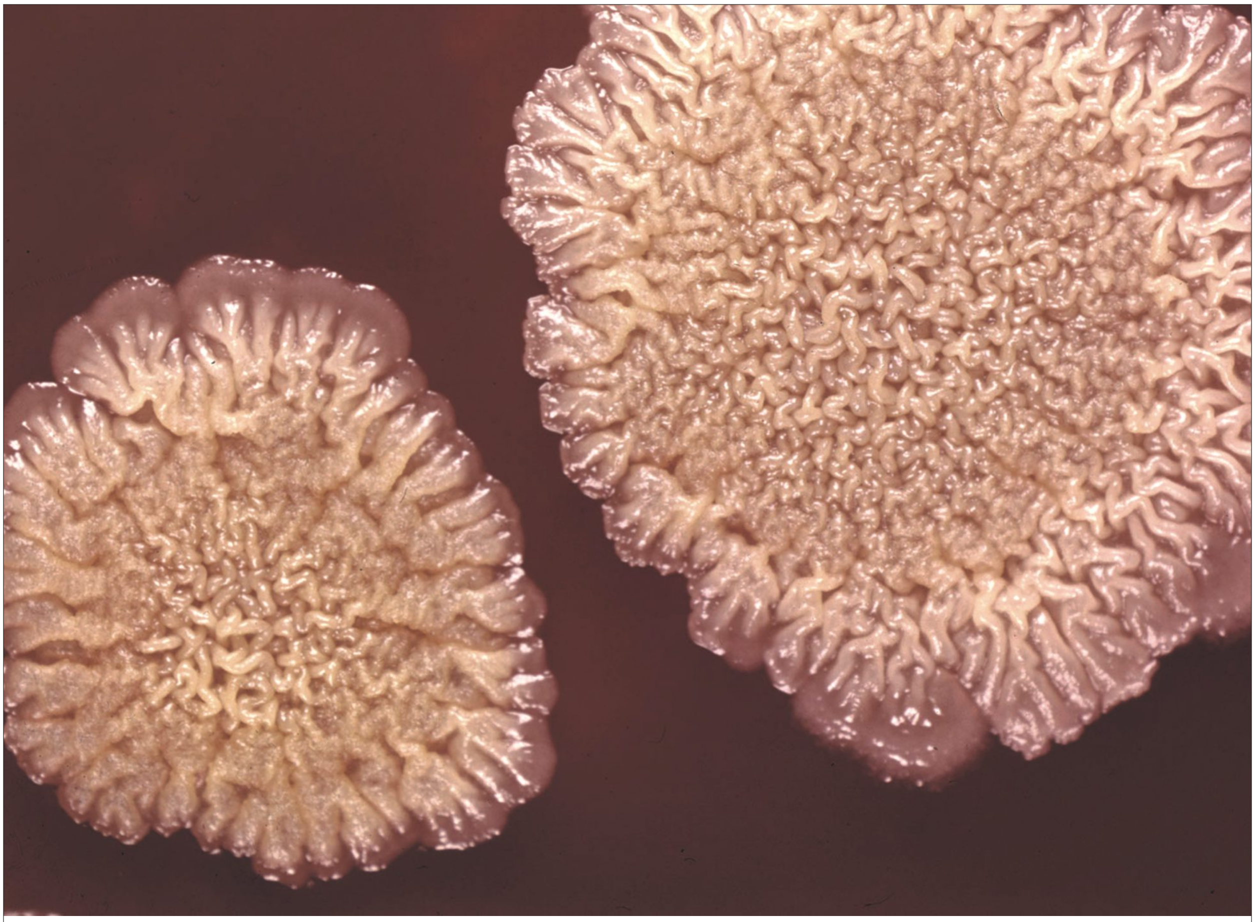

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Bioaccumulation of Heavy Metals by Zinc Resistant Bacteria Isolated from Agricultural Soils Irrigated with Wastewater

Munees Ahemad and Abdul Malik
Department of Agricultural Microbiology, Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh-202002, U.P., India

Corresponding Author: Munees Ahemad, Department of Agricultural Microbiology, Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh-202002, U.P., India Tel: +91-571-2702945

