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ISOLATION AND CHARACTERIZATION OF METAL INTERACTING BACTERIA BIO- PROSPECTING FOR PHYTOREMEDIATION

Mhalappa N. Jagtap[1], Mohan V. Kulkarni[2] and Pravin R. Puranik[3]
1.P.G.Department of Botany, D.B.F.Dayanand College Solapur 413002 (M.S),India
[2]Division of Biochemistry, Department of Chemistry, University of Pune, Pune 413 007, (M.S),India
[3] School of Life Sciences, North Maharashtra University, P.B. No. 80, Jalgaon 425 001, (M.S), India

ABSTRACT
Pollution of aquatic environment and soils by pesticides from agricultural use poses a major environmental and human health problem, which is still in need of an effective and affordable technological solution. Understanding of the plant uptake of organic chemicals is essential to assessing contaminant mobility in the ecosystem, exposure to humans, and phytoremediation technologies (Li *et al.*, 2002). Phytoremediation is an eco- friendly approach for remediation of contaminated soil, sludge, sediment, ground water, surface water, and wastewater using plants. The soil microorganisms play important role the present study focus on isolation of soil microorgamisms for phytoremediation . Metal tolerating bacterial cultures were obtained by soil enrichment method and gradient plate method . The minimum inhibitory concentration (MIC) of metals was checked for the cultures selected in metal gradient plate assay. The cultures were screened for metal biosorption The cultures were further characterized for metal tolerance in broth, growth curves, pH tolerance and metal biosorption. On the basis of experimental results cultures viz. MJ-Pb-13 and MJ-Cr-6 could be selected for phytoremediation trials.

KEYWORDS: Gradient plate method, Heavy metal, Phytoremediation

INTRODUCTION

It is of general observation that the increased concentrations of metals in soil decreases number of the soil microorganisms, their activity and diversity and structure of microbial community (Baath, 1989). Metal toxicity reduces the microbial counts due to disruption of essential functions in short term and in the long term that leads to changes their competitive ability (Giller *et al.*, 1998). There is considerable evidence that the activity of the soil microbiological community is decreased in the presence of metals if the concentration is high enough and this is confirmed by observed decreases in rates of litter decomposition, carbon mineralization, nitrogen transformations and enzyme activities (Baath, 1989). The reduction in microbial activity is attributed to elevated energy demand for metal tolerance mechanisms within microbial cells in comparison to steady state conditions (Giller *et al.*, 1998). The reduction in microbial diversity due to the addition of metals may decrease the resistance or resilience of the system to a new stress (Giller *et al.*, 1998). However, it has also been hypothesized that the overall functional diversity of the soil ecosystem does not change owing to other types of microbial species that can fill the same or a similar niche (Nannipieri *et al.*, 2003). Microbial populations are known to affect trace metal mobility and availability to the plant, through release of chelators, acidification and redox changes (Smith and Read, 1997; Abou-Shanab *et al*., 2003). Around plant roots there is a narrow zone of soil which is densely populated with microorganisms due to the release of root exudates, chemicals and elaborated metabolites, which supply a food source for this microbially-enriched zone of plant influence (Curl and Truelove, 1986).

Free living Plant Growth-Promoting Rhizobacteria (PGPR) are known to provide plants with a range of direct and indirect benefits, including the provision of nutrients and protection against pathogens . This results in increases in germination rates, root growth, yield, leaf area, chlorophyll content, magnesium content, nitrogen content, protein content, hydraulic activity, tolerance to drought, above ground biomass and disease resistance (Lucy *et al.*, 2004). There is also a range of benefits that specifically apply to plants growing in metal-contaminated soils and the main benefits cited are the synthesis of sideophores and 1-Aminocyclopropane-1-Carboxylate (ACC) deaminase (Glick, 2003). Sas-Nowosielska *et al.* (2008) examined the microbial populations of root free soil and rhizosphere soil and found inverse correlation between the number of sulfur amino acid decomposing bacteria and root mercury content. The presence of rhizosphere bacteria has been reported to increase the concentrations of zinc, copper, lead or chromium in plants (Abou-Shanab *et al.*, 2008). Improvement of the interaction between plants and beneficial rhizosphere microorganisms can enhance biomass production and tolerance of the plants to heavy metals and is considered to be an important component of phytoremediation technologies (Glick, 2003). Accumulation of heavy metals in the soil

environment and their uptake by both plant growth promoting rhizobacteria and plants is a matter of growing environmental concern (Khan *et al*., 2009). In the present study an attempt has been made to isolate rhizospheric microorganisms from metal contaminated soils and to screen their metal tolerance capacity so that they can be used to enhance the phytoremediation.

