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Assessment of Heavy Metals and Antibiotic Resistance in Rhizobacteria Isolated from Rhizosphere Soils Contaminated with Tannery Effluents in Bahir Dar, Ethiopia

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ABSTRACT: *Aims:* Pollution of the environment with toxic heavy metals is spreading throughout the world with industrial progress. Metal pollution in industrial areas is of serious environmental concern as these metals like chromium (Cr), nickel (Ni), mercury (Hg), cadmium (Cd), lead (Pb) and cupper (Cu) are known to cause damage to living organisms including human beings. The study investigated the level of heavy metals and antibiotics resistance in bacteria isolated from rhizosphere soils contaminated with tannery effluents.
Methodology and Results: Soil samples were collected from three selected rhizosphere soils of the Tannery effluent contaminated environments and heavy metals resistant rhizobacteria were isolated from soil. A total of twenty one rhizobacteria were isolated from Potassium dichromate supplemented nutrient agar. These isolates were categorized under *Pseudomonas species, Staphylococcus, Clostridium, Klebsiella species, Bacillus species, Listeria species, and Streptococcus species* after biochemical tests. Further, these isolates were assessed for resistance to other heavy metals and antibiotic resistance. Most of these isolate exhibiting maximum resistance against both metals and antibiotics.
Conclusion, Significance and Impact of study: These heavy metal resistant bacteria can be useful for the bioremediation of heavy metal contaminated environment including industrial effluents.

KEYWORDS: Antibiotics, Bacterial resistance, Heavy metals, Soil, Tannery effluents

1 INTRODUCTION

Heavy metal soil contamination is a significant environmental problem because of their toxicity, persistent and non-degradable conditions in the environment (Tam and Wong, 2000; Yuan *et al*., 2004). According to Altaf *et al*., (2008) tannery effluents are ranked as the highest pollutants among all industrial wastes. They are especially large contributors of chromium pollution. Unregulated disposal of chromium-containing effluent in both developing and developed countries has led to the contamination of soil, sediments, surface and groundwater (Altaf *et al*., 2008). Moreover, copper, chromium, cadmium and nickel are known to be the most common heavy metals used and the more widespread contaminants of the environment (Doenmez and Aksu, 1999).

The aim of this study was to investigate heavy metals and antibiotics resistant *rhizobacteria* isolated from tannery effluent contaminated soils. The identification of resistance against different metals may provide a useful tool for the simultaneous monitoring of several toxic metal pollutants in the environment.

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2 MATERIALS AND METHODS

2.1 STUDY AREA

This study was conducted in Bahir Dar tannery. Bahir Dar is the capital city of the Amahara Region, located in the North-west of Ethiopia on the southern shores of Lake Tana, the source of Abay River (Blue Nile). It is 578 KM (360 miles) south of Addis Ababa, having $11^0$36'N latitude and $37^0$23'E longtude with an elevation of 1840 meters above sea level (Adem and Lamma, 2012). Soil samples were collected from three places at depth of 20cm in plant rhizosphere in sterilized polyethylene bags with the help of a sterilized spatula (Tamer Akkan *et al*., 2013). Equal amount of soils from three sites were mixed well and these composite samples were used for further isolation.

2.2 ISOLATION AND IDENTIFICATION OF CHROMIUM RESISTANT RHIZOBACTERIA

For isolation of chromium resistant rhizobacteria, serial dilution of the soil sample was done. Ten gram soil from composite soil sample was added into 90 ml of distilled water and mixed well (Kasra *et al*., 2007). A serial dilution was made up to 10^{-6} from the soil sample. The concentration of $K_2Cr_2O_7$ was maintained in the stock solution. Nutrient agar plates were prepared and the medium was autoclaved at 121^0C for 15 min. The soil samples were spread plated on media supplemented with $K_2Cr_2O_7$ (100 µg ml^{-1}) and incubated at 28^oC for 3 days. The growth of the bacterial colonies was observed after 48hrs of incubation at 28°C. It was sub-cultured on nutrient agar plate containing 100 µg of potassium dichromate (K2Cr2O7). After incubation, rhizobacterial growth on the media was observed. To obtain pure culture of the isolates serial streaking of single colony was done on Nutrient Agar and preserved for further test. The bacterial isolates were screened on Nutrient Agar (NA) plates supplemented with various concentration of each metal one time by the standard spread plate method *(*Shrivastava *et al*, 2013). Identification of the isolates was done following standard biochemical tests. Biochemical tests like Gram staining, Thyoglycolate test, catalase activity were done for identification of isolates (Holt *et al*., 1994).

