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Assessment of Cadmium and Lead effect on the growth of phosphate dissolved bacterium *Bacillus polymyxa* and its efficiency *in vitro*

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Abstract. An incubation experiment was conducted to evaluate the effect of heavy metals (cadmium and lead) on growth of phosphate dissolved bacteria (*Bacillus polymyxa*). Cadmium nitrate and lead acetate were prepared at concentration of 0, 5, 10, 25 and 50 ppm in pvk media. The studies were conducted during different incubation periods 7, 14, 21, 28 and 30 days then estimated the efficiency of the bacteria to dissolve phosphorus fertilizer, adding rock phosphate (10% P) by determining mean of pH level, total acidity and soluble phosphorus in contaminated pvk medium. Results showed that the highest tolerance of *B. polymyxa* against the metals was found to be at 10 ppm for both cadmium and lead. The parameters affecting the bioadsorption of heavy metals; such as time, metal concentration and biomass concentrations have been investigated, Pb was attained within 14 days as compared with control treatment (no metals) that refers to higher tolerance of bacteria at lag phase and stationary phase than at late phase of growth. Results indicated that *B. polymyxa* has high ability to solve insoluble phosphorus (rock phosphate) to soluble state, rates (55% and 40%) at 10 ppm for both cadmium and lead respectively as compared to control (concentration = 0), hence efficiency of bacteria improved although contaminated conditions. From this study it is evident the efficiency and biomass are directly related to soluble phosphorus source supply to cells in lag phase than in late phase and live cells absorbed heavy metals more than dead cells.

Key Words: *Bacillus sp*., bioremediation, heavy metals, phosphorus fertilizer.

Introduction. Heavy metals are considered as one of main source of the environmental pollution (Damodaran et al 2011) which could be attributed to the anthropogenic activity, fertilizer producing plants and wastes left after mining and metallurgical processes all of these activities lead to increase the level of metals owing to atmospheric and industrial pollution accumulate in soil with a remarkable influence the ecosystem nearby (Zouboulis et al 2004). An increase in metal concentration also influence the soil microbial communities, especially respiration and enzymatic activity by blocking essential functional groups, displacing essential metal ions or modifying the active conforming of biological molecules that serve as good indicators of metal pollution (Doelman et al 1994). Several studies have shown the negative relationship between heavy metal concentrations and microbial activity. However, at relatively low concentrations, some heavy metal ions (e.g. Cd and Pb) are essential for microorganism's growth since they provide vital cofactors for metallo-proteins and enzymes (Eiland 1981; Doelman et al 1994).

In order to survive in heavy-metal polluted environments, many microorganisms have developed different means of resistance to the toxic metal ions (Nies & Silver 1995; Nies 1999). These mechanisms include: active transport of metal away from cell organism, enzymatic detoxification of metal to a less toxicity effect (Bruins et al 2000). Most microorganisms are known to have specific genes for resistance toxic of heavy metal by genes are found either on plasmids or on chromosomes (Nies 1999). Although some heavy metals are essential trace elements, most can be at high concentrations toxic to all forms of life including microbes like dissolved phosphate bacteria heavy metals may reduce the efficiency of *Bacillus sp*. to dissolve insoluble phosphorus and release it to liquid phase (Sanjotha et al 2011). Several studies on the application of

growing microbial cells for metal removal have shown that they are better than non–viable cells due to the microbe's ability of self – replenishment, continuous metabolic uptake of metals after physical adsorption, through development of resistant species and cell surface modification (Malik 2004). So the aim of this present study is to find out the effect of two heavy metals (cadmium and lead) on growth and efficiency of *Bacillus polymyxa*.

Material and Method

Bacterial isolate. *B. polymyxa* was isolated from rhizosphere of wheat root grown in Al-shattra at Al-Nassria province, Iraq, and identified at Soil Microbiology Laboratory, Soil Department, College of Agriulture, Basrah University.

