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RESEARCH ARTICLE

Effect of Different Concentrations of Metals Ions on *Bacillus* and *Pseudomonas* spp. Isolated from Industrial Effluents of Faisalabad

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ABSTRACT

With the intention to determine effects of different metals on growth of bacteria that were previously identified as heavy metal resistant, total six bacterial samples were selected, repurified and reidentified from the bacterial isolates. Different morphological and biochemical tests were performed that differentiated bacteria into two genera i.e. *Bacillus* sp. and *Pseudomonas* sp. Bacteria was grown at pH 7 and temp of 37ºC up to incubation time of 96 hours. Three metals (Na^+, Ca^{2+}, Zn^{2+}) were used with concentrations ranged between 25-200 mg/ml. It was found that as the concentrations of metals increased, growth of *Bacillus* and *Pseudomonas* spp. firstly increased and then decreased in case of all selected metals. Lowest growth occurred at 200 mg/ml of metal concentration. Optical density at 600 nm was considered as a measure of growth of bacteria. As growth increased, optical density increased and vice versa. Na^{+1} was found to have a more negative effect on growth of bacterial isolates as compared to other two metals that is, Ca^{2+}, Zn^{2+}. *Bacillus* sp. under investigation was found to be more resistant to these metals as compared to *Pseudomonas* sp. Both bacterial isolates showed their maximum growth after 72 hours of incubation in most of the cases, afterwards growth decreased. Decreasing resistance pattern for both of bacterial species was as follows; $Ca^{2+} > Zn^{2+} > Na^+$. The findings revealed the potential application of *Bacillus* and *Pseudomonas* spp. for the removal of heavy metals from industrial wastewaters under the conditions where salts of Sodium, Zinc and Calcium are present.

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INTRODUCTION

Global population is increasing, due to variation in natural and anthropogenic activities leading to contamination of various terrestrial and aquatic ecosystems with heavy metals, inorganic and organic compounds and radio nuclides (Rani, 2003). The effluents discharged by different industries contain higher values of physio-chemical parameters like temperature, pH, conductivity, hardness, alkalinity, chemical oxygen demand, total soluble salts, Nitrites and cations (Na, K, Ca and Mg) (Latif *et al*., 2008). Discharge of metals and some nonmetals into water bodies have serious environmental effects. In Pakistan, main contributors to the surface and ground water pollution are the byproducts of various industries such as textile, metal, dying chemicals, fertilizers, pesticides, cement, petrochemical, energy and power, leather, sugar processing, construction, steel, engineering, food processing, mining and others (Tariq *et al*., 2006). Main reason for this metal contamination is that 80% of the land is irrigated by wastewater generated from industry. The Indus River and its tributaries provide water to over 16 million hectares of land, situated in the mainly arid and semi-arid zones of the country. A rapidly growing population, saline groundwater, a poorly performing irrigation distribution system, and recurrent droughts have led to increased water shortages. Under these conditions, the use of untreated urban wastewater for agriculture has become a common and widespread practice (Ensink *et al.*, 2002). Heavy metals are major pollutants in marine, ground, industrial and even treated wastewater (Valdman *et al*., 2001). The alarming situation has aroused in the urban areas due to careless disposal of industrial waste and faulty drainage system that has badly affected the quality of the river water (Ugochukwu, 2004; Chindah *et al*., 2004; Emongor *et al.*, 2005). Major contributors to heavy metal water pollution are tanneries, brewery, textile, pottery, electroplating, metal finishing, mining, dyeing and printing industries, ceramic, photographic and pharmaceutical industries, scattered in the region (Azzaoui *et al*., 2002; Vutukuru, 2003).

