fig. 5.** For Cr **(Fig. 1)** the MIC value was 60 ppm for *Flavobacterium sps* the maximum MIC was 80 ppm for *Escherichia coli* and *Bacillus sps.* 80 ppm of Hg **(Fig. 2)** was the MIC of all isolates; 60 ppm of Cu **(Fig. 3)** was the MIC for *Bacillus,* 80 ppm for *Pseudomonas sps* and *Escherichia coli.* 80 ppm of Zn **(Fig. 4)** was the MIC of *Pseudomonas sps*, *Bacillus sps* and *Flavobacterium sps*. 50 and 60 ppm was the MIC of *Alcaligenes,* and *Escherichia coli* respectively. The MIC of Pb **(Fig. 5)** was found to be 60-80 ppm and MIC for *Escherichia coli* and *Alcaligenes sps* were 80 ppm respectively. 70 ppm of Pb was the MIC for *Pseudomonas sps* and 60 ppm for *Flavobacterium sps* and *Bacillus sps*. All the bacterial strains isolated were resistant to most of the antibiotics tested. Most of the bacterial strains were sensitive against Ciprofloxacin and *Pseudomonas sps*, *E.coli* were sensitive to Co-trimazole. Gentamycin, Kanamycin, Nalidixic acid, Amphicillin, and Streptomycin showed less resistance against bacteria. **(Table 3).** These antibiotics were chosen based on their application to control the bacterial infection [25]

Table - 3. Antibiotic resistance among bacterial isolates in terms of inhibition zone in mm diameter.

Antibiotics	Conc (µg/disc)	*Pseudomonas*	*E. coli*	*Bacillus*	*Flavobacterium*	*Alcaligenes*
Ampicillin	10	15 ±0.1	12 ±0.1	HR	14 ±0.1	13 ±0.1
Ciprofloxacin	30	35 (S) ± 0.2	45(S) ± 0.2	35(S) ±0.1	43(S) ± 0.2	45(S) ±0.1
Co-trimazole	25	20(S) ± 0.3	23(S) ± 0.1	HR	HR	HR
Gentamycin	10	14± 0.1	10 ± 0.1	11± 0.2	13 ±0.2	11± 0.2
Kanamycin	30	09± 0.1	12± 0.1	HR	10 ±0.1	HR
Nalidixicacid	30	HR	10 ±0.1	HR	15 ±0.1	12 ±0.2
Streptomycin	10	13 ± 0.2	15± 0.2	12 ±0.2	12± 0.1	10± 0.2

S Sensitive; HR Highly resistant.

DISCUSSION

In the present study, it was evident that different bacterial strains such as *Pseudomonas, E.coli, Alcaligenes, Flavobacterium*, and *Bacillus* species showing tolerance to five heavy metals, viz, Pb, Cu, Zn, Hg and Cr. The resistance of the isolates to heavy metals was neither lost nor altered when isolates were stored in nutrient agar at refrigerated temperature. The MIC determined for the five metals were between 60 to 80 ppm varied depending on the metal and bacterial isolate. . This study showed a high incidence of metal resistance for the bacterial isolates. Many bacterial species isolated from industrial zones had been shown to develop resistance to heavy metals [26-27]. In this study it is clearly seen that the bacterial isolates also show non vulnerability to different antibiotics **(Fig. 6).** Earlier bacterial strains resistant to Gentamycin and Penicillin were also resistant against to

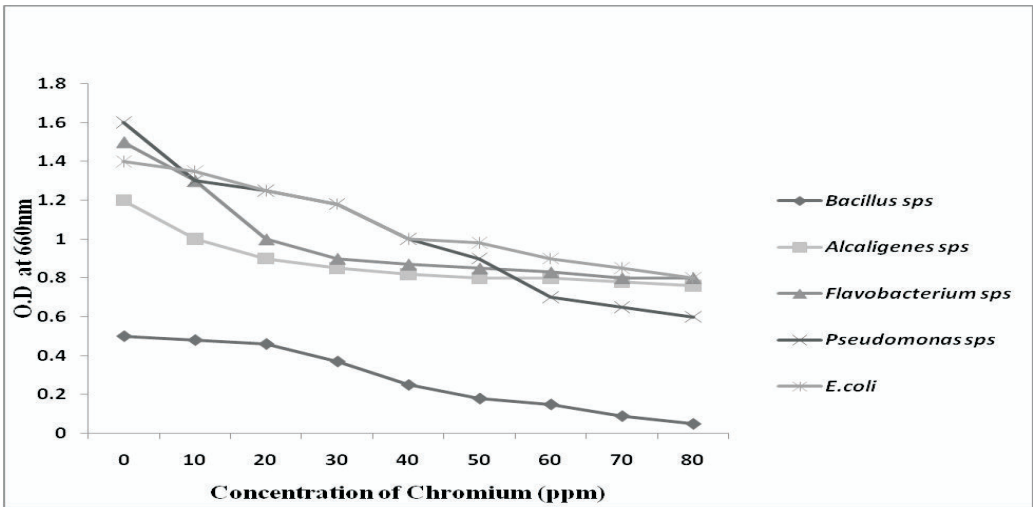

Fig 1: Minimum Inhibitory concentration of Chromium against *Bacillus, Alcaligenes, Flavobacterium, Pseudomonas* and *E.Coli.*