ABSTRACT

Due to global industrialization, heavy metals from industrial activities are accumulated in soils up to alarming concentrations. Microorganisms have developed several mechanisms to tolerate such high concentrations of heavy metals. One of these mechanisms dependent upon metabolic energy of microorganisms is the bioaccumulation of heavy metals. Therefore, this study was navigated to isolate the most promising zinc resistant bacteria from heavy metal contaminated soils and further, to assess their metal accumulating ability. A total of 34 bacterial isolates from agricultural soils irrigated with metal polluted wastewater were characterized and identified as *Pseudomonas* (23 isolates), *Bacillus* (5 isolates), *Staphylococcus* (6 isolates). The zinc resistant bacteria (*Pseudomonas* isolate SN7, *Pseudomonas* isolate SN28 and *Pseudomonas* isolate SN30) were selected because of exhibiting co-resistance against Cu^{2+}, Hg^{2+}, Cd^{2+}, Ni^{2+}, Pb^{2+}, Cr^{3+} and Cr^{6+} in addition to Zn^{2+} and displaying high values of Minimum Inhibitory Concentrations (MIC) for each heavy metal. Further, the three isolates were assessed for their ability to remove zinc and copper from medium amended with these metals. The zinc resistant bacterial isolates SN7, SN28 and SN30 accumulated zinc maximum 29, 25 and 26 mg g^{-1} dry weight of cells, respectively at the zinc concentration of 1.6 mM. Similarly, bacterial isolates SN7, SN28 and SN30 accumulated copper maximum 20, 25 and 22 mg g^{-1} dry weight of cells, respectively at 2.92 mM of copper. The results of present study showed that the metal resistant bacteria can be used for heavy metal bioremediation.

Key words: Bacteria, metal resistance, soil, wastewater, zinc

INTRODUCTION

Globally, environmental pollution is increasing ceaselessly on account of an unabated increment in population, industrialization, urbanization, anthropogenic activities and natural sources (Hanif *et al*., 2005; Khan *et al*., 2009). Among various pollutants, heavy metals are released into soils (Rajaganapathy *et al*., 2011) and water (Ansari *et al*., 2004; Benson, 2006) from industrial operations such as smelting, mining, metal forging, manufacturing of alkaline storage batteries and combustion of fossil fuel (Kumar *et al*., 2011). Moreover, the agricultural activities like application of agrochemicals and sewage sludge in agricultural fields also add a considerable amount of metals in the soils (Rajaganapathy *et al*., 2011). Moreover, heavy metals like zinc, copper, cadmium, lead, mercury and nickel have been reported as the most toxic pollutants (Cameron, 1992). The metal pollution is of great concerns, as these hazardous pollutants are accumulated in living organisms including microorganisms (Ogbo and Okhuoya, 2011), plants (Subroto *et al.*, 2007), animals (Bhattacharya *et al.*, 2008) and human (Hashem and Abed, 2002) and are responsible for many metabolic and physiological disorders (Rani and Goel, 2009; Matyar *et al.*, 2010). An enigma before the scientists is how to tackle the toxic contaminants that endanger the environmental health. Many novel approaches to clean-up the environment are being developed and many are already in practice (Kamaludeen *et al.*, 2003; Ahemad *et al.*, 2009) as alternatives to physical methods which are comparatively too costly (Strong and Burgess, 2008). Advancement in science and technologies parallel to industrial revolution has opened new vistas to exploit the inherent traits of natural resources including microorganisms to overcome the damage to the environment by pollutants (Khan, 2000; Ramadan, 2003). The ideal solution for pollution abatement is 'bioremediation' which is the most efficient strategy to manage and recover the contaminated environment (Ahemad and Khan, 2011). The term bioremediation has been introduced to exploit the biological traits of organisms for removing toxic species from the polluted sites (Vidali, 2001).