Heavy metals. Two heavy metals Cd and Pb were used as $Cd(NO_3)_2$ and $Pb(CH_3COO)_2$. Both of these metals were prepared at scale of concentrations of 0, 5, 10, 25 and 50 ppm and dissolved in 150 mL of broth media comprised of (in g/mL^{-1}): $(NH_4)_2SO_4$, 0.5 mL; $FeCO_3 \cdot 6H_2O$, 0.01 mL; $MgSO_4 \cdot 7H_2O$, 0.5 mL; Pepton, 2.5 mL; Sucrose, 5 mL; Glocose, 5 mL; Yeast extract, 2 mL; Tap water, 1000 mL (Pikovskaya 1948). The constituents of basal medium were modified by Nabilah (1977), then were inoculated with *Bacillus polymyxa* bacteria isolate at initial concentration of 1.5×10^8 CFU/mL^{-1} medium and incubated at 37°C in shaker incubator for different incubation periods 7, 14, 21, 28 and 30 days. Then estimated colony forming unit by dilution and plate's method was used to determine the effect of metals on biomass growth and efficiency of bacteria to dissolve phosphorus added as rock phosphate (10% P) at 1 gm/150 mL medium. Then was determined the changes in pH level by pH-meter, total acidity by titration with 0.01N of NaOH according to Sperber (1957) and soluble phosphorus in contaminated medium.

Statistical design and analysis. Data were statistically analyzed by ANOVA, according to (Al-Rawi & Khalaf 1980) by SPSS (1998) version 9 and treatment means were compared by New LSD test at 5% level of probability.

Results and Discussion

Effect of Cadmium on bacterial growth. The data in Table 1 show that the growth of *B. polymyxa bacteria* increased from 18.7×10^8 CFU/mL^{-1} at control treatment (without cadmium) to 2.7×10^9 CFU/mL^{-1} at 10 ppm of cadmium in medium, but at concentration of 50 ppm Cd growth, reduced to 54×10^5 CFU/mL^{-1}, as well as the data in Table 1 shows that effect of different incubation periods, its growth in contaminated medium with Cd increased during 7 and 14 days (0.05×10^9 CFU/mL^{-1} and 4.9×10^9 CFU/mL^{-1}) respectively. Then the growth reduced at 30 days (3.7×10^6 CFU/mL^{-1}), interaction effect of Cd concentration and incubation periods during 14 days and at 5 ppm Cd was 22×10^9 CFU/mL^{-1} while growth at 7 days and 50 ppm was 12×10^5 CFU/mL^{-1} (Table 1).

Table 1

The effect of Cadmium levels (ppm) and different periods (days) of incubation on growth of *Bacillus polymyxa*

Cd levels (ppm)	*Incubation periods (days)*					*Average*
	7	*14*	*21*	*28*	*30*	
no Cd	$10^7 \times 1.2$	$10^9 \times 1$	$10^7 \times 15$	$10^6 \times 36$	$10^9 \times 8.4$	$10^8 \times 18.7$
5 Cd	$10^8 \times 1.2$	$10^9 \times 22$	$10^6 \times 2$	$10^6 \times 9$	$10^6 \times 2$	$10^9 \times 4.4$
10 Cd	$10^9 \times 12$	$10^8 \times 15$	$10^7 \times 20$	$10^5 \times 35$	$10^5 \times 51$	$10^9 \times 2.7$
25 Cd	$10^7 \times 11$	$10^6 \times 9$	$10^7 \times 15$	$10^6 \times 29$	$10^4 \times 43$	$10^7 \times 5.9$
50 Cd	$10^5 \times 12$	$10^7 \times 0.9$	$10^7 \times 0.3$	$10^5 \times 31$	$10^5 \times 111$	$10^5 \times 54$
Average	$10^9 \times 0.05$	$10^9 \times 4.9$	$10^7 \times 1$	$10^6 \times 16$	$10^6 \times 3.7$	-

These results were in a good accordance with Wuana et al (2010) for growth of bacteria *Bacillus megaterium* at lower concentration of Cd in liquid culture due to heavy metal like Cd at lower concentration acts as stimulant growth element more than at higher concentrations, which is dependent on the cell's metabolism, it is often associated with an active defense system of the microorganism in the presence of toxic metal according to Huang et al (1990) and induce resistance/tolerance against harmful effects heavy metals besides toxicity of bacteria increasing populations significantly as in Table 1. The results of interaction between concentrates of (Cd and Pb) and incubation time on the growth *B. polymyxa* indicated high tolerance ability in low concentrates during the stage of lag phase and stationary phase, but has no tolerance at high concentrate of Cd metal especially during late stage of growth according to (Nies & Silver 1995).