MATERIAL AND METHODS

Isolation of bacterial cultures

Metal tolerating bacterial cultures were obtained by soil enrichment method. Garden soil sample (5 g) was added to the Erlenmeyer flasks containing 100 ml distilled water and one of the metals (copper, chromium, cadmium, lead, zinc and nickel) taken separately at the concentration of 10 mg/l. The flasks were incubated on rotary shaker at 120 rpm and room temperature for 5 days. The contents (5 ml) were transferred to 100 ml enrichment medium of composition; 5 g/l yeast extract, 10 g/l glucose, 2 g/l $NaNO_3$, 0.5 g/l KCl, 0.5 g/l $MgSO_4.7H_2O$ and 50 mg/l of metal and kept for incubation at room temperature for 5 days. At various stages of enrichment after incubation for five days contents were also spread on nutrient agar containing similar type and concentration of metal. Morphologically distinct bacterial colonies were picked up and preserved on agar slopes for further screening.

Screening of bacterial cultures for metal tolerance

The cultures obtained from soil enrichment were screened for metal tolerance capacity by following the gradient plate method. The metal concentration gradient was prepared by adding a base layer of 20 ml of nutrient agar to a 9 × 9 cm square Petri plate tilted at an angle of 30°. The agar was allowed to solidify at room temperature into a wedge shape layer. Onto the set base, another 20 ml of nutrient agar with metal (100 mg/l) was poured to give a metal concentration gradient across the plate surface. Bacterial cultures (16 h old) were streaked along the metal gradient using a sterile cotton swab. Plates were incubated at 28 ± 2°C for 2 days. After incubation, the length of bacterial growth along the gradient was recorded. Cultures showing the highest length of growth along the gradient were selected for further experiments.

Minimum inhibitory concentration assay for metal tolerance

The cultures showing highest tolerance in metal gradient plate assay were subjected to determine its metal tolerance in broth culture. The minimum inhibitory concentration (MIC) of metals was checked for the cultures selected in metal gradient plate assay. Loopful of 16 h old culture was inoculated in series of tubes containing 10 ml of broth amended with metals (copper, chromium, cadmium, lead, zinc and nickel) ranging from 10-500 mg/l. Two growth media viz. nutrient broth and synthetic medium containing (in g/l) yeast extract, 1; sodium chloride, 10; sodium glycerophosphate, 30; $MgSO_4.8H_2O$, 5; glucose, 20 at pH 6.5. Metal concentrations were adjusted by adding appropriate volumes of metals stocks (100 mg/l). The tubes were incubated at 30°C for 24 h. The metal tolerance of cultures was noted in terms of visible growth. The lowest metal concentration causing no visible growth was recorded as MIC value.

Growth curve of cultures

The cultures selected from metal gradient plate assay and MIC broth assay were grown in presence and absence of metals. The growth performance in terms of optical density at 600 nm was plotted against time so as to obtain the growth curve.

Screening of cultures for metal biosorption

The set of cultures were grown in nutrient broth in absence of metals. The cell mass were harvested by centrifugation at 5000 rpm for 10 min. The biomass was washed twice with distilled water. The cell suspension was prepared using glass homogenizer. Cell dry weight was calculated by drying a known volume (1 ml) of suspension in a glass container at 80°C to a constant weight. Approx. 50 mg of cell dry weight was added to the 50 ml of solution containing 100 mg/l of metals and incubated for 1 h on rotary shaker at room temperature. The contents were centrifuged for separation of biomass and the supernatants were collected for metal analysis.

The amount of metal sorbed was calculated by the following equation:

$$Q = V(C_i - C_{eq})/1000M$$

Where, Q is metal adsorbed (mg/g), V is the volume of the metal solution (ml), C_i and C_{eq} are the initial and equilibrium concentrations of metal in the solution (mg/l), and M is the dry weight of the biomass (g).

Metal solutions

Metal stock (1000 mg/l) solutions were prepared in acidified Milli-Q water with appropriate amounts of analytical grade nitrate salts of the test metals, viz. cadmium, chromium and zinc. Working metals solutions were prepared from stock solutions and the pH of each metal solution was adjusted using 0.1 M NaOH or HCl.

Metal analysis

Metal analysis of all digested samples was performed by atomic absorption spectrometry (Model: S2, Make: Thermo, USA) using the 'SOLAAR' software. Analyses were performed using hollow cathode lamps for cadmium (Cd), chromium (Cr), nickel (Ni), lead (Pb), copper (Cu) and zinc (Zn) at 228.8, 357.9, 232.0, 217.0, 324.8 and 213.9 nm, respectively. Air-acetylene flame was generated using a fuel flow rate of 0.8 to 1.1 l/min. All analyses were replicated three times. All the reagents used were analytical grade (Thermo Fischer Sci. India Pvt. Ltd., and HiMedia Lab. Pvt. Ltd., India).