Some metals such as, zinc, copper, nickel and chromium are indispensable or useful micronutrients for the growth and development of organisms (Nies, 1999). In contrast, other metals e.g., cadmium, mercury and lead have no known physiological functions (Gadd, 1992). However, the higher concentration of these metals above threshold levels has deleterious impact on the microbial communities and their functional activities in soils. Otherwise, microorganisms exposed to the higher concentrations of toxic heavy metals may develop resistance against the elevated levels of these metals (Habi and Daba, 2009). In addition, microorganisms inhabiting in metal polluted soils have evolved various methods to resist themselves against metal stress. One of the microbial processes as a bioremediation tool to remove heavy metals from soils is bioaccumulation (Zolgharnein *et al.*, 2010) which is an active process dependent upon metabolic energy of microorganisms (Rani and Goel, 2009). Therefore, this study was designed with the objective to isolate the metal (zinc) resistant bacteria from heavy metal contaminated agricultural soils and to determine their feasibility on the removal of metals (zinc and copper) through bioaccumulation process.

MATERIALS AND METHODS

Collection of soil samples: Composite surface soil samples from the top 15 cm were collected from agricultural fields of Aligarh (27°29' and 72°29' longitude), Uttar Pradesh, India that had been irrigated with wastewater about a decade. The wastewater comprised both industrial wastewater from factories and domestic sewage. This city is famous for its lock manufacturing, steel and electroplating industries. However, it is noteworthy that there is no infallible separation of industrial effluents from domestic wastes and the two get mixed because (a) small scale industries are house-based and (b) proper compartmentalization of different sewerages has not gained popularity due to lack of strict laws and their enforcement procedures. Soil samples were collected in sterile polythene bags with the help of sterilized spatula as described by Reddy *et al.* (1986).

Enumeration of bacteria from soils: The enumeration of bacteria was done by the method as described by Reddy *et al.* (1986) and Malik and Ahmad (2002). Bacterial isolates were identified as per the standard methods following Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994).

Determination of relative growth and growth pattern: Degree of zinc resistance of the selected bacterial isolates was also evaluated in nutrient broth containing 25, 100 and 250 μg mL^{-1} of $ZnCl_2$. The growth of the isolates in nutrient broth was determined by measuring the optical density at 540 nm with uninoculated broth as control. Relative growth of the isolates was expressed as the percent of those obtained in untreated control which was taken as 100%. To assess the growth pattern of the bacterial isolates, exponentially grown cultures were inoculated into liquid nutrient broth treated with 0 (control), 25, 100 and 250 μg mL^{-1} of $ZnCl_2$ and incubated at 28±2°C in a rotary shaker (150 rpm) for 48 h. Growth was determined turbidimetrically after interval of each 6 h by measuring optical density (OD) at 540 nm.

Determination of minimum inhibitory concentration of heavy metals against bacterial isolates: Minimum Inhibitory Concentration (MIC) of metals [Hg^{2+}, Cd^{2+}, Cu^{2+}, Cr^{3+}, Zn^{2+}, Ni^{2+}, Pb^{2+} and Cr^{6+} in the form of $HgCl_2$, $CdCl_2$, $CuSO_4$, $CrCl_3.6H_2O$, $ZnCl_2$, $Pb(CH_3COOH)_2$ and $K_2Cr_2O_7$ in varying concentration from 3.12 to 2400 μg mL^{-1}] against each bacterial isolate was determined by the plate dilution method as adopted by Summers and Silver (1972)and Malik and Ahmad (1995). In this experiment, AB11157 strains of *E. coli* K-12 were used as control.

Bioaccumulation of heavy metals: Bacteria were grown in nutrient broth containing different concentrations of zinc and copper for 24 h. The bacterial cells were harvested by centrifugation and suspended in 1 mL of distilled water. The metal content of bacterial cells was determined after acid dissolution of bacterial cells according to the method of Ganje and Page (1974). Metal concentrations were measured by an atomic absorption spectrophotometer (GBC, Australia).

Statistical analysis: Each individual experiment was replicated three times at different time intervals. The Standard Deviation (SD) among the replicates was calculated using software Sigma Plot 10.