2.3 DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC) OF THE HEAVY METALS

The MIC of the metal for each bacterial isolate was determined on nutrient agar plate supplemented with heavy metals. The heavy metals: Cu^+, Zn^+, Cr^+, Cr^{+3}, Ni^+, Cd^+, Hg^{+2} and Pb^+ were used in the form of ($CuSO_4.5H_2O$, $ZnSO_4.7H_2O$, $K_2Cr_2O_7$, $CrCl_3$, $NiSO_4.6H_2O$, $(CH_3COO)_2Cd.H_2O$, $HgCl_2$ and $(CH_3COO)_2Pb.3H_2O$), respectively; in varying concentration. Stock solutions of the metal salts were prepared by mixing 100ml of distilled water with 10g of metal salt and were added to the respective media in varying concentrations. The concentration of heavy metals gradually increased in nutrient agar until the isolate failed to grow on the metal amended plate. The concentrations varied from 50 to 2000 µg/ml. The MIC was recorded when the isolates failed to show growth on the plates (Shakoori, 1998).

2.4 ANTIBIOTIC SUSCEPTIBILITY TEST

The antibiotic sensitivity of isolated rhizobacterial strains was conducted using the conventional Kirby Bauer disk diffusion method (CLSI, 2007). The rhizobacterial colonies were taken from an agar plate cultures. The plates were incubated at 37 ^{o}C for 24 hours. Then the inoculum were transferred into a tube containing 5 ml of sterile nutrient broth medium and mixed gently to form homogeneous suspension. The broth culture was incubated at 37°C until it achieves or exceeds the turbidity of the test suspension. The broth culture was incubated at 37^oC to examine the zone of growth inhibition. Finally the turbidity was adjusted to 0.5 Mac Farland standards (CLSI, 2007). The results were classified as resistant or sensitive (CLSI, 2007).

2.5 GROWTH PATTERN OF THE MOST PROMISING BACTERIA

The most promising rhizobacterial isolate exhibiting maximum resistance to both metals and antibiotics was selected for growth study under chromium stress. To assess the growth pattern of the rhizobacterial isolate, exponentially grown culture was inoculated into nutrient broth treated with different concentration of chromium (Cr^{3+} and Cr^{6+}) and incubated at 28 ^{o}C. Growth was determined turbid-metrically after interval of 6 hours by measuring optical density at wavelength 540nm (Ahemad and Malik, 2011).

3 RESULTS AND DISCUSSIONS

In the present study, soil samples were collected from the rhizosphere region of the plants grown, Mango (*Mengifera indica*) and Cabbage (*Brassica oleracea*), in chromium contaminated soils in Bahir Dar tannery. Soil sample was taken 6 times from each selected plant rhizosphere. The soil samples collected from the rhizosphere of plants grown in metal polluted environment contain heavy metals resistant bacteria (Soumitra Nath, 2012). Further, a total of 21 rhizobacterial isolates different in colony morphology and pigmentation were picked up randomly from nutrient agar plates. All the isolates further tested for their morphological, cultural and biochemical characteristics.

Rhizobacterial isolates were tested for resistance to heavy metals, Hg^{2+}, Cd^{2+}, Cu^{2+}, Cr^{3+}, Zn^{2+}, Ni^{2+}, Pb^{2+} and Cr^{6+} [in the form of ($HgCl_2$, $(CH_3COO)_2Cd.H_2O$, $CuSO_4.5H_2O$, $CrCl_3$, $ZnSO_4.7H_2O$, $NiSO_4.6H_2O$, $(CH_3COO)_2Pb.3H_2O$, and $K_2Cr_2O_7$), respectively. The rhizobacterial resistance at each concentration of heavy metal was depicted by growth on agar plate containing heavy metals. There was a decline in the growth of the rhizobacteria with increasing concentrations of the heavy metals in the media in contrast to the situation in the control i.e., 0.0 µg ml^{-1} of the metals where there was a profuse/heavy growth by all the organisms. The rhizobacterial load decreased with the increase in concentration of heavy metals indicating toxic effect of the heavy metals on the growth of rhizobacterial isolates (Table3).

