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Range determination for resistance/tolerance and growth kinetic of indigenous bacteria isolated from lead contaminated soils near gas stations (Iran)

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ABSTRACT

Lead (Pb) is one of the most widely heavy and toxic metal in the environment. Since it is used at the petroleum production, its abundance has been developed around the world. Application of the resistant bacteria to this toxic metal could be utilized in bioremediation process. The goal of this study is to isolate and characterize the lead-resistant bacteria and evaluate their ability in lead bioremediation and evaluate their growth kinetic. Sampling was conducted from the soil around the three gas stations in Jahrom city. The amount of lead in the samples was measured by atomic absorption spectroscopy. The numbers of bacteria were counted at two medium with and without lead acetate. The isolation of bacteria was conducted by primary enrichment and then culturing on the nutrient agar with lead acetate. The bacteria were characterized by the common biochemical tests. The MIC test was utilized in order to obtain the minimum concentration lead essential for preventing bacteria growth. These bacteria were cultured in different concentrations of lead acetate on the LB broth medium in order to evaluate their growth kinetic. The logarithm average of the number of bacteria at the medium without lead was 6.605 and more than medium with lead. The maximum number of lead-resistant bacteria was 7.289 at station C, and the minimum number was 5.179 at the station A. The Bacillus sp, Pseudomonas sp, Corynebacterium sp, Staphylococcus sp and E.coli were isolated as the lead-resistant bacteria. Most of isolated bacteria from the soil of gas stations were highly capable to eliminate lead. Bacillus sp, Pseudomonas sp, Corynebacteriumsp, Staphylococcussp and E.coli eliminated 89.66% , 87.97 %, 86.64 %, 64.82 % and 60.35% of lead, respectively. The results of this study showed that Bacillus sp, Pseudomonas sp, Corynebacterium sp are highly resistant to lead and are sufficient options for lead bioremediation.

Keywords: Lead-Resistant Bacteria, Soil, Atomic spectroscopy, *Bacillussp, Pseudomonas sp*. Bioremediation.

INTRODUCTION

Heavy metals have been recognized as the deleterious contaminators which have negative effect on the microorganisms of soils[1]. The pollution of the ecosystem by heavy metals has been considered as real threat to the environment, since heavy metals cannot be naturally degraded like organic pollutants and could accumulate in different parts of food chain [2]. These metals are poisonous for organisms and have effect on their growth, morphology, and biochemical activities so that finally cause decrement of biomass and variety of them [3]. Bacteria are the most plentiful organisms which are existed on the earth. Heavy metals are generally found in bacteria which have several natural processes of anthropogenic. Genes related to tolerance mechanism have been found both on chromosomes and plasmids. There are some mechanisms in bacteria which capable them to tolerate heavy metals: one method is sending heavy metal out the cell and the other one by using heavy metals as terminal electron

acceptors in anaerobe respiration. However, the most studies have been paid on mechanism in which metallic ions sending out of cell [4].

Lead (Pb) is one of the most known harmful heavy metals among environmental contaminators. It has unexpected effects in the human body which are included of : disorder in biosynthesis of hemoglobin and anemia , increasing of blood pressure , kidney disease , abortion and baby immaturity, nervous system's disorder, brain damage , male infertility, decreasing the learning power and behavioral disorders such as remonstration in children. The primary sources of Pb-contamination come from the mining and smelting activities, combustion of leaded gasoline, land application of sewage sludge , battery disposal and Pb-bearing products . The toxicity and bioavailability of Pb are affected by soil pH, redox-potential and Pb compounds. Pb compounds in soil mainly exist in exchangeable, carbonate-bound, Fe/Mn oxid-bound, organic and residual phases [5]. Among different pollutant resources of soil, the lead particles which are exhaust from vehicles are so important. They were identified in 1920 which made from burning gasoline and became popular rapidly. During this period, a much of leaded gasoline were consumed and the automobiles became the dominant resources of lead through emitting the lead particles in the environment . Studies proved that the lead density in air, soil, and plants surrounding streets and highways has been increased; while this density is decreased by being far from streets and highways .it has also been detected that the amount of this metal is decreased in soils as depth increase [6]. Considering the poisonous of lead and its effect on human health, recognizing resistant bacteria to this metal is so important. The objectives of this study were isolation and evaluation of lead-resistance bacteria, determination of the minimum inhibitor concentration (MIC) and growth kinetic of these strains. The biological elimination rate of this heavy metal in strains with high MIC and possibility of using these resistant strains in eliminating this pollutant in soils surrounding gas-station in Jahrom city were investigated.