Bioremediation, which is essentially the use of microbial metabolism, seems to offer a viable, safer, more efficient and less expensive alternative to physiochemical methods for pollution treatment (Pandey and Jain, 2002). Microorganisms are of primary importance in bioremediation of soils and waters contaminated with heavy metals, essentially because of their ability to alter the chemical status of the metal ions and in turn the metal ion mobility through processes such as reduction, accumulation, mobilization and immobilization. In the treatment of waste water microorganisms (mainly bacteria) including both the gram-positive types, such as *Bacillus* sp. and the gram negative types such as *Pseudomonas* sp. use the soluble organic compound and convert them into carbon dioxide, water and energy to produce new cell (Minna *et al*., 2006). Interestingly, microorganisms do require certain metal ions, like those of Cu^{+2}, Zn^{+2}, Co^{+2}, Ni^{+2}, in very low concentration as essential micronutrients as components of important cofactors in enzymatic reactions. However at higher concentrations, researchers have reported that these metals may cause cellular death by intervening with nucleic acids and enzyme activation sites (Sani *et al*., 2001).

High levels of TDS (Total dissolved oxygen) and Sodium Chloride are typically present in tannery effluent. The TDS concentration can reach 7,000 mg/l and in some cases more than 15,000 mg/l. Effluent of brewery industry, sugar industry, effluent of the fluoride removal process of the electronics industry, fleshing wastes, textile industry, oil industry, marble industry, metal finishing company contain high concentration of calcium (Barefoot et al., 2002). Zinc is an important trace metal used in numerous biological processes, however, at higher concentration, Zinc becomes a biological hazard. Therefore there is significant interest regarding Zinc removal from wastewater and its toxicity for humans at levels of 100–500 mg/day. Addition of trace amounts of heavy metals to the environment of microbial cells often stimulates microbial growth (Gikas, 2007; 2008). However, higher concentrations result in severe reduction of microbial activity, which is reflected by reduction of apparent growth rate and increase in lag time. Heavy metal toxicity on microorganisms has been modeled by Utgikar *et al*. (2003) who studied the effects of Cu and Zn ions toxicity on sulfate reducing bacteria.

The aim of this study was to assess the resistance and effect of different concentrations of Na^{+1}, Ca^{+2} and Zn^{+2} on growth of *Bacillus* and *Pseudomonas* spp and to check effect of different incubation periods on resistance to metal by *Bacillus* and *Pseudomonas* spp.

MATERIALS AND METHODS

Sample collection

The bacterial samples were obtained from Research Laboratory of Zoology Department at GC University, Faisalabad. These bacterial samples had already been identified and categorized as heavy metal resistant isolates and were preserved on nutrient agar slants during previous work at GCUF (Umber, 2008).

Culturing of preserved heavy metal resistant bacteria

Fresh cultures of preserved bacterial isolates were obtained by growing them on nutrient agar.

Purification and preservation of heavy metal resistant bacteria

Colonies of *Bacillus and Pseudomonas* spp. were picked and grown again and again on nutrient agar plates to remove contaminations. These two genera (*Bacillus* and *Pseudomonas*) were separated on the basis of morphological and biochemical tests, and preserved on slants at about 8°C.

Biochemical Tests

Following biochemical tests were performed for the identification of bacteria. Gram’s Staining, Catalase Test, Oxidase Test, Indole Test, Motility test, Vogas Proskauer Test.

Determination of effect of different concentration of metals (Zn^{+2}, Na^{+1}, Ca^{+2}) on growth of bacteria

(Oladipo *et al*., 2010) 100 ml of distilled water was taken in 250 ml flasks, and added 2.5 grams of nutrient broth in it. Metallic salts $ZnCl_2$, NaCl, $CaCl_2$ were used as source of Metals ions. These Metals ions (Zn^{+2}, Na^{+1}, Ca^{+2}) were used in varying concentrations 25, 50, 75, 100, 125, 150, 175, 200 mg/ml, added these concentration in nutrient broth solution. The pH of the solutions was checked by pH meter and heats them till boiling. They were sterilized for 15 minutes at 121°C in autoclave. After cooling, the flasks were inoculated with the test organism under laminar flow and incubated at 37 °C in incubator on shaker at 160 rpm. Growth was detected using atomic absorption spectrophotometer in terms of optical density at 600 nm after 0, 24, 48, 72, 96 hours of incubation time. Increase in turbidity of the medium was recorded as positive for growth while a negative result showed no turbidity. Flasks without metals served as control. All the experiments were conducted in triplicates.