Many previous studies reported that many bacterial species isolated from industrial zones had been shown to develop resistance to heavy metals (Osborn *et al*., 1997; Ansari and Malik, 2007). The study by Valentina, (2006) reported that there was considerable contents of Ag, Au, Cr, Cu, Ni, Pb, Zn, As, Bi, Cd, Co, Hg, Mo and Sn were present in industrial soil and bacteria from such soil are frequently tolerant to higher levels of metals than those isolated from unpolluted areas. Also, Ersoy *et al*. (2011) reported that high levels of Ni, Cr, Cu, Co, and Zn were present in the industrial soils as compared with the levels of those metals in forest soil and Cr and Ni values in these locations were 5 times higher than the legal limits. Heavy metals could be spread to these locations in different probable ways. For example, high concentrations of Cr and Ni might be due to the application of waste waters from factories to soil for irrigation purposes, because the drainage channels are so close to the open storage zone of the solid wastes of the chromium factory. Second, the heavy metals might be transferred from the open metallic waste storage zone to the soils by means of the wind (Ersoy *et al*., 2011). In the present study, the total viable count of rhizobacteria was found to be 11.1 x 10^6 in the rhezosphere soils. In a study reported by Abou-Shanab (2007), the number of culturable bacteria in the rhizosphere were 6.6 x 10^6CFU, 14.2- and 9-fold higher than that in the sediment and water, respectively. In the present study, the bacteria count on nutrient agar supplemented with chromium decreased from 11.1 x 10^6 $CFUg^{-1}$ of soil with increasing concentration of chromium from 0 to 100 µg ml^{-1} (Table 1).

Table 1. Bacterial count on chromium supplemented media† from tannery effluent contaminated Rhizosphere soil

Concentration of chromium (µg ml^{-1})	Total viable count (CFU g^{-1} of soil)
0	11.10 x 10^6
50	8.75 x 10^6
100	5.00 x 10^6

Twenty one rhizobacterial isolates which differed in colony morphology and color were selected from potassium dichromate supplemented nutrient agar plates randomly. All the isolates further tested for their morphological, cultural and biochemical characteristics. Further, the rhizobacterial isolates were identified as five *Pseudomonas* Spp., three *Staphylococcus* Spp., four *Clostridium* Spp., three *Klebsiella* spp., two *Bacillus* Spp., two *Listeria* Spp., and two *Streptococcus* spp., (13 gram-positive and 8 Gram- negative), following *Bergey's Manual of Determinative Bacteriology* (Holt *et al.,* 1994).

The isolated bacteria were showed very high degree of resistance to all heavy metals as, MIC values of rhizobacterial isolates varied from 50 to 2000 µg ml^{-1} (table 2). Responses of rhizobacterial isolates to heavy metals were very heterogeneous. The resistance levels in the form of MIC indicated that among the eight selected heavy metals, lead was a less toxic heavy metal showing the growth of rhizobacterial isolates up to 2000 µg ml^{-1}, followed by nickel on which the isolates grew up to 1600 µg ml^{-1}. In contrast to these results previous study by Mohd *et al*., (2012) reported that most of the bacterial isolates showed high resistance to zinc and iron compared to lead. On the other hand, in this study among all the eight heavy metals selected mercury and cadmium were the most toxic inhibiting the growth of isolates in lower concentration, showing no growth above 800 µg ml^{-1} (Table 3).