MATERIALS AND METHODS

In this investigation, soil samples were collected around three gas stations.

Sampling: Soil samples were collected from 0-10 cm depth of ground surface and 3 different positions (3 repetitions from each position). The samples were put in sterilized plastic dishes and kept into ice. They were sent to laboratory two hours after sampling for bacterial culture and measuring of the rate of lead.

Preparing samples and measuring rate of lead

Before measuring the lead rate, the samples were dried in oven in temperature 103°C in order to put probable moisture aside. The dried samples were then pounded in mortar and, 1 gram of each sample was mixed with a mixture of 6mm Nitric acid and chloridric acid and heated. The samples were then filtered with watman filter 42 micrometer [1]. After preparing, the rate of lead was measured in samples via atomic absorption spectrophotometer system (Varian model) followed flame atomic absorption method.

Counting bacteria

Counting of bacteria was done using total viable plate count method. dilutions of 10^{-1} to10^{-10} was prepared by physiological serum and was used for a surface plate method in Nutrient agar medium (made in Germany Merk Company) having 0.4 $gr.lit^{-1}$ lead acetate and medium without lead acetate. The plates were incubated for 48 h in 30°c. The number of bacteria was then counted in culture mediums with and without lead [7] .

Isolation and identification of bacteria

Isolation of resistant bacteria to lead was conducted via primary enriching, and the culture on solid medium. In primary enriching, 5 gr of each sample was added to 90 ml luria-Bertani broth medium (made in Germany Merck Company) having $0.4gr.lit^{-1}$ lead acetate , and were incubated for 48 h in 30 °c . Then, 0.1 ml of medium having bacteria was cultured in LB agar medium through surface plate method and incubated. Then, a pure culture was provided from formed colonies [4]. Identification of pure bacteria was done using Gram's reaction and bio-chemical tests (catalase activity, oxidase activity, acid production from glucose , oxidation fermentation reaction (OF) etc.) according to book "Bergey's Manual of systematic Bacteriology [3].

MIC test

This test was done in order to obtain a density of lead which prevents the growth of bacteria. a bacterial suspension equal to Macfarland standard was provided and 0.1 ml of it was inoculated into LB broth medium (made in Germany Merek Company) including different concentrations of metal (100 , 50 , 25 , 12.5 , 6.25 , 3.12and 1.56 $mmol.lit^{-1}$) . After incubating for 24 h in 30°C, the last concentration of metal which had inhibited the bacterial growth was considered as the minimum inhibitor concentration or MIC [8].

Determination the effect of lead on bacterial growth
In order to determine the growth speed of isolated resistant bacteria the growth kinetic of these bacteria was investigated in presence of lead a concentration equal to Macfarlanf Standard was prepared from bacteria growth in LB broth medium and 1ml was added to related mediums in order to determine kinetic of growth . The growth curve of these bacteria was investigated in three conditions:
First : bacteria culture in LB broth medium acetate (control) , Second: bacteria culture in LB broth medium including different concentrations of lead acetate (0.2 , 0.4 and 0.7$gr.lit^{-1}$) and third : adding acetate lead from half phase of bacteria logarithm growth (the time that optical density of bacteria reaches to *0.5* in wavelength 600 nm). Bacterial growth was measured in terms of optical density at 600 nm for a day at 12 hour intervals using the optical absorption spectrophotometer system . The growth curves of bacteria was then drawn [9].

Preparing bacterial samples
Before measuring the eliminating rate of lead by resistant bacteria, bacterial samples were prepared. Bacteria were cultured in LB broth medium including 0.4 gr lit^{-1} acetate lead and were incubated for 24 h in 30°c, then mediums having bacteria was centrifuge for 15 minutes in 6000 rpm and the cellular mass was completely separated from the supernatant liquid . The obtained cellular mass was washed with distilled water and located for 24 h in 105 °c. The obtained dry mass was digested by nitric acid 0.5 ml and was located for 1 hour in 100 °c . The volume was then reached to 5 ml with distilled water . The supernatant liquid was also filtered with watman filter 42 micrometers [10].

Measuring the rate of lead elimination
The rate of lead elimination was measured via atomic absorption spectrophotometer system following flame atomic absorption method.