RESULTS

Enumeration, isolation and characterization: In the present study, the total viable count was found to be 8.0 ×10^6 CFU g^{-1} in agricultural soils irrigated with wastewater. The bacterial count on nutrient agar supplemented with zinc was decreased from 5.5 ×10^6 to 3.8 ×10^6 CFU g^{-1} of soils with increasing concentrations of metal from 50 to 250 μg mL^{-1} (Table 1). A total of 34 bacterial isolates which differed in color and morphology were selected randomly from zinc supplemented nutrient agar plates. All the isolates further tested for their morphologies, cultural and biochemical characteristics. Based on their properties, these isolates were identified as *Pseudomonas* [23 isolates (SN2, SN3, SN4, SN7, SN8, SN11, SN12, SN13, SN15, SN17, SN18, SN19, SN20, SN21, SN22, SN23, SN24, SN27, SN28, SN29, SN30, SN31and SN33)], *Bacillus* [5 isolates (SN1, SN9, SN14, SN26 and SN34) and *Staphylococcus* [6 isolates (SN5, SN6, SN10, SN16, SN25 and SN32).

Table 1: Bacterial count on zinc supplemented media

Zinc concentration (Mg mL^{-1})	Total viable count (CFU g^{-1} of soils)
0 (control)	$8.0\times 10^6 \pm 2.8\times 10^5$
50	$5.5\times 10^6 \pm 1.7\times 10^5$
100	$4.0\times 10^6 \pm 1.0\times 10^5$
250	$3.8\times 10^6 \pm 2.5\times 10^5$

Relative growth and growth pattern under metal stress: Further, the relative growth with respect to controls was ascertained turbidimetrically for all the bacterial isolates. It was observed that the growth of each bacterial isolate was declined with increasing concentration of zinc. Among the bacterial isolates, the isolate SN7, SN28 and SN30 showed the maximum relative growth up to 71, 77 and 69%, respectively at 250 zinc μg mL^{-1}. Thus, SN7, SN28 and the isolate SN30 were found to be the most promising (Table 2). The growth pattern of the three bacterial isolates SN7, SN28 and SN30 at different time intervals in the presence of 0, 50, 100 and 250 μg mL^{-1} of zinc are shown in Fig. 1. Similar to the relative growth, the growth of each bacterial isolate was also decreased progressively with increasing concentration of zinc in the medium.

Table 2: Percent zinc tolerance of bacterial isolates from wastewater irrigated soils

	Relative growth (%)		
Bacterial isolates	50 μg mL^{-1}	100 μg mL^{-1}	250 μg mL^{-1}
SN1	51	44	17
SN2	74	53	46
SN3	73	63	52
SN4	81	74	45
SN5	84	62	40
SN6	68	47	37
SN7	88	79	71
SN8	73	68	55
SN9	71	44	33
SN10	73	70	43
SN11	80	77	38
SN12	59	30	22
SN13	77	70	60
SN14	65	57	24
SN15	84	71	68
SN16	82	71	28
SN17	65	32	13
SN18	83	66	33
SN19	83	50	45
SN20	80	52	43
SN21	71	66	63
SN22	75	50	25
SN23	60	40	20
SN24	82	61	55
SN25	71	69	40
SN26	79	64	35
SN27	80	71	64
SN28	90	82	77
SN29	78	68	55
SN30	87	73	69
SN31	73	51	28
SN32	72	40	28
SN33	79	55	45
SN34	60	50	20