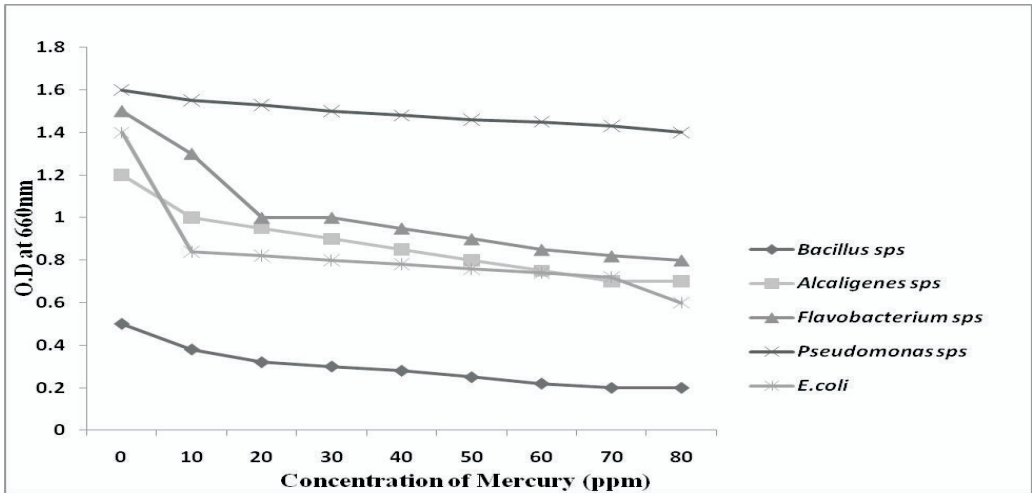

Fig 2: Minimum Inhibitory concentration of Mercury against *Bacillus, Alcaligenes, Flavobacterium, Pseudomonas* and *E.Coli.*

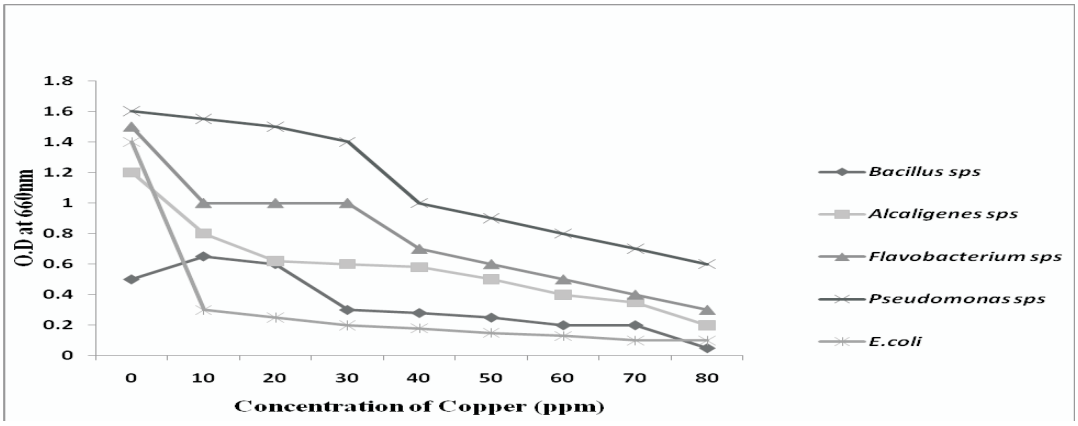

Fig 3: Minimum Inhibitory concentration of Copper against *Bacillus, Alcaligenes, Flavobacterium, Pseudomonas* and *E.Coli.*

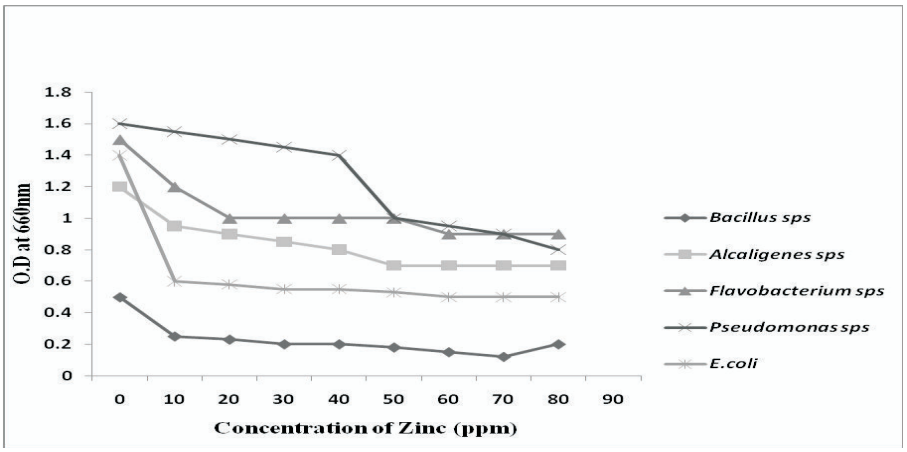

Fig 4: Minimum Inhibitory concentration of Zinc against *Bacillus, Alcaligenes, Flavobacterium, Pseudomonas* and*E.Coli.*