Statistical Analysis

The data was subjected to statistical analysis in which uptake of various metals (Na^{+1}, Ca^{+2}, Zn^{+2}) by *Bacillus* and *Pseudomonas* spp. at pH 7 and temperature 37 °C was compared using student’s t test.

RESULTS

Identification of Bacterial Isolates

On the basis of morphological and biochemical studies two bacterial genera were isolated namely *Bacillus* and *Pseudomonas* spp.

Table 1: Morphological and biochemical results

Bacteria identified	*Bacillus* sp.	*Pseudomonas* sp.
Colony morphology	Creamy white irregular	Creamy white round
Gram's staining	+Ve	-Ve
Catalase test	+Ve	+Ve
Oxidase test	+Ve	+Ve
Indole test	+Ve	-Ve
Motility test	+Ve	+Ve
Vogas Proskaure test	+Ve	-Ve

Bacillus sp. and Metal resistance pattern

Na^{+1}: Figure 1 shows effect of Sodium metal on growth of *Bacillus* sp. cultivated at 37 °C and pH 7. A significant increase in growth with time of incubation with metal concentrations ranged between 25 - 75 mg/ml was observed. However, very little increase in growth was observed with Sodium metal concentration ranged between 100-150 mg/ml. The concentration of 175 mg/ml of Na^{+1} metal had no effect on *Bacillus* sp. growth. Whereas, a gradual decrease in growth of isolate was found at 200 mg/ml. Moreover, *Bacillus* sp. showed increase in growth up to 72 hours of incubation with almost all Na^{+1} concentration except 200 mg/ml, while death phase occurred at 96 hours.

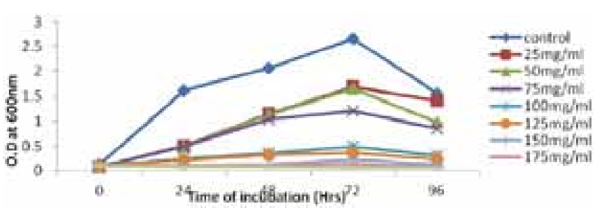

Fig. 1: Effect of different concentrations of Na^{+1} on growth of *Bacillus* sp. on different incubation periods.

Ca^{+2}: It was noticed that 25, 50, 75 and 100 mg/ml of Ca^{+2} had a positive effect on *Bacillus* sp. and increased the growth from 24 to 72 hours of incubation, afterwards at 96 hours, growth significantly decreased. While, 125, 150, 175 and 200 mg/ml of Ca^{+2} had a negative effect and decreased the growth with increase in time of incubation (Fig. 2).

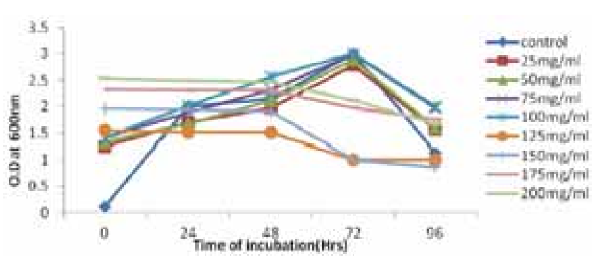

Fig. 2: Effect of different concentrations of Ca^{+2} on growth of *Bacillus* sp. on different incubation periods.

Zn^{+2}: It was observed that 25 and 50 mg/ml of Zn^{+2} of metal concentration in the medium had a positive effect on *Bacillus* sp. It caused increase in the growth significantly till 72 hours of incubation while, decrease after 96 hours of incubation. Zn^{+2} metal concentrations of 75 and 100 mg/ml in medium had no effect on *Bacillus* sp. However, at the concentration of 125, 150, 175 and 200 mg/ml of Zn^{+2}, growth decreased with increase in time of incubation (Fig. 3).