RESULTS

Isolation of bacterial cultures

Isolation of bacterial cultures was followed by metal enrichment of samples collected from 10 diverse sites in and around Solapur city. These sites included metal exposed site like Common Effluent Treatment Plant at MIDC area, Solapur city. Metal non-exposed sites such as farms sites were included to obtain wild varieties of metal interacting microorganisms. Samples were enriched in presence of 10 mg/l of various metals in separate flasks. After 10 days of incubation samples were plated on nutrient agar plates containing 10 mg/l of respective metal concentrations. In total, 94 different colonies were picked either on the basis of different colony morphology or source and type of metal (Table 1). These cultures were given codes containing the name of metal used in enrichment.

Table 1. Number of bacterial isolates obtained from metal enrichment experiments

Site	No. of bacterial cultures isolated in presence of 10 mg/l					
	Copper	Chromium	Cadmium	Lead	Zinc	**Nickel**
Firing range	2	1	1	1	1	**1**
Kamber lake	1	2	1	-	2	**2**
Lature farm	2	1	2	2	1	**2**
CETP-MIDC	4	2	2	3	1	**2**
Garad farm	1	1	2	2	2	**1**
Rupa Bhawani	2	1	1	1	1	**2**
Shelke farm	3	2	2	1	2	**1**
Dindigave farm	2	1	2	2	1	**1**
Kapse farm	1	2	2	2	2	**2**
Hotagi lake	2	1	1	1	1	**1**
Total	**20**	**14**	**16**	**15**	**14**	**15**

CETP-MIDC, Common Effluent Treatment Plant in Maharashtra Industrial Development Corporation at Solapur.

Screening of bacterial cultures for metal tolerance in metal gradient plate

The set of isolates was subjected screening wherein their metal tolerance capacity was assessed using metal gradient plate assay. All the cultures were grown in nutrient broth and 16 h old active cultures were cross inoculated along the metal gradient on square plates. Although, the isolates had an exposure to only single metal during enrichment, the whole set of cultures was tested against six metals in gradient plate. The summary of results of metal gradient plate assay has been presented in Table 2.

Seven isolates could be selected on the basis of highest tolerance to metals in terms of length of growth on metal gradient plate (Table 2). It can be seen that cultures adapted to a Cd, Cr, Pb and Ni during enrichment exhibited highest tolerance to the same metal on metal gradient plate. However, in case of copper and zinc the highest tolerance was

shown by the cultures with exposure history of chromium and cadmium, respectively. The growth performance of representative cultures is shown in Figure 1. The colony characters and microscopic observations of selected bacterial isolates are given in Table 3.3.

Table 2 Growth performance of bacterial cultures in metal gradient plate assay

Isolate	Habitat	Length of culture growth across the metal gradient (cm)					
		Copper	Chromium	Cadmium	Lead	Zinc	**Nickel**
MJ-Cd-6	Firing range	6.3	8.6	4.5	3.4	8.2	**8.3**
MJ-Cd-4	CETP-MIDC	5.4	6.2	8.8	2.6	8.2	**7.0**
MJ-Cd-14	Kamber lake	6.7	7.2	5.8	4.4	8.9	**8.3**
MJ-Cr-6	CETP-MIDC	8.4	8.6	7.4	6.2	8.0	**7.8**
MJ-Cr-11	Rupa Bhawani	4.3	6.8	6.3	4.6	8.3	**8.5**
MJ-Pb-13	Firing range	8.2	6.8	6.4	6.4	8.2	**8.5**
MJ-Ni-2	**Shelke farm**	**5.1**	**4.6**	**6.2**	**2.4**	**7.8**	**8.6**

Table 3. Colony characteristics, morphology and Gram's nature of bacterial cultures

Isolate	Colony characters isolates grown on nutrient agar					Cell morphology	Gram's nature
	Shape	Size (mm)	Texture	Color	Opacity		
MJ-Cd-6	Circular	2	Smooth	Cream white	Translucent	Rods	Negative
MJ-Cd-4	Circular	1	Rough	Yellowish	Opaque	Thick rods	Positive
MJ-Cd-14	Circular	3	Smooth	Buff white	Translucent	Rods	Negative
MJ-Cr-6	Circular	2	Rough	Crimson white	Opaque	Rods	Negative
MJ-Cr-11	Oval	4	Smooth	White	Translucent	Short rods	Negative
MJ-Pb-13	Circular	3	Smoot	White	Translucent	Thin rods	Negative
MJ-Ni-2	Irregular	2	Rough	Cream white	Opaque	Thick rods	Positive