Table 2. MIC of rhizobacteria in µg ml^{-1} for eight heavy metals

Rhizobacterial isolates	Hg^{+}	Cd^{+}	Cu^{+}	Zn^{+}	Ni^{+}	Pb^{+}	$Cr3^{+}$	$Cr6^{+}$
***Klebsiella* sp. MB1**	400	1000	1400	1200	1600	1400	1200	1200
***Klebsiella* sp. MB2**	500	200	1200	500	1000	1600	1600	600
***Klebsiella* sp. MB3**	600	600	400	400	600	1400	600	600
***Staphylococcus* sp. MB4**	800	600	800	600	1400	1000	600	600
***Staphylococcus* sp. MB5**	100	300	400	200	1000	1800	600	500
***Staphylococcus* sp. MB6**	500	600	800	50	300	1400	600	1200
***Pseudomonas* sp. MB7**	50	50	600	50	1000	1800	1600	500
***Pseudomonas* sp. MB8**	800	1000	800	400	1600	2000	1000	1200
***Pseudomonas* sp. MB9**	200	100	800	1000	1000	1600	600	1000
***Pseudomonas* sp. MB10**	500	500	300	600	1000	1400	1000	1400
***Pseudomonas* sp. MB11**	100	50	800	100	100	1600	600	600
***Clostridium sp*. MB12**	500	1000	800	600	1000	1000	600	1400
***Clostridium sp*. MB13**	800	600	1200	1000	1200	1600	1000	1400
***Clostridium sp*. MB14**	300	400	1200	500	1200	1600	800	800
***Clostridium sp*. MB15**	100	300	500	400	1000	1800	1600	500
***Streptococcus* sp. MB16**	50	100	800	1000	1600	1200	1000	500
***Streptococcus* sp. MB17**	600	500	100	100	1200	1600	600	500
***Listeria* sp. MB18**	300	400	800	1000	1200	1800	1000	1000
***Listeria* sp. MB19**	600	1000	800	1200	1200	1400	600	600
***Bacillus* sp. MB20**	50	400	1200	500	1200	1600	1000	1000
***Bacillus* sp. MB21**	600	100	800	400	1000	600	800	1000

Table 3. Pattern of metal resistance in bacterial isolates from tannery effluent contaminated rhizosphere soil

Metal	Sensitive range				Resistant range				
Mercury	MIC(µg ml^{-1})	50	100	200	300	400	500	600	800
	No. of isolates inhibited	3(14.3)	3(14.3)	1(4.8)	2(9.5)	1(4.8)	4(19.0)	4(19.0)	3(14.3)
Cadmium	MIC(µg ml^{-1})	50	100	200	300	400	500	600	100
	No. of isolates inhibited	2(9.5)	3(14.3)	1(4.8)	2(9.5)	3(14.3)	2(9.5)	4(19.0)	4(19.0)
Copper	MIC(µg ml^{-1})	100	300	400	500	600	800	1200	1400
	No. of isolates inhibited	1(4.8)	1(4.8)	2(9.5)	1(4.8)	1(4.8)	10(47.6)	4(19.0)	1(4.8)
Zink	MIC(µg ml^{-1})	50	100	200	400	500	600	1000	1200
	No. of isolates inhibited	2(9.5)	2(9.5)	1(4.8)	4(19.0)	3(14.3)	3(14.3)	4(19.0)	2(9.5)
Nickel	MIC(µg ml^{-1})	100	300	600	1000	1200	1400	1600	
	No. of isolates inhibited	1(4.8)	1(4.8)	1(4.8)	8(38.1)	6(28.6)	1(4.8)	3(14.3)	
Lead	MIC(µg ml^{-1})	600	1000	1200	1400	1600	1800	2000	
	No. of isolates Inhibited	1(4.8)	2(9.5)	1(4.8)	5(23.6)	7(33.3)	4(19.0)	1(4.8)	
Chromium (Cr^{3+})	MIC(µg ml^{-1})	600	800	1000	1600				
	No. of isolates inhibited	9(42.8)	2(9.5)	7(33.3)	3(14.3)				
Chromium (Cr^{6+})	MIC(µg ml^{-1})	500	600	800	1000	1200	1400		
	No. of isolates inhibited	5(23.6)	5(23.6)	1(4.8)	4(19.0)	3(14.3)	3(14.3)		

†Total number of isolates = 21; Values in parenthesis indicate percentage of the total isolates; MIC = minimum inhibitory concentration

Previous study by Hassen *et al*., (2006), found that copper and chromium were tolerated heavy metals whilst mercury was the most toxic heavy metal for the bacteria. In the same manner, a study by Abou-Shanab *et al.*, (2007) reported that Mercury was the most toxic heavy metals inhibiting bacterial isolates at 0.01 mM followed by cadmium. Also the same results were reported by Laur-Dorina *et al.* (2011) as mercury had the highest toxicity on the bacteria as no colonies developed on 3 mM Hg. In contrast to this finding, Tamer *et al*., (2013), reported that heavy metal resistance of Cd and Cu in isolates was 100% that is less toxic than Mn and Pb in which 90.7% and 67.7% of isolates respectively where resistant.