Statistical analysis
Data analysis was performed using ANOVA and Duncan tests. All statistical analysis was done using SPSS software with significance based on 0.05 in most of the cases.

RESULTS

Lead rate in samples: There was a significant variation ($p<0.05$) between amounts of lead in different stations .The highest rate of lead (0.400 ppm) was obtained in soil samples of gas station No.3 (station C) while the lowest rate of lead (0.127 ppm) was observed in soil samples of gas station No. 1 (station A) .

Counting bacteria: The logarithmic average of bacteria number in the culture medium including lead (2.844) was lower than logarithmic average of bacteria number in control culture medium (6.605). There was a significant variation ($p<0.05$) between the logarithmic average of bacteria number in medium with lead and without lead. Comparing stations in viewpoint of logarithmic average of bacteria number resistant to lead; the highest number of bacteria resistant to lead (7.289) was observed in gas station No.3 (station C) and the lowest number of bacteria resistant to lead (5.179) was obtained in gas station No.1 (station A). The logarithmic average of resistant bacteria number had significant variation ($p<0.01$) in different stations.

Isolation and identification of bacteria: The percentage of gram positive bacteria was higher than gram negative bacteria. They showed significant variation ($p<0.05$) so that 67% of the isolated resistant bacteria were gram positive and 33% of the bacteria were gram negative. The highest percentage was belonged to *Bacillus sp* (100%) and the lowest was belonged to *Escherchia coli* (33%) .

MIC test: The most resistant bacteria were *Bacillus sp, corynebacteriumsp, Pseudomonas sp* and having 12.5 $mmol.lit^{-1}$MIC from lead acetate while two bacteria *E.coil* and *staphylococcus sp* having lower MIC and 6.25 $mmol.lit^{-1}$from lead acetate.

Determination of effect of lead on bacterial growth: Through kinetic investigation of resistant bacteria's growth during 12 hours, the growth speed of bacteria was evaluated in the presence of different concentrations of lead acetate. The growth curve of these bacteria showed that *Bacillus sp* and *corynebacterium sp* bacteria had their maximum growth in concentration 0.7 $gr.li^{-1}$ lead acetate in comparison to 0.2 and 0.4 $gr.lit^{-1}$ (Figures 1 and 2), *pseudomonas sp* with concentration 0.4 $gr.lit^{-1}$ showed a better growth proportionate to other two concentrations (Figure 3), but *staphylococcus sp* and *E.coli* showed lower growth in the presence of lead acetate 0.4 $gr.lit^{-1}$ than mentioned bacteria (Figure 4 and 5). Adding lead in the half of logarithmic phase growth caused a short pause in the growth of all bacteria however the bacteria adapted themselves to the new conditions very fast .