The effect of Cadmium on pH, total acidity and soluble phosphorus. Data in Table 2 proved that the Cd significantly affected each of pH level, total acidity and soluble phosphorus in contaminated medium compared to control, so the different increasing of Cd concentration from 5 ppm to 50 ppm, pH level and total acidity increased from 3.8, 0.16 meq/mL^{-1} both of them to 6.8, 0.75 meq/mL^{-1} compared to control 5.3, 0.23 meq/mL^{-1} respectively. Data in Table 2 concluded that there is no significant affect of Cd concentration on pH level and total acidity at all periods of incubation as compared to control pH was 5.9, 5.8, 5.8, 6 and 6.3 and total acidity was 0.46, 0.42, 0.45, 0.45 and 0.49 meq/mL^{-1} at 7, 14, 21, 28 and 30 days respectively. Results show that significant interaction between Cd concentration and incubation periods on pH and total acidity during reduced pH to 3 at 5 ppm Cd during 14 days also increase total acidity to 0.96 meq/mL^{-1} at 50 ppm Cd during 30 days we observed that pH level reduced while total acidity increased with increasing Cd concentrates compared to control in contaminated medium (Table 2). That may indicate that *B. polymyxa* has high capacity to produce organic acids accompanied by acidification of medium (Mahdi et al 2011), also Alam et al (2002) and Kumari et al (2008) reported that organic acids like citric acids, tartaric acids educed by species of *Bacillus* like *B. megterium* led to reduce pH level and increasing total acidity. Data in Table 2 shows that significant increase in soluble phosphorus from 30.35 mg/L^{-1} at control to (39 and 47) mg/L^{-1} at concentration of 5 ppm and 10 ppm respectively, but soluble phosphorus reduced at high concentration Cd (25 ppm and 50 ppm) to (20.8 mg/L^{-1} and 15 mg/L^{-1}) respectively. In Table 2 data shows that soluble phosphorus in contaminated medium is reduced significantly from 28.3 mg/L^{1} during 7 days to 17 mg/L^{-1} at 30 days. The results indicate that significant interaction between Cd concentration and periods of incubation during increase soluble phosphorus to 78 mg/L^{-1} at 10 mg/L^{-1} Cd during 21 days incubation. These results demonstrated the significant effect of Cd concentration on soluble phosphorus in contaminated pvk medium, when soluble phosphorus concentration increased at 10 ppm and reduced at 50 ppm Cd that is referred to metal toxicity leds to reduce capacity of bacteria to dissolve phosphorus at high concentration, these results are in accordance with those reported by Omer & Farag (2012) that any solubilization of phosphorus in medium was associated with secretion of reducing pH level and different organic acids which depend on pH and total acidity in incubated medium with *B. magterium* and Chen et al (2006) were observed a strong relationship between pH level and soluble phosphorus concentration indicates that organic acids production by *Bacillus* sp. strains play significant role in acidification of medium facilitate the solubilization. Data in Table 1 show that significant effect incubation periods on soluble phosphorus, that maximum increase during 7 days and minimum decrease during 30 days for Cd that explain high activity to dissolved insoluble phosphorus by bacteria at first periods incubation in accordance with Chen et al (2006).

Effect of Lead on bacterial growth. The data in Table 3 shows that growth of *B. polymyxa* increased from 18.6 x 10^{8} CFU/mL^{-1} at control treatment (Pb = 0 ppm) to 30 x 10^{9} CFU/mL^{-1} at 10 ppm Pb in pvk medium, but high concentrate of Pb (at 50 ppm) leds to reduce the growth to 11.8 x 10^{5} CFU/mL^{-1} in inoculated medium. Data presented in Table 3 shows increases of bacteria growth during different periods of incubation from 56 x 10^{8} CFU/mL^{-1} during 7 days to 3.4 x 10^{9} CFU/mL^{-1} and 2 x 10^{9} CFU/mL^{-1} during 21 and 30 days of incubation periods respectively. Suggesting the interaction between Pb concentration and incubation periods at 10 ppm during 14 days (23 x 10^{9} CFU/mL $^{-1}$) in an incubation medium.