Table 4. Minimum inhibitory concentration of metals for selected cultures in broth assay

Isolate	Minimum Inhibitory Concentration (mg/l)											
	Copper		Chromium		Cadmium		Lead		Zinc		**Nickel**	
	NB	SM	NB	SM	NB	SM	NB	SM	NB	SM	NB	**SM**
MJ-Cd-6	80	40	90	90	40	30	60	10	120	80	60	**40**
MJ-Cd-4	60	40	70	50	60	40	70	30	80	60	70	**70**
MJ-Cd-14	80	30	60	30	50	30	70	10	120	110	80	**80**
MJ-Cr-6	80	60	100	80	80	50	100	20	100	80	80	**70**
MJ-Cr-11	60	40	80	50	40	20	50	10	80	60	60	**40**
MJ-Pb-13	100	70	100	80	60	40	90	30	120	100	100	**90**
MJ-Ni-2	**60**	**40**	**80**	**50**	**40**	**30**	**40**	**<10**	**80**	**60**	**60**	**40**

NB, Nutrient broth; SM, Synthetic medium

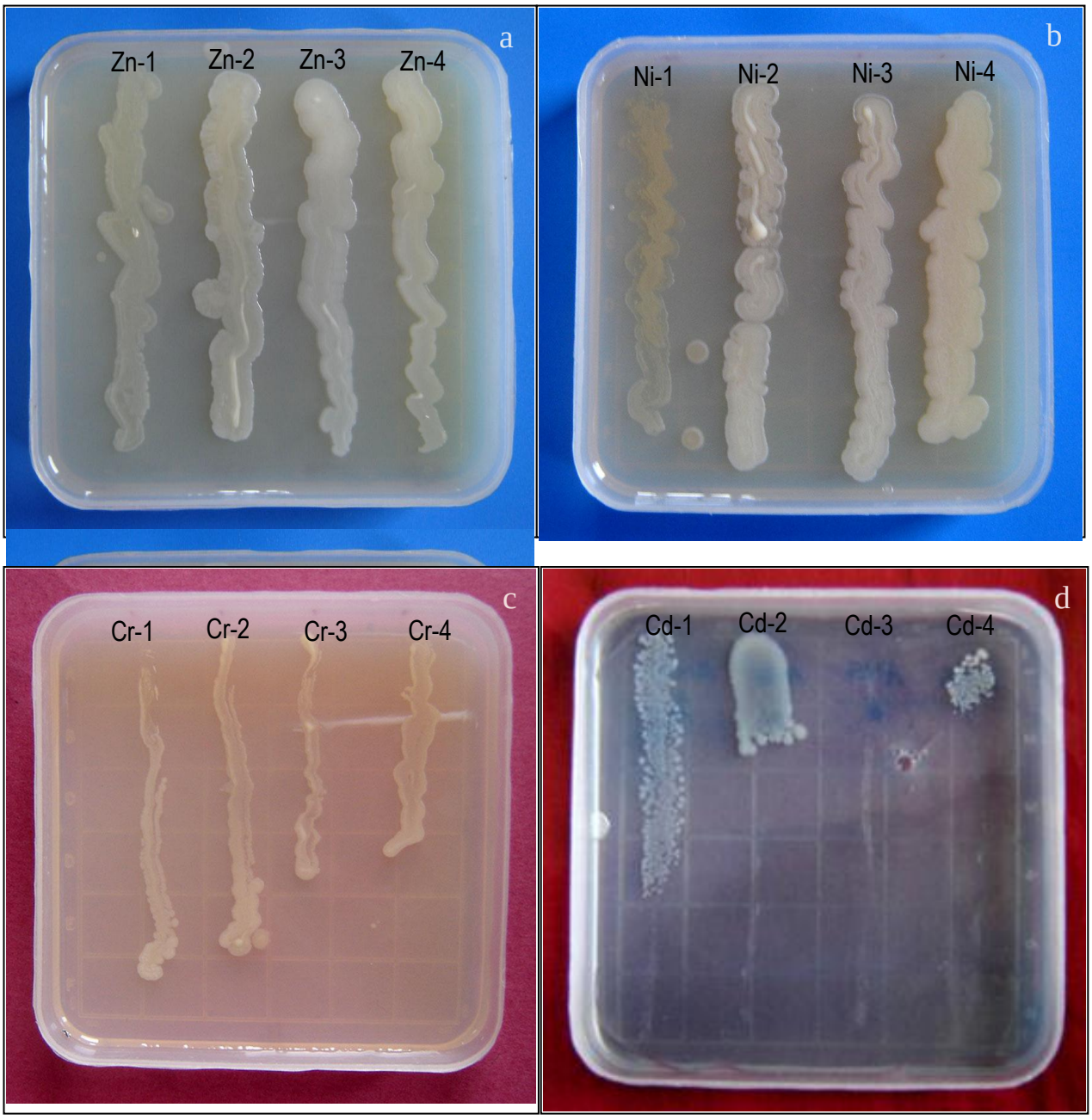


Figure 1. Testing the metal tolerance of bacterial isolates using metal gradient plate technique. Growth of bacterial isolates on plates containing a, zinc; b, nickel; c, chromium; d, cadmium