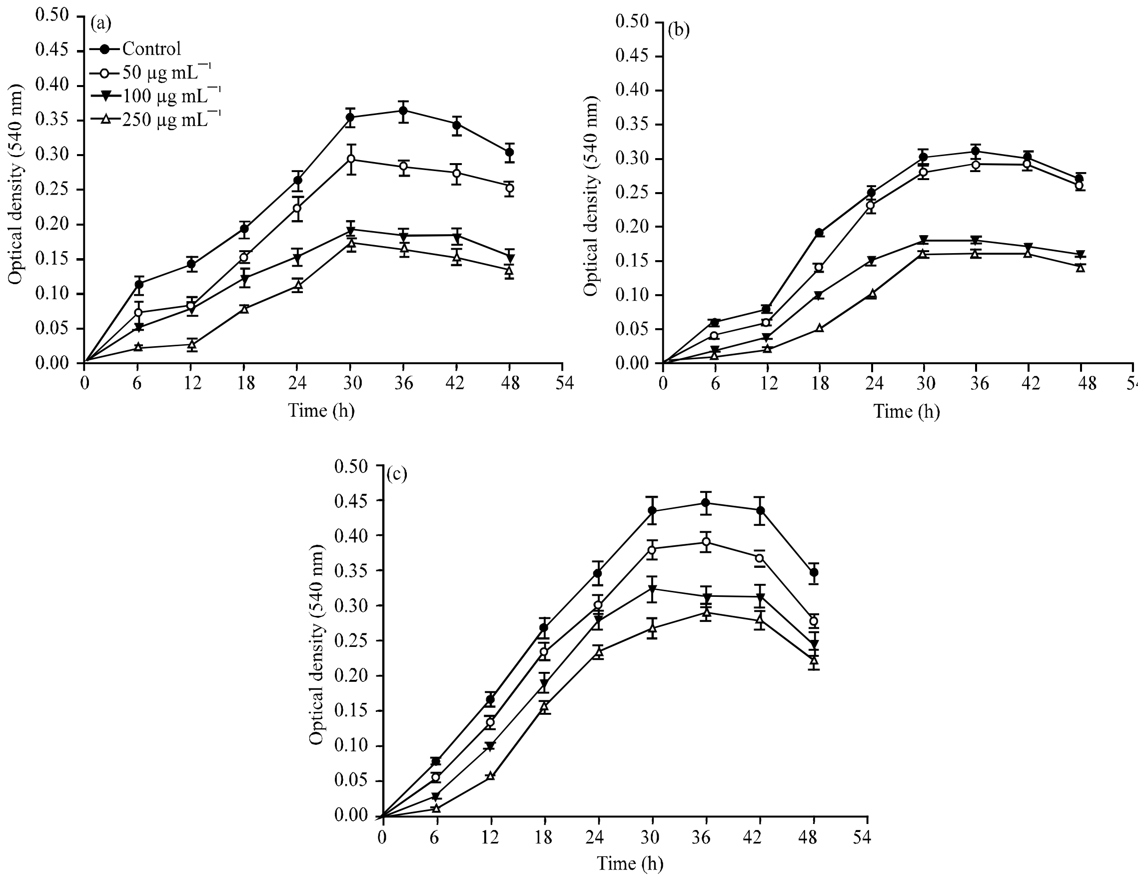

Fig. 1: Growth pattern of the bacterial isolates, SN7 (a), SN28 (b) and SN30 at different concentration of Zn^{2+}

Minimum inhibitory concentration of the bacterial isolates against heavy metals: The bacterial isolates SN7, SN28 and SN30 were tested for the resistance against metals Zn^{2+}, Cu^{2+}, Hg^{2+}, Cd^{2+}, Ni^{2+}, Pb^{2+}, Cr^{3+} and Cr^{6+} in varying concentration from 50 to 2400 µg mL^{-1}. A great deal of variation was observed in resistance pattern of the promising isolates against the selected heavy metals. Moreover, the bacterial isolates also displayed substantial co-resistance against other heavy metals in addition to zinc. The isolates SN7 and SN28 showed the MIC levels against both Zn^{2+} and Cu^{2+} up to 1600 µg mL^{-1} while SN30 exhibited MIC against Zn^{2+} and Cu^{2+} up to 800 and 1600 µg mL^{-1}, respectively. Generally, each of the three isolates showed the maximum resistance against Pb^{2+} (MIC= 2400 µg mL^{-1}) and Cr^{3+} (MIC= 2400 µg mL^{-1}) and the least resistance against Hg^{2+} (MIC= 50 µg mL^{-1}) (Table 3).

Bioaccumulation of zinc and copper by zinc resistant bacterial isolates: All the three promising zinc resistant bacterial isolates (SN7, SN28 and SN30) exhibiting also co-resistance against copper were tested for the zinc and copper accumulating ability. The isolates accumulated zinc and copper substantially when zinc and copper were added in the medium at sub-inhibitory concentrations. In general, accumulation of zinc and copper by the zinc resistant bacterial isolates increased progressively when the concentration of zinc in the medium increased from 0.4 to 1.6 mM (Fig. 2a-c) and that of copper augmented from 0.73 to 2.92 mM (Fig. 3a-c). The

Table 3: Minimum inhibitory concentration (MIC) of the selected bacterial isolates from agricultural soils irrigated with wastewater