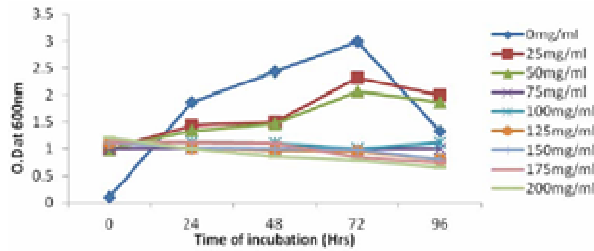

Fig. 3: Effect of different concentrations of Zn^{+2} on growth of *Bacillus* sp. on different incubation periods.

Pseudomonas sp. and Metal resistance pattern

Na^{+1}: A significant and constant raise in growth of *Pseudomonas* sp. was noticed at 25 and 50 mg/ml concentration of Na^{+1} metal even after 96 hours of incubation. In addition, growth of bacterial isolate increased at 75 and 100 mg/ml of Na^{+1} metal concentration up to 72 hours of incubation, afterwards it declined. However, no effect of Na^{+1} metal concentration of 125 and 150 mg/ml were observed. A further increase of metal concentration (i.e. 175 and 200 mg/ml) had caused gradual decline in the growth of *Pseudomonas* sp. (Fig. 4).

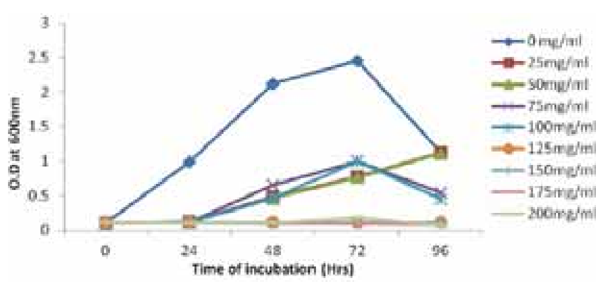

Fig. 4: Effect of different concentrations of Na^{+1} on growth of *Pseudomonas* sp. on different incubation periods.

Ca^{+2}: It was noticed that 25 and 50 mg/ml of Ca^{+2} had a positive effect on *Pseudomonas* sp. and increased the growth from 24 to 72 hours, afterwards growth significantly decreased at 96 hours of incubation. At 75-125 mg/ml concentration of Calcium metal, growth remained unaffected. Whereas, the concentrations of 150, 175 and 200 mg/ml of Ca^{+2} Metal ion had a negative effect and decreased the growth.

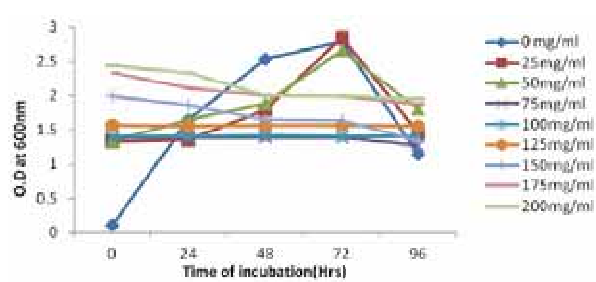

Fig. 5: Effect of different concentrations of Ca^{+2} on growth of *Pseudomonas* sp. on different incubation periods.

Zn^{+2}: It was observed that 25 and 50 mg/ml concentrations of Zn^{+2} had a positive effect on *Pseudomonas* sp., it increased the growth significantly till 72 hours but decreased after 96 hours of incubation. At 75 mg/ml of Zn^{+2} growths remained unaffected till 72 hours afterwards it decreased. In addition, 100 - 200 mg/ml of Zn^{+2} decreased the growth with increase in time of incubation. It could be concluded that this *Pseudomonas* sp. can tolerate Zn^{+2} up to 75 mg/ml.

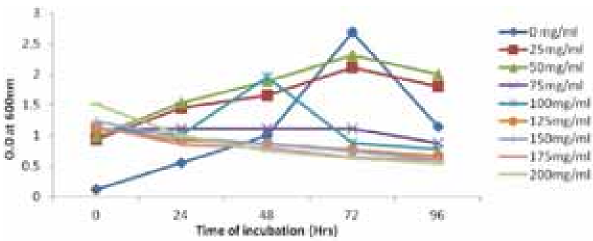

Fig. 6: Effect of different concentrations of Zn^{+2} on growth of *Pseudomonas* sp. on different incubation periods.