Table 2

Effect Cd levels (ppm) and different incubation periods (days) on pH, total acidity and soluble phosphorus in contaminated medium

Cd level (ppm)	*Incubation periods*														*Average*		
	7			*14*			*21*			*28*			*30*				
	pH	*Total acidity*	*Soluble P*	*pH*	*Total acidity*	*Soluble P*	*pH*	*Total acidity*	*Soluble P*	*pH*	*Total acidity*	*Soluble P*	*pH*	*Total Acidity*	*Soluble P*	*pH*	*Total acidity*	*Soluble P*
0 Cd	5.9	0.3	30.39	5	0.25	21.35	4	0.28	20.25	5.1	0.2	20.25	6.4	0.11	30.5	5.3	0.23	30.35
5 Cd	3	0.11	63	3	0.15	32	4.9	0.2	19	4	0.19	40	4.1	0.15	41	3.8	0.16	39
10 Cd	5.6	0.5	48	6	0.45	49	6	0.44	78	6.2	0.65	49	7.1	0.7	11	3.98	0.55	47
25 Cd	6.7	0.6	22	8	0.67	25	7	0.66	27	6	0.5	21	6	0.55	9	6.5	0.59	20.8
50 Cd	8.1	0.8	8.9	7	0.6	17.8	6.9	0.7	10.6	8.8	0.69	15.2	7.8	0.96	24	6.8	0.75	15
Average	5.9	0.46	28.3	5.8	0.42	25	5.8	0.45	26.9	6	0.45	25.1	6.3	0.49	17	-	-	-
RLSD0.01	pH	Total acidity	Soluble P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cd con(c)	1.35	0.46	12.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
period (p)	0.55	0.08	10.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C×p	0.51	0.19	20.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 3

Effect of the Pb levels (ppm) and different incubation periods (days) on bacterial growth in contaminated media

Pb levels (ppm)	*Incubation periods (days)*					*Average*
	7	*14*	*21*	*28*	*30*	
0 Pb	1.2×10^7	1×10^9	15×10^7	36×10^6	8.4×10^9	18.6×10^8
5 Pb	12×10^8	15×10^8	25×10^8	72×10^7	69×10^7	13.9×10^8
10 Pb	14×10^9	23×10^9	12×10^9	82×10^7	93×10^7	10.15×10^9
25 Pb	3×10^9	14×10^8	25×10^8	32×10^8	25×10^7	2.7×10^9
50 Pb	1×10^6	28×10^5	21×10^5	1.5×10^4	1×10^4	11.8×10^5
Average	56×10^8	53×10^8	3.4×10^9	0.95×10^9	2×10^9	-

These results may be refer to higher level of resistance and detoxification of *B. polymyxa* with increased Pb concentration and its resistance is continuously till 30 days. Same observations were reported by Sabyasachi et al (2012) and Cavicchioli & Thomas (2000), that many bacteria like *Bacillus* has developed resistance to toxic heavy metal by the bacterial cell acquire a gene or genes which normally code for proteins and enzymes that perform specific functions either to protect the bacterial cell, or block or alter the incoming toxic metal or both. Malik (2004) has reported that an excellent growth capability of two bacteria strains increased with periods of incubation at low concentration (0 – 1 mM) of Pb in the first 12 hours of incubation, but at high concentration Pb (5 – 10 mM) growth was inhibited rapidly in all the periods of incubation.

The effect of Lead on pH, total acidity and solouble phosphource. Data in Table 4 shows that pH level and total acidity is affected significantly with increase of Pb concentrates from (7, 0.04 meq x mL^{-1}) compared to control (4.9, 0.68 meq x mL^{-1}) at 25 ppm Pb respectively. The result shows that pH level reduced from 7 (initial period) to 5.98, 4.98, 5.3, 6 and 5.8 during 7, 14, 21, 28 and 30 days of incubation respectively when total acidity increased from 0.39 meq x mL^{-1} during 7 days to 0.5 meq x mL^{-1} during 14 days. As it shown in Table 4, there is a significant interaction between incubation periods and Pb concentration at 25 ppm. During 14 days the pH was 3.6 and total acidity 0.89 meq x mL^{-1} at 10 ppm Pb. The results differed according to amount of heavy metal (Pb), may be referred to metal like Pb more soluble in acidic conditions than alkaline, hence toxicity problems are more sever in acidic medium, although, bacteria has high tolerance for these conditions that may explain the increase of bacteria growth (biomass) in contaminated medium according to (Zhang et al 2009).