	Minimum inhibitory concentration (μg mL^{-1})		
Metals	SN7	SN28	SN30
Zinc	1600	1600	800
Copper	1600	1600	1600
Mercury	50	50	50
Cadmium	200	100	100
Nickel	1600	1600	1600
Lead	2400	2400	2400
Chromium (3^+)	2400	2400	2400
Chromium (6^+)	800	400	400

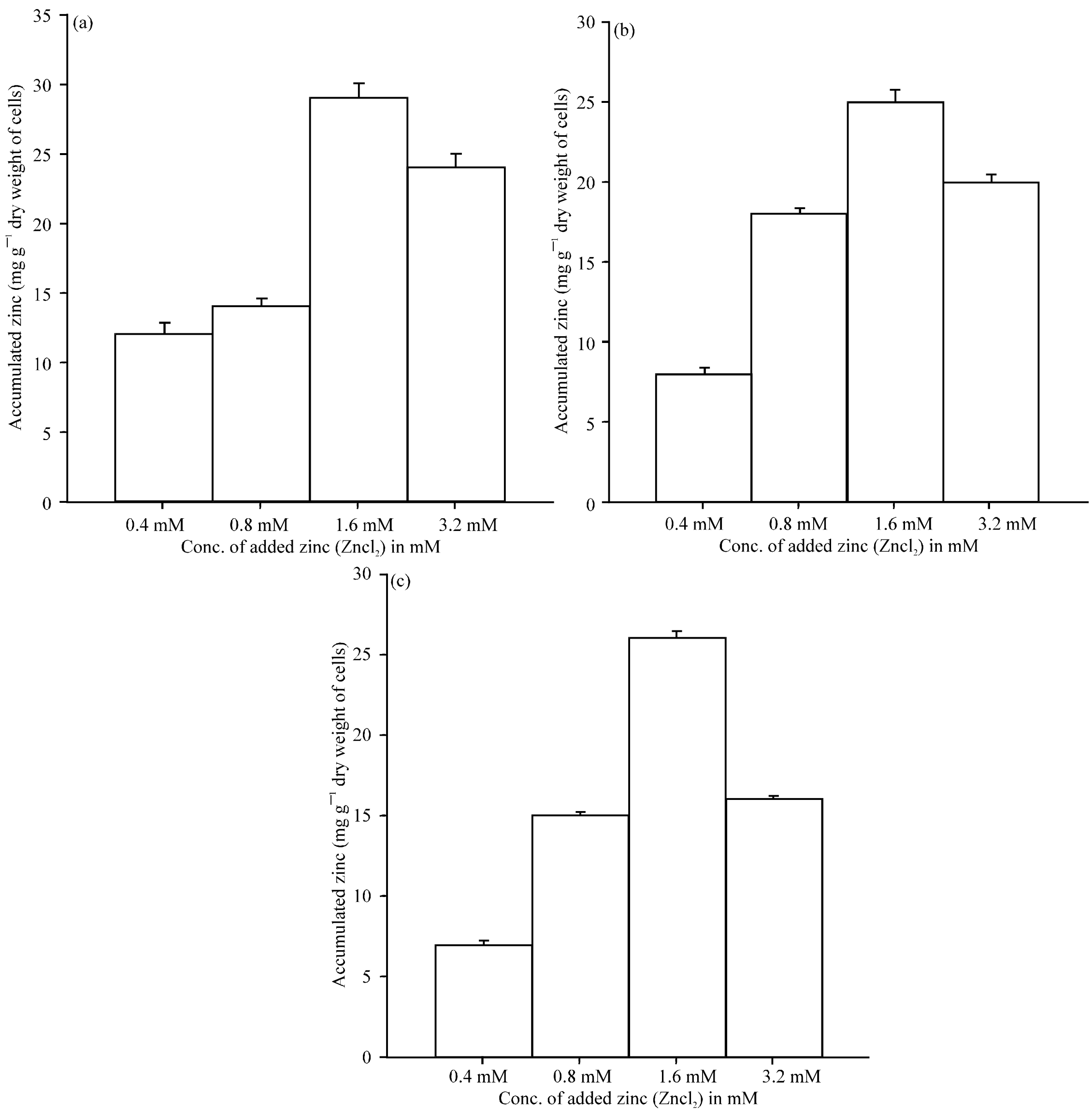

Fig. 2 (a-c): Accumulation of zinc by the isolate SN7, (b): Accumulation of zinc by the isolate SN28, (c): Accumulation of zinc by the isolate SN30