Minimum inhibitory concentration assay for metal tolerance

The set of bacterial isolates were further tested for metal tolerance in broth assay (MIC assay). Presence of organic compounds and inorganic phosphates in broth may complex the heavy metals thus reducing their bioavailability. To test the role of bioavailability of metals due to complex nature of growth, the bacterial cultures were also grown in synthetic broth containing organic phosphates i.e. sodium β-glycerophosphate that does not complex metals maintaining the bioavailability. The data of MIC assay are presented in Table 3.4. It can be seen that highest MIC of 120 mg/l was reported for metal zinc. Comparatively, the MIC of cadmium was found to be lower among the metals tested.

In all cases the metal tolerance of test cultures was found to be decreased when grown in synthetic medium. The difference between MIC in nutrient broth and synthetic broth was highest in case of lead than any other metals. Among the cultures tested, isolate numbers MJ-Pb-13 and MJ-Cr-6 could be considered to be the most metal tolerating cultures. However, on the basis of MIC values for all the metals the isolates viz. MJ-Cr-11 and MJ-Ni-2 could be considered as least effective metal tolerating cultures. These two cultures were not considered for further characterization studies.

Growth curve of cultures

In addition to metal tolerance growth rate of culture can be taken into consideration for the selection of cultures. Growth curves were obtained for the set of five cultures grown in nutrient broth and are depicted in Fig. 2. Minimum lag phase (<3 h) and rapid growth phase could be seen in case of culture, MJ-Pb-13. Growth performance in terms of lag phase, log phase and highest cell density of MJ-Cd-4 was slowest amongst all. The microbial tolerance to metal has been reported to be dependent of pH of medium. In order to check the pH tolerance and its effect on growth performance, the five cultures were grown in nutrient broth at varying pH from 4.0 to 8.0. The data are presented in Fig. 3. It can be seen that the culture growth increased with an increase in pH of medium from acidic (4.0) to neutrality (7.0).

The optimum pH for growth of MJ-Cd-6, MJ-Cd-14 and MJ-Pb-13 was found to be at neutrality (7.0), while, cultures, MJ-Cd-4 and MJ-Cr-6 grew well in acidic pH (6.0-7.0). On the basis of growth curve, (Fig. 2) and pH tolerance (Fig. 3), cultures MJ-Pb-13 and MJ-Cr-6 could be selected for further characterization.

The growth curve experiments were carried out for these two cultures in nutrient broth containing sub lethal concentrations of metals (50 mg/l). The data obtained for culture MJ-Pb-13 are presented in Fig. 4. The cell densities were found to be decreased in presence all metals except for zinc at 50 mg/l. Delay in lag phase could be noticed for all metals. The data obtained for culture MJ-Cr-6 grown in presence of 50 mg/l of metals are presented in Fig. 5. The cell densities were found to be decreased in presence all metals. Change in initial lag phase could be noticed for all metals. However, in presence of zinc, culture grew well with no delay in lag phase. The recovery from metal shock was seen in presence of copper and chromium. Lead and cadmium appeared to be most toxic metals than other metals at 50 mg/l.

Table 5. Metal adsorption capacity of isolates at 100 mg/l initial concentration of metals, pH 5.0 at 30°C

Sr. No.	Isolate	Metal adsorbed, *Q* (mg metal/g dry wt of cells)					
		Cu	**Cr**	**Cd**	**Pb**	**Zn**	**Ni**
1	MJ-Cd-6	15.60	17.26	38.20	42.33	14.20	16.90
2	MJ-Cd-4	32.66	23.18	34.20	51.83	17.14	26.14
3	MJ-Cd-14	28.00	15.50	18.22	43.22	29.55	15.15
4	MJ-Cr-6	26.72	33.31	45.12	66.16	26.36	21.36
5	MJ-Cr-11	24.17	25.42	23.04	45.12	28.81	17.12
6	MJ-Pb-13	25.14	38.66	42.34	71.45	25.48	23.44
7	MJ-Ni-2	21.22	28.72	16.15	28.08	22.10	26.04

Fig. 2 Growth curve of bacterial isolates, MJ-Cr-6 and MJ-Pb-13 in presence and absence of metals