Comparison of effects of metals between *Bacillus* sp. and *Pseudomonas* sp.

Figures 7, 8 and 9 Show that *Bacillus* sp. was more resistant to Na^{+1}, Ca^{+2} and Zn^{+2} as compared to *Pseudomonas* sp.

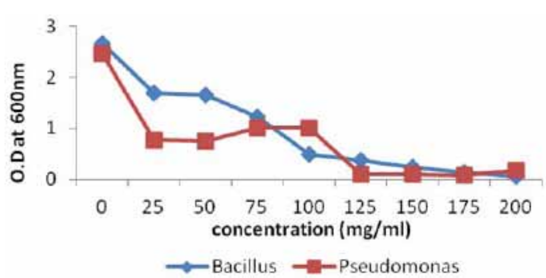

Fig. 7: Comparison of effects of Na^{+1} between *Bacillus* and *Pseudomonas* spp.

Comparison of effect of heavy metal (Zn^{+2}) with two non heavy metals (Na^{+1}, Ca^{+2})

It was observed during the present investigation the minimum uptake of metal take place for Sodium metals as compared to other two metals; Ca^{+2} and Zn^{+2}. It was also found that highest growth values obtained in case of Calcium. This shows order of decreasing resistance which is as follows; Calcium > Zinc > Sodium.

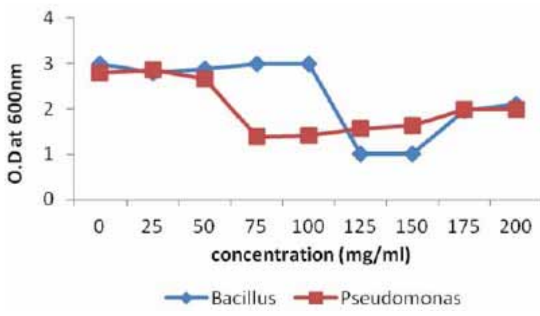

Fig. 8: Comparison of effects of Ca^{+2} between *Bacillus* and *Pseudomonas* spp.

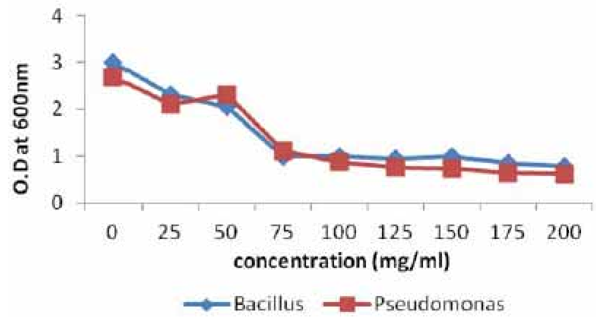

Fig. 9: Comparison of effects of Zn^{+2} between *Bacillus* and *Pseudomonas* spp.

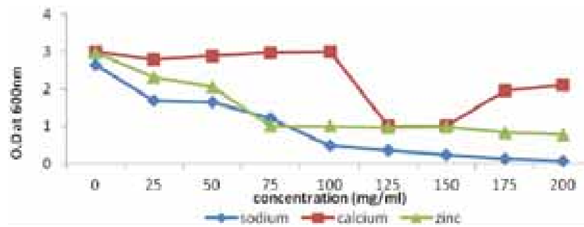

Fig. 10: Comparison of effect of heavy metal (Zn^{+2}) with two non heavy metals (Na^{+1}, Ca^{+2}) on *Bacillus* sp.

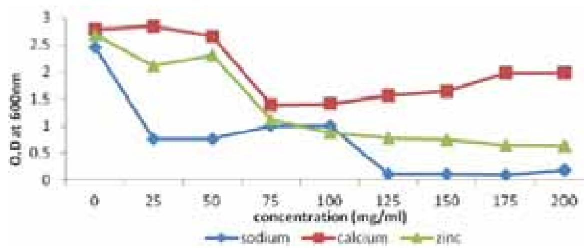

Fig. 11: Comparison of effect of heavy metal (Zn^{+2}) with two non heavy metals (Na^{+1}, Ca^{+2}) on *Pseudomonas* sp.