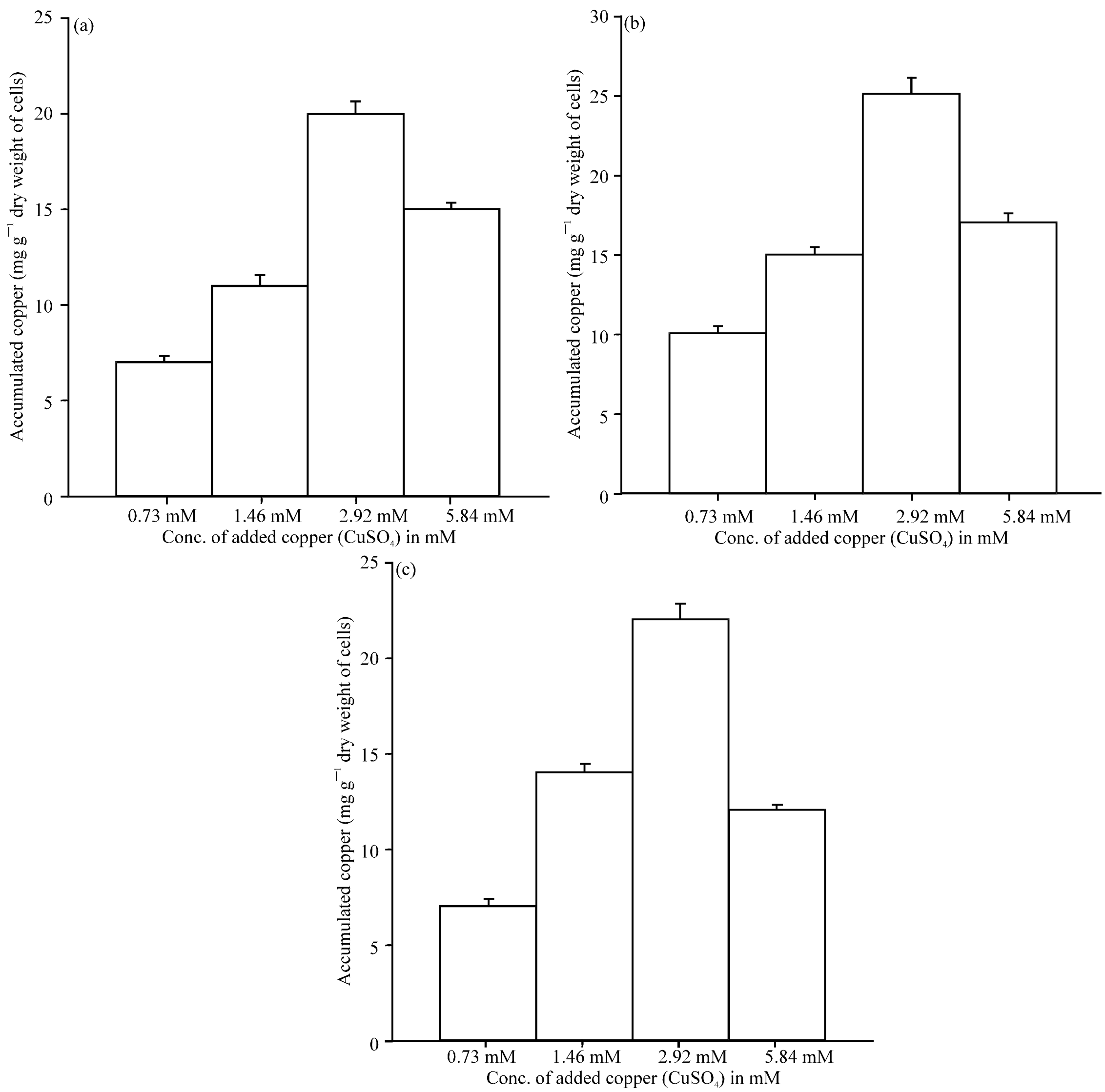

Fig. 3 (a-c): Accumulation of copper by the isolate SN7, (b): Accumulation of copper by the isolate SN28,(c): Accumulation of copper by the isolate SN30