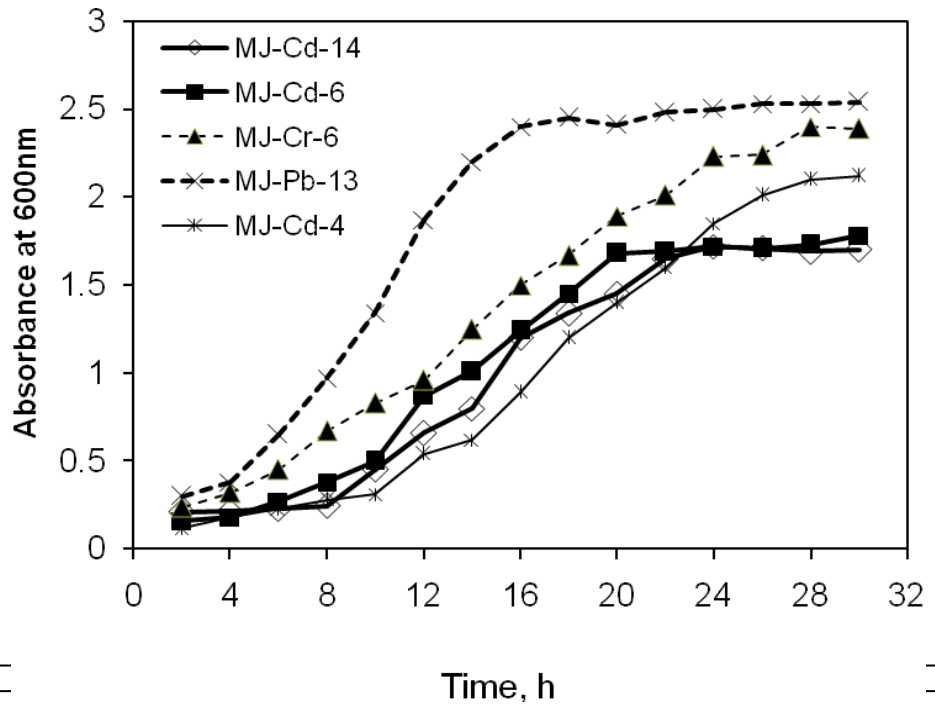


Fig. 3 Effect of pH on growth performance of the isolates in nutrient broth

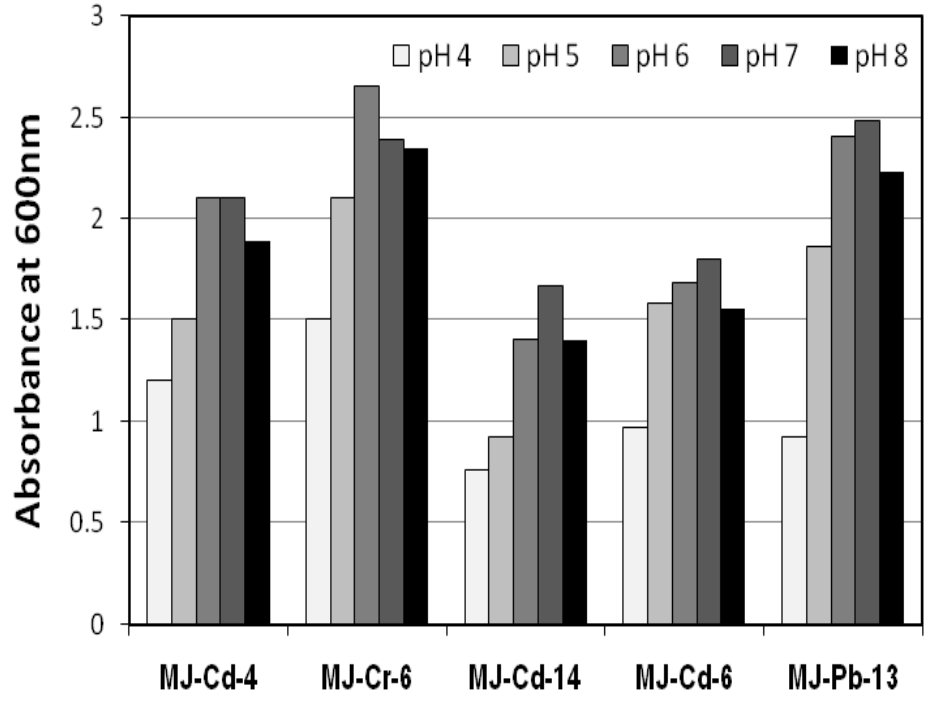


Fig. 4 Growth performance of the isolate, MJ-Pb-13 in presence of sublethal concentrations of metals (50 mg/l)