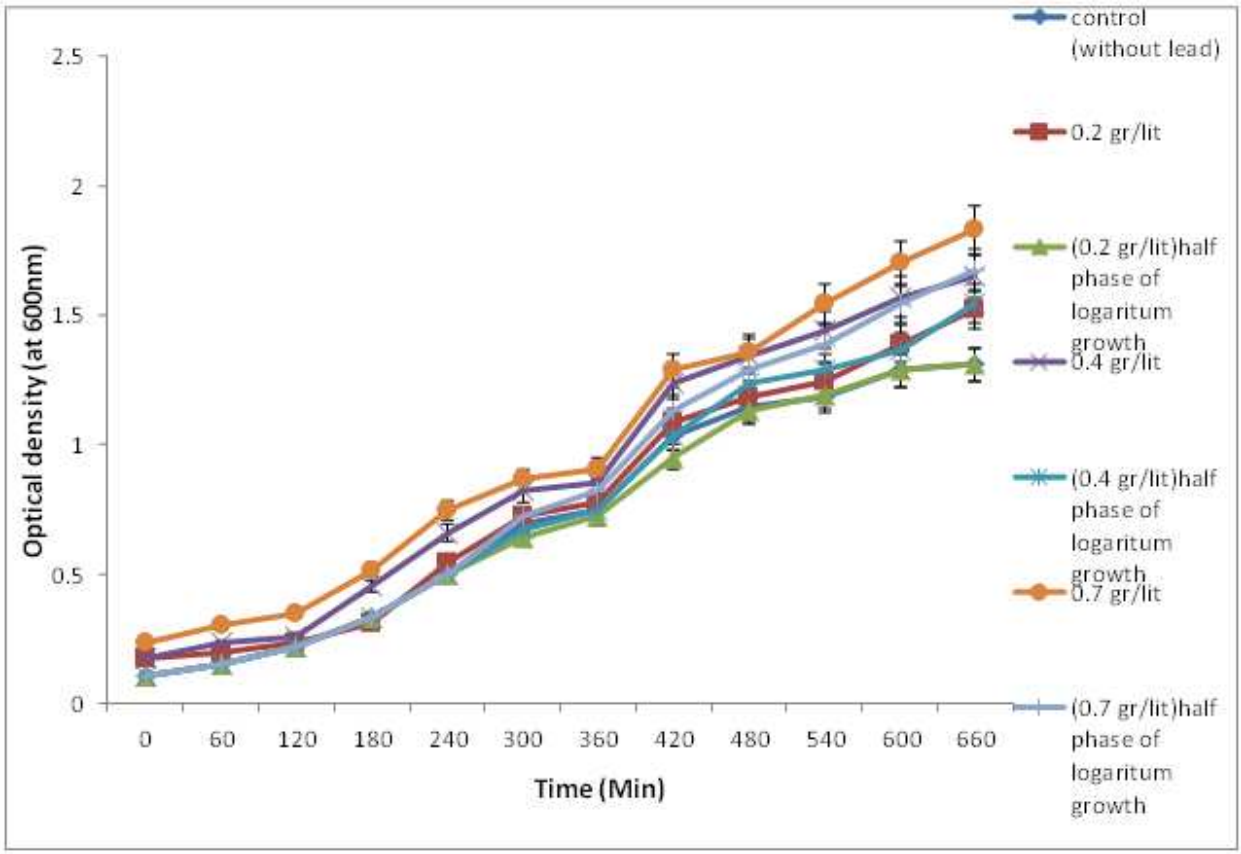

Fig. 1 : Growth curves of Bacillus sp

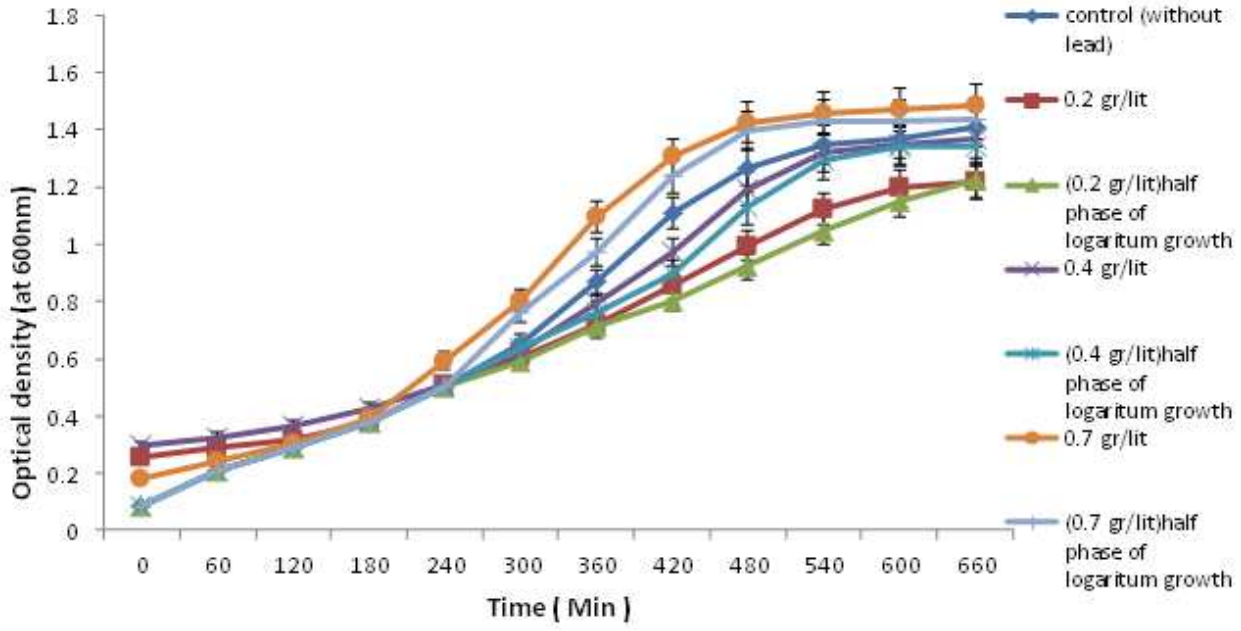

Fig. 2 : Growth curves of *Corynebacteriumsp*

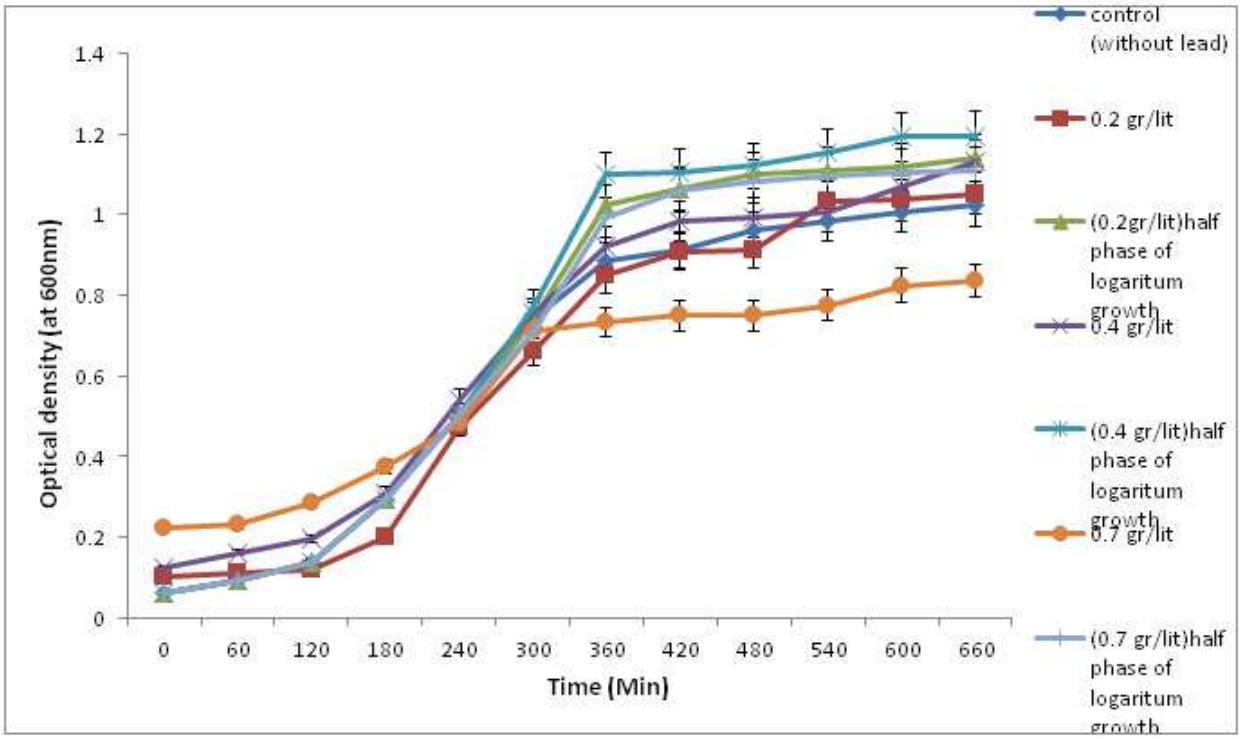

Fig. 3 : Growth curves of *Pseudomonas sp*

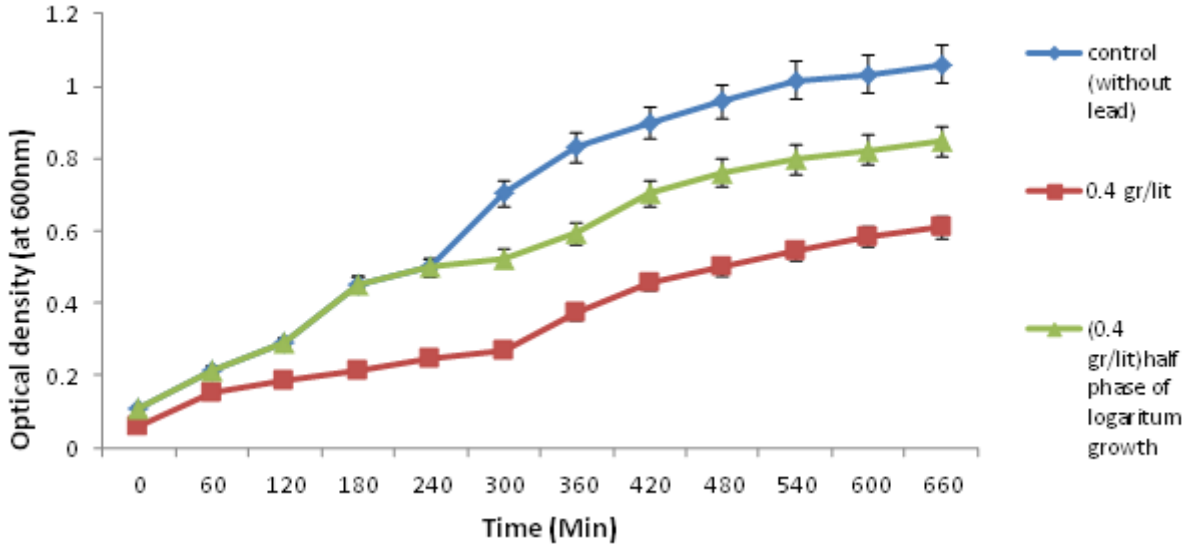