zinc resistant bacterial isolates SN7, SN28 and SN30 accumulated zinc maximum 29 (Fig. 2a), 25 (Fig. 2b) and 26 mg g^{-1} dry weight of cells (Fig. 2c), respectively at the zinc concentration of 1.6 mM and accumulated copper maximum 20 (Fig. 3a), 25 (Fig. 3b) and 22 mg g^{-1} dry weight of cells (Fig. 3c), respectively at 2.92 mM of copper. In contrast, zinc and copper accumulated by the bacterial isolates decreased as the concentration of zinc and copper was raised beyond 1.6 mM and 2.92 mM, respectively (Fig. 2, Fig. 3). Thus, zinc resistant bacterial isolate SN7, isolate SN28 and isolate SN30 showed substantial zinc and copper accumulating ability.

DISCUSSION

In the present study, the bacterial isolates SN7, SN28 and SN30 out of 34 bacterial isolates from agricultural soils irrigated with metal polluted wastewater were selected owing to their maximum relative growth and MIC. These results are consistent with the screening for multi-metal resistant bacteria performed in previous studies (Vullo *et al.*, 2005; Vullo *et al.*, 2008) where Zn resistant bacterial isolates from soils were also resistant to Cd. Two factors can be considered to account for metal toxicity: (1) at low concentrations, metals (e.g. cobalt, copper and zinc) are essential for microorganisms, since they provide vital co-factors for metalloproteins and enzymes. At high concentrations, heavy metals however, exert inhibitory action on microorganisms by blocking essential functional groups or modifying the active conformations of biological molecules (Nies 1999; Li *et al.*, 2004) and (2) the low complexing capacity of the culture medium used in this assay may leave heavy metals almost completely bioavailable (Ceretti *et al.*, 2006).

The three selected isolates in our study were not only resistant against zinc and copper but also, had the extensive capability of accumulating these heavy metals. It is well reported that microorganisms have affinity for metals and can accumulate them by various mechanisms (Rani and Goel, 2009). Several principal sites of metal-complex formation in biological systems have been proposed (Vieira and Volesky, 2000). These include accumulation in the cell wall, carbohydrate or protein polyphosphate complexes and complexion with carboxyl groups of the peptidoglycan in the cell wall or entering into cells. An enhanced metal uptake by the metal resistant bacteria on increasing the metal concentration in medium in our study suggested that the metal uptake may involve diffusion phenomenon. Higher is the concentration gradient, the more rapid is the movement of molecules or ions. Our study also showed that metal accumulating ability of the bacterial isolates diminished beyond the specific concentrations of zinc and copper. This might be because of the saturation of the isolates with metals or due to increased toxicity of metals at high concentrations (Gadd, 1988; Kaewehai and Prasertson, 2002; Al-Garni, 2005).

Similar to our study, Zolgharnein *et al.* (2010) studied the uptake of heavy metal ions by *Pseudomonas aeruginosa* strain MCCB 102 isolated from the Persian Gulf. The highest adsorption was observed for Cu, Zn, Cd and Pb. They also reported that heavy metal uptake involves both surface phenomena and diffusion. In another study, Transmission Electron Microscopy (TEM) analysis of *Pseudomonas putida* 62BN demonstrated intracellular and periplasmic accumulation of cadmium. The intracellular and periplasmic cadmium accumulation in *P. putida* 62BN suggested the presence of metal-binding and/or efflux mechanisms inside the cells mediating resistance against metal toxicity (Rani *et al.*, 2009). Cytoplasmic (Yoshida *et al.*, 2002) and periplasmic (Naz *et al.*, 2005) accumulation of heavy metal ions as a result of metallothioneins expression has been reported in *E. coli*.

CONCLUSION

This experimentation of zinc and copper accumulation by the zinc resistant bacterial isolates from heavy metal contaminated agricultural soils revealed a positive indication for its use in bioremediation of zinc and copper in contaminated soils. Although this study was carried out under idealized laboratory conditions, the results illustrate some general considerations that are important for the use of the metal accumulating bacteria for soil bioremediation under the field conditions.

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