DISCUSSION

In the present study 6 samples of preserved bacterial isolates were collected from research laboratory of G.C University Faisalabad. These bacterial isolates were originally isolated from industrial effluents of Faisalabad region by Umber (2008) in the present study, these bacterial isolates were reidentified and reclassified into two bacterial genera namely *Bacillus* and *Pseudomonas* spp. As reported by Umber (2008).The present study was carried out to explore the effects of Na^{+1}, Ca^{+2}, Zn^{+2}, on growth of *Bacillus* and *Pseudomonas* spp. In the present study reidentified, purified and characterized isolates were preserved on fresh nutrient agar slants, stored at 4ºC for 24 hours and later incubated at 37ºC for 24 hours prior to each study. Nutrient broth was used for the cultivation of *Bacillus* and *Pseudomonas* spp. This was also reported by Ibezim *et al*. (2006).

It was found that *Bacillus* and *pseudomonas* spp. grew well between 24 to 72 hours of incubation period as reported by Prescott *et al.* (2005) and Ynte *et al*. (2004). Increase in turbidity of the medium was recorded as positive for growth while a negative result shows no turbidity, previously this was also reported by Oladipo *et al*., (2010).

The use of NaCl salt as a sourse of Na^{+1} metal is also reported by Abdulkarim (2009) and Oladipo *et al*. (2010). Decrease in growth of *Bacillus* and *Pseudomonas* spp. with increase in Na^{+1} metal concentration found in present study, has also been recorded by Komives *et al*. (2005); Okanlawon *et al*. (2010); Oladipo *et al*. (2010); Veenagayathri and Vasudevan (2010). The decrease in total growth due to increase in the salt concentration was probably as a result of hyper osmotic effect.

During the present study Calcium Chloride supplementation was found to have a positive influence on *Bacillus* and *Pseudomonas* spp. in terms of growth rate at lower concentrations (25-100 mg/ml) as previously mentioned by Saranya and Shenbagarathi (2010) and Yuangklang *et al.* (2010). While higher concentrations of Calcium (125 to 200 mg/ml) had a negative effect on growth.

Zinc is an essential trace element for growth and enzyme activities of bacteria. However, excess load of Zn shows toxicity and inhibition to microbial processes. In Pakistan, several zinc resistant strains were isolated. *Pseudomonas aeruginosa* CMG 103 was isolated from a metal polluted river and displayed a high level of zinc and cadmium stress. Similarly, two Zinc resistant strains resistant to $ZnSO_4$ up to 12.5 mg/ml were isolated by Hasnain and Sabri (1992) from industrial effluents. These isolates also showed tolerance to other heavy metals such as Ni^{+2}, Ba^{+2}, Fe^{+3} and Cd^{+2}. $ZnCl_2$ was used as a salt supplement during the present research work and it was found that *Bacillus* and *Pseudomonas* spp. were resistant to low concentrations of Zinc (25-100 mg/ml) and growth increased with increase in time of incubation. However, higher concentrations (125-200 mg/ml) decreased the growth of both bacterial isolates. Similar findings were reported by El-Bstawy (2000) and Bong *et al*. (2010).

Account on comparison of effects of Na^{+1}, Ca^{+2}, Zn^{+2} on growth of *Bacillus* and *Pseudomonas* spp., it was found that calcium had a positive effect on growth while sodium had negative effect on growth. Similar results were reported by Tarakci *et al*. (2004); Gomaa and Azab (2007); Alshiyab *et al*. (2008). During the present study it was also found that *Bacillus* sp. was more resistant to metals under investigation (Na^{+1}, Ca^{+2}, Zn^{+2}) as compared to *Pseudomonas* sp., this was also reported by Leung *et al.* (2000); Ravikumar *et al.* (2007).

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