Fig.4 : Growth curves of *Staphylococcus sp*

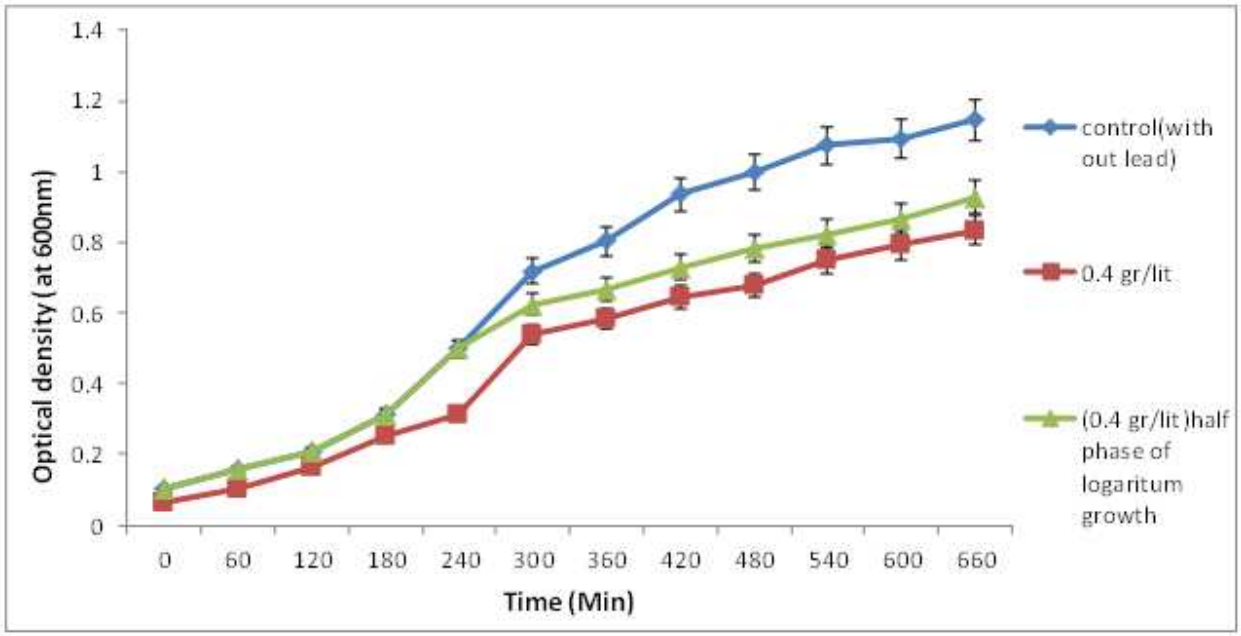

Fig.5 : Growth curves of *E.coli*

Measuring the elimination rate of lead by bacteria: The result obtained from atomic absorption spectrophotometer analysis showed that the highest percentage of eliminating metal were belonged to bacteria such as *Bacillus sp* (89.66%), *Pseudomonas sp* (87.97%) and *Corynebacteriumsp* (86.64%) while the lowest percentage of eliminating this metal were belonged to *Staphylococcus sp* (64.82%) and *E.coli* (60.35%) respectively.