Most of the bacterial isolates grew well in the presence of low concentrations of the heavy metals, while no bacteria could grow at higher concentrations. Moreover 95.2% of the isolates showed growth when the concentration of Pb^{2+} was above 1000 µg ml^{-1} and for Ni^{2+}, 85.7% of the isolates were resistant, while no isolates could tolerate Hg^{+} above 800 µg ml^{-1}

and for Cd^{+} 4(19.0) were grown on 1000 µg ml^{-1} (Table:4). Further, all the rhizobacterial isolates were also tested for antibiotic susceptibility to nine different antibiotics (vancomycin, co-trimoxazole, ciprofloxacin, nalidixic acid, erythromycine, tetracyclin, cefoxitin, chloramphenicol and gentamicin). In this study, most of the rhizobacterial isolates showed resistance to the antibiotics. Metal resistance has been reported to be associated with antibiotic resistance (Verma *et al*., 2001).The present study indicated that 80.9% of the isolates were resistant to nalidixic acid while 71.4% of the isolates showed resistance to cefoxitin. In contrast, the bacterial sensitivity was maximum to chloramphenicol (19.0%) rhizobacterial isolates were resistant and erythromycin (28.6%) isolates were resistant (Table 5). In the study by Nath *et al*., it was reported that the isolates having high MIC values for a set of metals exhibits high resistance pattern towards a group of antibiotics (Nath *et al*., 2012). A study in India by Tamil *et al*., (2012) reported that the bacterial isolates from tannery showed highest resistance to both heavy metals and antibiotics.

Table 4. Number and Percentage of bacterial isolates resistant to heavy metal at concentration 1000 µg ml^{-1} and above

Heavy metals	No. of bacteria
Pb^{+}	20 (95.2)
Ni^{+}	18 (85.7)
Cr^{3+}	10 (47.6)
Cr^{6+}	10 (47.6)
Zn^{+}	6 (28.6)
Cu^{+}	5 (23.6)
Cd^{+}	4 (19.0)
Hg^{2+}	0 (0.0)

Total number of isolates = 21

Table 5. Percent resistance to antibiotics in rhizobacterial isolates† from tannery effluent contaminated rhizosphere soil

Antibiotics	Number of isolates	Percent resistance
Nalidixic acid (NA)	17	80.9
Cefoxitin (CX)	15	71.4
Co-trimoxazole (COT)	12	57.1
Tetracycline (TE)	6	28.6
Chloramphenicol (C)	4	19.0
Ciprofloxacin (Cip)	9	42.9
Erythromycin (E)	6	28.6
Vancomycin (Va)	11	52.4
Gentamycin (Gen)	10	47.6

†Total number of isolates = 21.

Hundred percent of the bacteria isolates were resistant to at least one of 9 antibiotics tested. In the same manner, Mohd *et al*., 2012, reported that 100% of 24 isolated bacteria were resistant to at least one of 10 antibiotics tested. Multi-resistance was observed in 9.5% of the isolates, for up to 8 antibiotics. The observed multi-resistance pattern to 7 antibiotics agents was 9.5 %, followed by 4.8% and 23.8% respectively to 6 and 5 antibiotics, 19.0% to 4 antibiotics, 9.5 % to 3 antibiotics, 9.5% to 2 antibiotics and 14.3% to 1 antibiotic. In general, a total of eight combinations of the selected antibiotic resistant patterns were observed. For example, 23.8% of the isolates were resistant to five antibiotics in four different combinations where as 19.0% of the isolates exhibited resistance to four antibiotics in four different combinations. In this study among 21 rhizobacterial isolates only 9.5% of the isolates were resistant to eight antibiotics and no isolate showed resistance to all the nine antibiotics (Table 6).