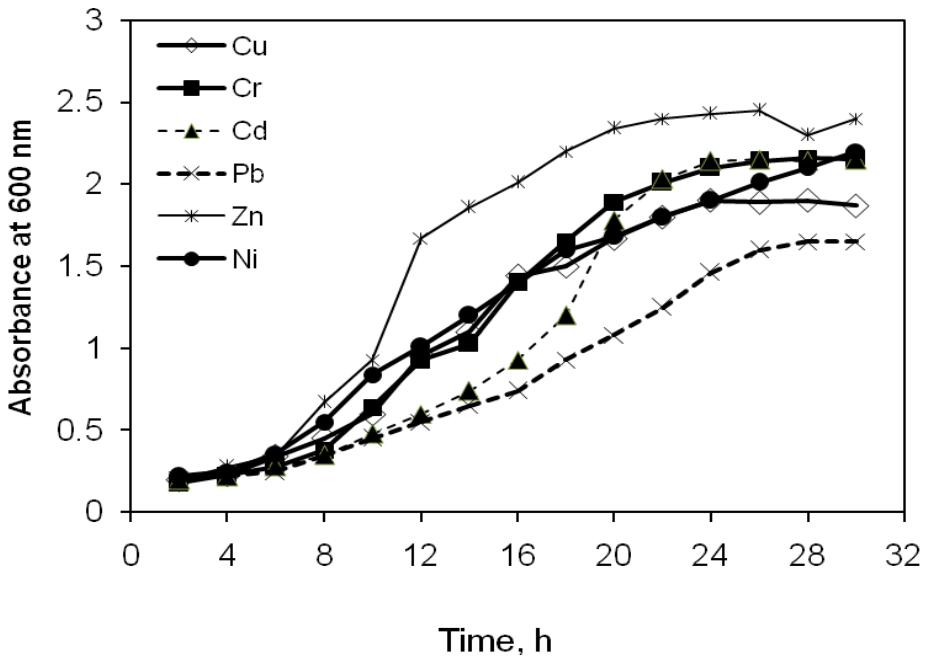
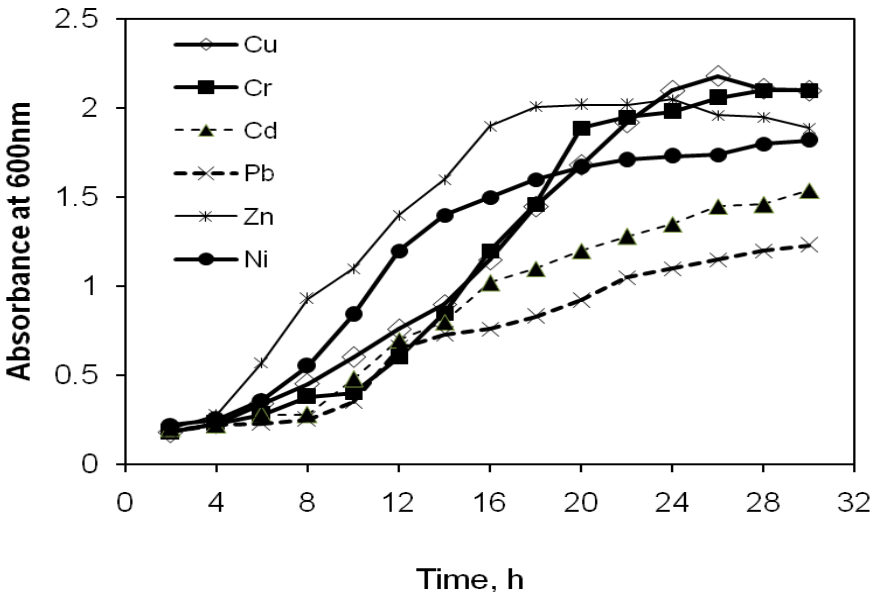


Fig. 5 Growth performance of the isolate, MJ-Cr-6 in presence of sublethal concentrations of metals (50 mg/l)



Screening of cultures for metal biosorption

The set of 7 metal tolerant bacterial isolates were tested for biosorptive capacity for metals viz. copper, chromium, cadmium, lead, zinc and nickel. The screening data are presented in Table 5. Highest metal sorption of 71.45 mg/g was obtained for lead by culture MJ-Pb-13. Cultures MJ-Cr-6 and MJ-Pb-13 had exhibited highest biosorption of cadmium (45.12 mg/g) and chromium (38.66 mg/g), respectively. Culture, MJ-Cd-14 was found to be effective biosorber of copper (32.66 mg/g) and nickel (26.14 mg/g). Maximum adsorption of zinc (29.55 mg/g) was seen in case of culture, MJ-Cd-14. On the basis of amount of metal adsorbed by bacterial a preferential series could be prepared as Lead > Cadmium > Chromium > Copper > Zinc > Nickel.