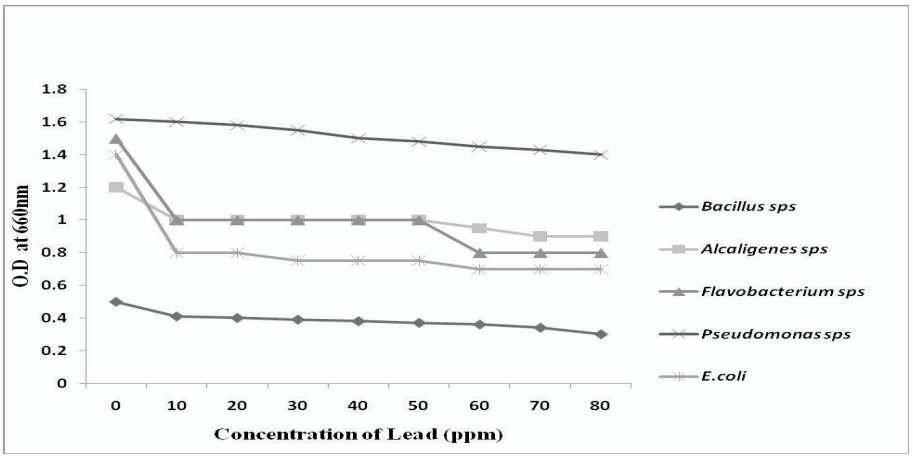

Fig 5: Minimum Inhibitory concentration of Lead against *Bacillus, Alcaligenes, Flavobacterium, Pseudomonas* and *E.Coli.*

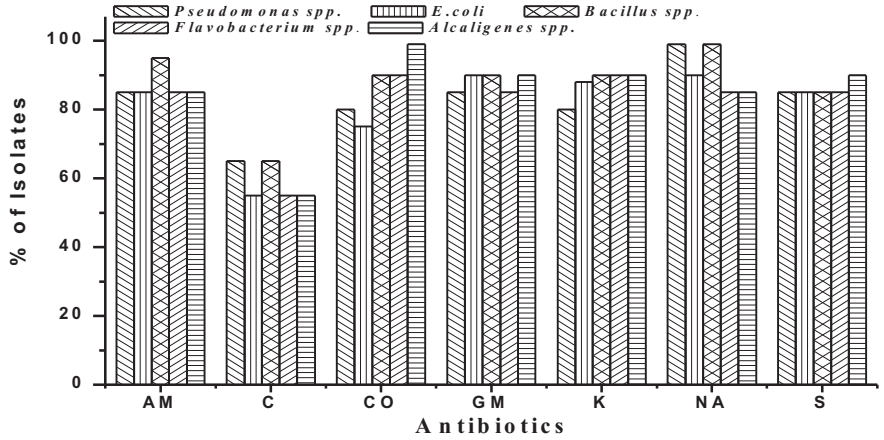

Fig 6: Antibiotic resistance of various isolates against the antibiotics such as Ampicillin (AM),Ciprofloxacin (C), Cotrimazole (CO), Gentamycin (GM), Kanamycin (K), Nalidixicacid (NA) and Streptomycin (S).

heavy metals [28- 29]. The spread of multiple antibiotic resistant bacteria has been the most serious threat to the successful treatment of disease [30-31] The 5 bacterial isolates used in the study were resistant to the heavy metals viz., Cu, Zn, Hg, Cr and Pb. This increase in the MIC of metals as well as the antibiotic resistance 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 [28]. Among 90% of *Achromobacter* bacteria were tolerant to Co-trimazole, Gentamycin and Streptomycin. Multiple antibiotic resistances with tolerance to Cu, Zn, Hg and Pb were observed for these species. The tolerance to Cu, Zn, Hg and Pb were expressed by *Flavobacterium sps* and *Alcaligenes sps* at 70% level. 90% of enteric isolates showed tolerance against Co-trimazole, Gentamycin, Kanamycin, Nalidixic acid and Amphicillin and 70% of *E. coli* and 50% of *Bacillus sps* expressed tolerance against Pb, Cr, and Zn. Among *Pseudomonas sps*, 80% showed tolerance against all antibiotics except Ciprofloxacin. The tolerance of *Pseudomonas sps* against to Pb is 90% and Cu, Zn, Hg and Cr were 80%.

CONCLUSION

The industrial effluents are enriched media to grow and spread microbial population, which are resistant to different metals. The identification of resistance against different metals may provide a useful tool for the simultaneous monitoring of several toxic pollutants in the environment. It is clearly indicated that domestic waste and industrial waste are responsible for the development of bacterial resistance along with the risk of human health and environment. Among all the isolates *Pseudomonas sps* showed resourceful tolerance against all the heavy metals used. Hence, this species can be used as a bioremediation tool for the treatment of effluent from leather and other industries handling heavy metals.

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