The data indicated a significant effect of Pb concentration on soluble phosphorus in treated medium; the increase was from 30.4 mg x L^{-1} at control to 42.4 mg x L^{-1} at 10 ppm Pb, while increasing Pb concentration to 50 ppm in medium leds to decrease soluble phosphorus to 20.9 mg x L^{-1} (Table 4). There a significant increasing of soluble phosphorus appeared during 7 days up to 37.4 mg x L^{-1}, then decreased to 27, 25.6 and 27 mg X L^{-1} during 21, 28 and 30 days respectively, data presented also significant interaction effect between Pb concentration and incubation periods. There is 70 mg x L^{-1} at 10 ppm Pb during 7 days, and 13 mg x L^{-1} of dissolved phosphorus at 50 ppm Pb during 30 days of incubation. These results are in accordance with Chen et al (2006) and El-Komy (2005) which have reported that there is significant effect for incubation periods on amounts of soluble phosphorus in incubated medium with *B. magaterium* bacteria and release soluble phosphorus to medium being the first time when was indicated the importance of this nutrient for bacterial life. Significant interaction between Pb concentration and incubation periods especially at 10 ppm Pb during 7 days refers to high efficiency for bacteria to dissolve phosphorus although in contaminated conditions.

Table 4

Effect of Pb levels (ppm) and different periods (days) on pH, total acidity and soluble phosphorus in contaminated medium

Pb level (ppm)	*Incubation periods*														*Average*		
	7			*14*			*21*			*28*			*30*				
	pH	*Total acidity*	*Soluble P*	*pH*	*Total acidity*	*Soluble P*	*pH*	*Total acidity*	*Soluble P*	*pH*	*Total acidity*	*Soluble P*	*pH*	*Total acidity*	*Soluble P*	*pH*	*Total acidity*	*Soluble P*
0 Pb	5.9	0.3	30.4	5	0.25	21.35	4	0.28	20.33	5.1	0.2	20.25	6.4	0.11	30.5	7	0.04	30.4
5 Pb	6.2	0.3	31	5.4	0.36	36	5	0.36	40	6.8	0.44	20	5	0.4	33	5.7	0.37	32
10 Pb	6.4	0.45	70	5.3	0.89	44	5.2	0.75	29	5.6	0.8	37	5.1	0.7	32	5.7	0.72	42.4
25 Pb	4.7	0.55	35.5	3.6	0.65	38	5.9	0.65	34	5.1	0.7	37	5.3	0.86	27	4.9	0.68	34.3
50 Pb	6.7	0.33	20	5.6	0.45	37	6.7	0.19	19	7.6	0.23	13.9	7.2	0.19	13	6.8	0.28	20.9
Average	5.98	0.39	37.4	4.98	0.5	32.8	5.3	0.45	27	6	0.47	25.6	5.8	0.45	27	-	-	-
RLSD 0.01	pH	Total acidity	Soluble P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pb conc	0.48	0.06	6.7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Period (p)	0.16	0.03	5.05	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C×p	1.77	0.09	10.27	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Conclusions. Bacteria of *B. polymyxa* were showed varying the degrees of resistance to different concentrations of metals (Cd and Pb). It is tolerant to low concentrate of Cd and Pb after 14 days of incubation, but bacteria has no tolerance at high concentrations (50 ppm of Cd or Pb). Efficiency study has been carried out by depending on pH level and total acidity in contaminated liquid medium, the results revealed that *B. polymyxa* has high ability to dissolve phosphorus in medium at early periods of incubation, that means *B. polymyxa* has better tolerance in contaminated medium with Cd and Pb and it has good potential of accumulating within 14 days. Thus bioremediation of metals contaminated medium using *B. polymyxa* can be considered as the most effective way to remediate medium providing better technique aid to isolate the organism from the soil. Hence, it would be necessary to determined accumulated metals and the kinetic properties of biosorption, which are significant to determine the time required to reach equilibrium and to evaluate the maximum adsorption capacity of microorganisms in contaminated conditions.

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