Table 6. Multiple resistant pattern in rhizobacterial isolates to antibiotics † from tannery effluent contaminated rhizosphere soil

Number of antibiotics	Number of resistant isolates (%)	Resistance pattern
2	2 (9.5)	NA, VA (1)
		NA,CIP (1)
3	2 (9.5)	NA,CIP,C (1)
		COT,CX,Gen(1)
4	4 (19.0)	NA, VA, CIP, Gen (1)
		NA,Cip,CX,C (1)
		NA,COT,CX,C (1)
		NA,COT,Cip,CX (1)
5	5 (23.8)	VA,TE,COT,CX,Gen(2)
		NA, VA,COT,E,CX (1)
		NA,COT,CiP,CX,GEN(1)
		NA,VA,Cip,CX,Gen(1)
6	1 (4.8)	NA,VA,COT,Cip,CX,E(1)
7	2 (9.5)	NA,VA,COT,TE,CX,E,Gen(2)
8	2 (9.5)	NA,VA,COT,Cip,TE,CX,E,Gen(1)
		NA,VA,COT,TE,CX,C,E,Gen(1)

†Total number of isolates = 21; Values in parenthesis indicate percentage of the total isolates.
Note: NA = Nalidixic acid, CX = Cefoxitin, COT = Co-trimoxazole, TE = Tetracycline, C =Chloramphenicol, E = Erythromycin, Cip = Ciprofloxacin, VA = Vancomycin, Gen = Gentamycin

In agreement of these findings, many authors have reported extensive metal resistant traits in rhizobacterial isolates belonging to diverse genera and evidenced their implications in bioremediation processes (Wani *et al*., 2007; May *et al*., 2009). Bacterial isolate, *Klebsiella* sp. MB1 was selected for further study due to exhibiting highest resistance to antibiotics and most of the selected heavy metals including Cr^{6+} and Cr^{3+}. A growth pattern study of the *Klebsiella* sp. MB1 with respect to controls was carried out in NB medium supplemented with $K_2Cr_2O_7$ (Cr^{6+}) and $CrCl_3$ (Cr^{3+}) in three different concentrations. The growth of the isolate in nutrient broth was determined by measuring the optical density at 540nm with uninoculated broth as control after interval of each 6 hours by measuring optical density at 540 nm. It was observed that the growth was declined with increasing concentration of both Cr^{6+} and Cr^{3+}. The growth pattern of bacterial isolate MB1 at different time intervals in the presence of 0, 50, 100 and 200(µg ml^{-1}) of $K_2Cr_2O_7$ and $CrCl_3$ are shown in fig. 1 and 2 respectively.

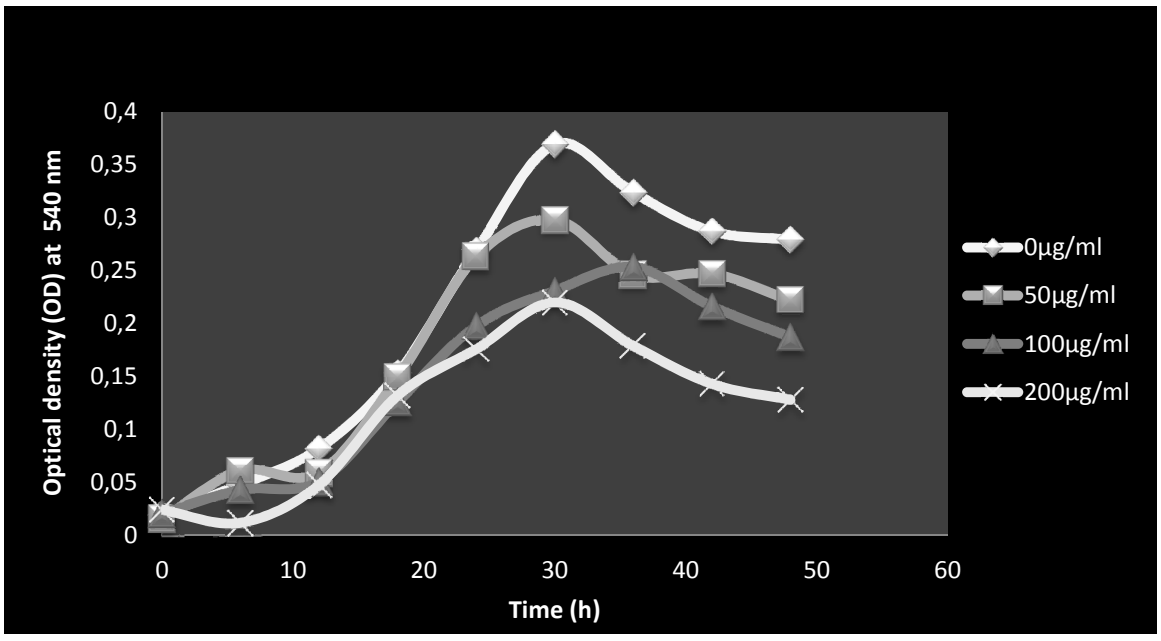

Fig. 1. Growth pattern of Klebsiella sp.MB1 at different concentration of Cr^{6+} ($K_2Cr_2O_7$).