DISCUSSION

In the present research, the number of resistant bacteria to lead was lower than the number of bacteria in control medium. It is in agree with the Smejkalova et al. researches in 2003, which reported that the number of bacteria in the culture medium of lead is less than the number of bacteria in the control culture medium [11] . The existence of lead in the culture medium causes the death of bacteria. For this reason, the number of bacteria in the medium having lead is lower than the control culture medium. In the study by Zhang et al. (2007), it was suggested that the tolerate strains to lead are much more in soils having this metal than soils without lead [12]. The obtained results from current study confirmed the hypothesis that the increase of environment pollution with lead is related with the increase of resistant bacteria number to lead . However, other environmental factors could affect abundance and availability of many metals, and change their effective concentrations in different places. The microorganisms for being alive should control their environment and the effect of metals in their cytoplasm. The bacteria could adsorb adequate amounts of necessary metals, and avoid their accumulation. This reaction includes absorption systems, genes that omit extra metals and proteins which absorb the metal [13]. In the current investigation, the resistant bacteria to lead were isolated through enriching method in the presence of 0.4 $gr.lit^{-1}$ acetate lead. In the subsequent steps, these bacteria showed a better growth, higher resistant and also higher ability in eliminating lead from culture medium. Affan et al. (2009) isolated resistant bacteria using enriching bacteria in the LB borth medium having lead. Those bacteria had the ability to resist against high concentrations of lead [4] . In the present research, the gram positive and gram negative bacteria were recognized as resistant bacteria to lead . The abundance percentage of gram positive bacteria was higher than percentage of gram negative bacteria. Ashraf et al (2007) reported the resistance to lead in *Bacillus sp*, *Pseudomonas sp*, *Corynebacterium sp* and *Staphylococcus sp* bacteria [2]. In the present research also, all the mentioned bacterial genus were also recognized as the resistant bacteria against lead. Wyszkowska et al (2008) isolated and recognized the *Pseudomonas sp* and *Arthrobacter sp* resistant bacteria to lead [14] . In the research which was done in 2005 by Lugauskas and his colleagues, the *Bacillus sp* bacteria were recognized as the most abundant gram positive bacteria in the soil [1] . In the current research, not only *Bacillus sp* was isolated from all the stations, but also it was recognized as one of the most resistant bacteria against lead . Several bacterial species use intra-and extracellular binding of Pb^{2+} to avoid its toxicity. *Staphylococcus aureus* and *Bacillus megaterium* decrease the concentration of free lead ions by precipitating lead as a phosphate salt . *Pseudomonas marginalis* avoids lead toxicity by precipitating it as an extracellular polymer [15,16,17]. However, the molecular mechanisms responsible for the formation of lead precipitates in these strains are not known yet.

The first step for recognizing the ability of bacteria which could clean the environment is to recognize resistant and tolerant bacteria to high concentrations of heavy metals. This could help us to select prior strains which eliminate these poisonous compounds. Amoozgar et al. (2007) used MIC test for determining the minimum inhibitor concentration of this metal. They used different concentrations of 1,2,3,4,5,6,7,8,9,10,15,20 and 30 mmol of lead, to select the most resistant bacteria [8]. In the current research , the MIC test with different concentrations of laed acetate (100 , 50 , 25 , 12.5 , 6.25 , 3.12 ,1.56 $mmol.lit^{-1}$) was also used to isolate resistant bacteria . The most resistant bacteria had MIC 12.5 $mmol.lit^{-1}$ from lead acetate.