DISCUSSION

Phytoremediation is a fast expanding technology for remediation of contaminated soils but it usually takes several years to remove metals from soil partly because of their low bioavailability (Braud *et al*., 2006). In addition to use of synthetic chelators to enhance the phytoextraction synergistic use of plants and microorganisms is gaining importance (Glick, 2003). Soil bioaugmentation with microorganisms which increase the bioavailability of metals at the plant's disposal and its coupling with phytoextraction is a promising process for the soil cleaning-up (Braud *et al*., 2006). For successful development of technological approach, the most limiting factor would be availability of microorganisms that establish in metal laden soils. Appearance of metal in the soil most often inhibits the soil microbial activity. Certain organisms are always known to develop tolerance to increasing concentrations of toxic heavy metals. However, their number is scanty and needs to be enriched. In present study, an enrichment of soils with metals leads to inhibition of most of the microflora and allow the metal tolerating minor population to grow. The plating of metal enriched soil samples have indicated towards the survival of limited types of cultures. Metal toxicity reduces the microbial diversity thus less diverse types of cultures could be obtained. Bacterial isolates were known to have exposure to one type of metals but the gradient plate assay indicated that these cultures could tolerate other metals at considerable concentrations. This has indicated towards presence of some general mechanisms of metal tolerance than any metal specific mechanisms. Methods used so far for the determination of metal resistance capacity of isolated cultures were found to be laborious, required huge number of glass ware and chemicals. If the number of cultures to be screened is sufficiently more, which is always desired for getting most efficient metal interacting microorganisms, the expenses for screening experiments may limit the experimental scope.

This poses major problem before the researchers for searching new efficient organisms. In screening, the major objective is to choose appropriate organism as well as to eliminate the high number of less or non-efficient cultures. In screening experiments, we have used a simple and reliable method viz. gradient plate method. This method provides a wide range of metal concentrations across the gradient and can help to characterize the set of cultures for tolerance/resistance capacity. Gradient plate method was used in this study as a primary screen to handle a large number of isolates. This method proved successful in yielding the seven cultures possessing metal tolerance. The gradient plate assay was developed at Eli Lilly and Company in 1976 as a preliminary screen for the identification of direct-acting and activation dependent mutagens (Rexoat *et al*., 1995). Two distinct advantages of the gradient plate assay are (i) it requires a smaller amount of the test article than any other conventional assays, and (ii) many cultures can be screened on a single plate. The gradient plate provides exposure to continuously increasing concentrations of metals, whereas, broth MIC assays provides only step wise rise in metal concentrations. The gradient plate does not give quantifiable tolerance to metal concentrations rather it can be used to distinguish the performance of set of cultures under same set of conditions.

The results of metal gradient plate assay were reconfirmed using broth assay. The broth environment provides easy access to metals for microorganisms. The MIC assay helps in quatifying the metal tolerance of cultures. Use of synthetic media containing sodium glycerophosphate ensures the solubility and thus the availability of metals. The decrease in MIC values in synthetic medium than nutrient broth could be due to the increased availability of metals. Biosorption and immobilization are major mechanisms utilized by animals and plants to limit the concentrations of internal reactive metal species (Eccles, 1999). Biosorption using microbially produced synthetic phytochelatins has been shown to be a promising technique for ameliorating heavy metal contamination (Wu *et al.*, 2006). All the metal tolerant bacterial isolates when studied for their biosorptive capacities showed promising results. This has pointed towards the role of biosorption as one of the leading mechanisms of metal resistance. Bacteria might transform toxic trace element to forms that are more readily taken up into roots (Zayed *et al*., 1998). To survive adverse conditions the

microbes develop mechanisms that confer upon them resistance against these heavy metals. These microorganisms combat the metal stress by metal complexation and precipitation and/or resorting to efflux mechanisms (Ahuja *et al*., 2001).

CONCLUSIONS

The present investigation was undertaken to isolate and characterize metal tolerating microorganisms which can be suitably used in phytoremediation techniques. Bacterial cultures were isolated from the soil samples collected from diverse metal exposed and unexposed habitats. Metal enrichment technique has yielded 94 isolates tolerating various metals. A simple method i.e. metal gradient plate assay was adopted to screen the isolates. Seven cultures were selected on the basis of growth performance on gradient plate. The cultures were further characterized for metal tolerance in broth, growth curves, pH tolerance and metal biosorption. On the basis of experimental results cultures viz. MJ-Pb-13 and MJ-Cr-6 could be selected for phytoremediation trials.

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