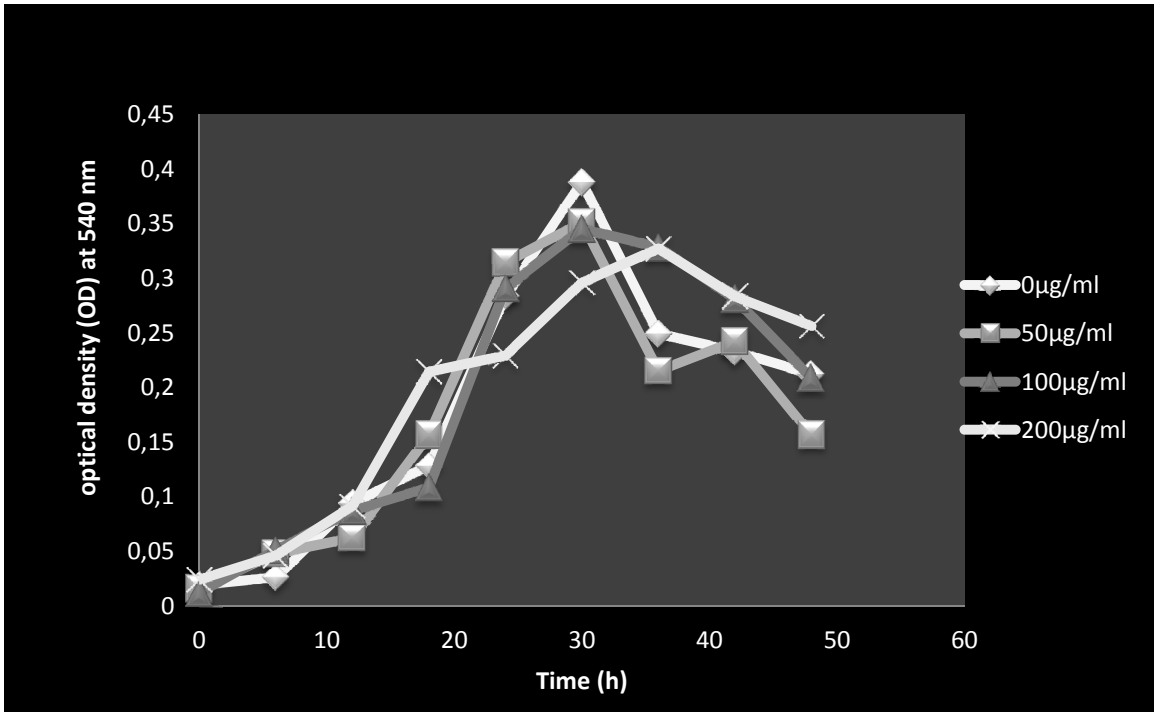

Fig. 2. Growth pattern of Klebsiella sp. MB1 at different concentration of Cr^{3+} ($CrCl_3$).

4 CONCLUSIONS

The industrial effluents are enriched media to grow and spread microbial population, which may be 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. The present study revealed that the most resistant bacteria towards selected heavy metals and different antibiotics were isolated from tannery effluent contaminated rhizosphere soils. Microbes have adapted to tolerate the presence of metals or can even use them to grow. This study showed that the rhizobacteria isolated from rhizosphere soils contaminated with tannery effluents had considerable resistance to chromium in addition to other metals. Along with heavy metal resistance, bacterial isolates also possessed significant resistance to various antibiotics along with heavy metal resistance. From the results, it could be concluded that the bacterial strains isolated from industrial effluent possessed potential to be used in bioremediation activities. The identification of resistance against different metals in bacterial isolates may provide a useful tool to assess the extent of metal pollution in the environment.

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