In the present investigation, *Bacillus sp*, *Pseudomonas sp* and *Corynebacterium sp* bacteria had high resistance. Resistant bacteria to lead could survive in polluted regions with this heavy metal using a transporter protein and phosphatas. Rathnayak et al. (2010) investigated the effect of different concentrations of lead on the growth of *Bacillus thuringeinsis* and *Paenibacillus* bacteria. These showed a better growth in higher concentrations of lead than in lower concentrations [3]. In the current investigation, the resistant bacterium *Bacillus sp* showed a better growth in higher concentrations of lead acetate. The growth of this bacterium increases by adding lead to the culture medium from the half of logarithmic growth phase. However this increase starts suddenly after a short pause. This indicates the ability of this bacterium for eliminating the lead existed in the culture medium. It seems that continuous contact with lead in the medium and using enriching method has adapted this bacterium with stressful conditions caused by the poisonous material. On the other words, it has changed it to the metal-lover bacterium; in the way that, the existence of lead causes this bacterium's growth to be motivated [12]. The results obtained from present research conforms the findings from Affan et al. which evaluated the growth curve of metal resistant bacteria [4]. Xie et al. (2010) investigated the effect of different concentrations of lead on the growth of resistant strains of bacteria. They showed that poisonous concentrations of lead have a little effect on the growth curve of resistant bacteria to lead while this effect is much more on the sensitive bacteria to lead [18]. The results obtained from current research also showed that the bacteria which have not high growth in the presence of this metal are not resistant to lead. They are only tolerating poisonous effects of lead. Lugauskas et al. (2005) investigated the elimination percentage of lead by isolated resistant bacteria. He shows the direct relation between the increase of lead's pollution and the increase of elimination percentage of lead by the resistant bacteria [1] . Smejkalova et al. (2003) and Ahmad et al (2005) , measured the rate of lead elimination by the resistant bacteria . They showed that resistant bacteria had a high ability for eliminating this poisonous metal from the culture medium [11,7]. Amoozgar et al. (2007) reported that the rate of lead elimination by the isolated resistant strain equal to 90.71% [8].

CONCLUSION

The result of this study showed that the isolated bacteria from polluted environments with lead have high potential for eliminating this heavy metal. This investigation also showed that bacteria such as *Bacillus sp*, *Pseudomonas sp* ,*Corynebacteriumsp* , *Staphylococcus sp* and *E.coli* are capable of eliminating lead. As a result, they could be used for eliminating this heavy metal from the environment by providing suitable conditions and bed for their growth.

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REFERENCES

[1]. A. Lugaskas, L. Levinskaite, D. Peeiulyte, J. Repeekiene, A. Motuzas, R. Vaisvalavieius, I. Prosyeevas, *Ekologija*., **2005**., 1, 61-69.
[2]. R. Ashraf., T. Adam ali, *Pak. J. Bot*., **2007**., 39(2),629-36.
[3]. V.N. Rathnayak, M. Megharaj, N. Bolan, R. Naidu, *World. Acad. Sci. Eng. Tech.*, **2009**., 53, 1185-1189.
[4]. Q. Affan, E. Shoeb, U. Badar, J. Akhtar, *J. Basic. Appl. Sci*, **2009**., 5(2):55-60**.**
[5]. D.L. Huang, G.M. Zeng, X.Y. Jiang, C.L. Feng, H.Y. Yu, G.H. Huang, H.L. Liu, *J. Hazard. Mater*, **2006**., 134(1-3), 268-276.
[6]. K.E. Juracek, A.C. Ziegler, *J. Environ. Qual*, **2006**., 35, 2092-2102.
[7]. I. Ahmad, S. Hayat, A. Ahmad, A. Inam, *J. Appl. Sci. Environ*, **2005**., 9(1), 115-121.
[8] M.A. Amozegar, N.S. Ghazanfari, *J. Environ. Sci. Technol*, **2009**., 11(4), 479-491.
[9]. D.H. Nies, *Appl. Microbiol. Biotechnol*, **1999**., 51(6), 730-750.
[10]. R. Lovely, J.D. Coates, *Curr. Opin. Biotechnol*, **1997**., 8(3), 285-289.
[11]. M. Smejkalova, O. Mikanova, L. Boruvka, *Plant. Soil. Environ*, **2003**., 49(7), 321-326.
[12]. H.B. Zhang, M.X. Yang, W. Shi, Y. Zheng, T. Sha, Z.W. Zhao, *Microb. Ecol*, **2007**., 54(4), 705-712.
[13]. A. Hynninen, T. Touze, L. Pitkanen, D. Mengin-Lecreulx, M. Virta, *Mol. Microbiol*, **2009**., 74(2), 384-394.
[14]. J. Wyszkowska, J. Kucharski, A. Borowik, E. Boros, *J. Elementol*, **2008**., 13(3), 443-453.

[15]. H.S. Levinson, I. Mahler, P. Blackwelder, T. Hood, *FEMS. Microbiol. Lett*, **1996**., 145(3), 421-5.
[16]. H.S. Levinson, I. Mahler, *FEMS. Microbiol. Lett*, **1998**., 161(1), 135-8.
[17]. T.M. Roane, *Microb. Ecol*, **1999**.,37(3), 218-24.
[18]. X. Xie, J. Fu, H. Wang, J. Liu, *Afr. J. Biotechnol*, **2010**., 9(26), 4056-4066 .