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STUDYING THE EFFICIENCY OF BIOREMEDIATION IN REMOVING LEAD FROM CONTAMINATED SOILS USING THE PSEUDOMONAS OLEOVORANS BACTERIUM

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Abstract

The current study included a laboratory biological experiment, which was conducted in the laboratories of the Department of Life Sciences–College of Education for Pure Sciences / University of Anbar from November to January 2021.The purpose of this experiment was isolating and purifying bacteria and improving the environmental conditions for the growth and tolerance of bacteria to the element of lead added to the culture medium. A total of (10) bacterial isolates were obtained and isolated from soil contaminated with heavy elements. These were subjected to primary and secondary screening and one of them was selected as efficient and potential isolation for concentration of 750 mg/L of lead element and was diagnosed using the Vitek compact2 system.

Results for improved environmental conditions for the growth of lead-induced elected bacterial isolation *Pseudomonas oleovorans* (250,500,750, 1,000 mg/L) showed that the best bacterial growth density was at pH 6 and using 3 ml/100 ml of the agricultural medium which had a vaccine size (25 × 105 cells/mL of bacterial cells). Best incubation time was obtained at 96 hours and a temperature of 30 °C was the best conditions for elected bacterial isolation, while the highest concentration was at 750 mg/l.

Introduction

Research on the pollution of soil, vegetation, water, and food with heavy metals (Heavy metals) has received wide international attention due to its great danger. In recent years, pollution of these metals has increased, which have become a threat to human health and life in general due to their high toxicity. The heavy metals we are talking about are not biodegradable. They may accumulate and raise their levels in the soil to form a serious problem that requires treatment and avoidance of their effects on food chains linked to human life, which may be a cause of chronic or severe diseases (Mustafa,2015).

Interests tended to adopt biological treatment through the use of plants and microorganisms, which are called (Bioremediation), which has gained great attention recently for several reasons, the most important of which is its effectiveness, low cost and environmental friendliness, as certain types of plants can accumulate these heavy metals at different concentrations. (Tayang & Songachan, 2021)

One of the minerals that efforts have been focused on addressing its harmful effects is lead. It is found in nature and its effect on the environment is an important subject that has attracted the interest of environmental researchers around the world because it has entered into many industries,

the most important of which are batteries, pesticides, dyes, and lead smelting furnaces, to name but a few. It is, at the same time, one of the most toxic metals, and has a negative effect on most living organisms, including humans.

Methods

Soil Sample collection

Ten samples of soil contaminated with heavy elements were collected from different areas of the city of Ramadi (Iraq, west of Baghdad) for the purpose of isolating potential bacteria for heavy elements. One kilogram of soil was taken at a depth of 25 cm after removing two cm from the soil surface.

Bacteria Isolation

Primary Screening

The bacteria were isolated from soil samples collected from different regions of Ramadi city by taking1 g of soil and a series of decimal dilutions were made on it.Then, the NA-nutrient agar medium was prepared and sterilized using an autoclave.The method of pouring plates was used, as 1 ml was taken from the fourth and fifth dilutions and placed in sterile Petri dishes. Then, the sterile nutrient agar medium was added and after this medium got hardened, it was incubated in the incubator at a temperature of 30 ° C for 24 hours.

The growing colonies were examined on the medium with the naked eye and bacterial colonies were observed, after which the isolates were re-purified by sub-culturing and transferring the developing bacterial colony. This in turn is done through planning method on N.A medium and incubating the dishes at 30 ° C for 24 hours to obtain pure single colonies.

Secondary Screening

After obtaining the pure isolates, the efficiency of the bacteria to tolerate lead was tested by preparing a concentrate of lead nitrate.The isolates added from the fourth and fifth dilutions were re-cultivated on N. A. medium by the planning method, then the element of lead was added in the form of lead nitrate at concentrations of 250, 500, 750 and 1000 mg/L and the pH was set to 7 as well.

Preparation of the liquid inoculum

1000 ml of liquid nutrient medium N.B was prepared and sterilized with a temperature of 121 ° C and pressure (15 pounds / ang^2) for 15 minutes, then left to cool and inoculated with selected bacterial isolate and incubated the medium in the incubator at 30 ° C for 24 hours.To calculate the number of bacteria per milliliter from the agricultural medium, a nutrient medium was prepared from N.A., sterilized in the autoclave, poured into the plates until hardened, and 1 mL of the vaccine was administered by Sprite, and the number of developing colonies was calculated with the Colony Counter according to the following equation:

Number of cells /ml = number of colonies reverse dilution.

Application of optimal conditions for the growth of lead-tolerant bacteria

The media were inoculated with the selected bacterial isolate and incubated in an incubator at 30°C for a period of time.24 hours using different parameters, including pH, quantity of inoculum, incubation time, shaking speed, and temperature stabilization, as follows:

pHpH

pH of the growth medium was adjusted on each of the following numbers (8, 7, 6, 5, 4) to determine the optimal pH for growth on NB medium.

Volume of inoculum

Different volumes of the selected inoculum were used to inoculate the liquid medium to find out the effect of the volume of the added inoculum in removing the lead element. The volume of the inoculum used was (1, 2, 3, 4, 5) ml/100 ml medium which had a bacterial density of 25×510 cells/100 ml medium.

Incubation time

The selected isolates were tested with the application of the previous optimal conditions with different incubation periods (1, 2, 3, 4, 5) days to obtain bestincubation time to get rid of the largest amount of lead.

Applying optimal conditions to selected bacteria to remove lead

100 ml of the nutrient mediumNB was prepared and the best growth PH was set and sterilized by autoclave at (15 pounds/inch^2) for 15 minutes, then inoculated with the optimum inoculum size for growth and incubated with the optimal incubation time for growth at a temperature of (30) °C and at a growth density of 25×510 cells / 100 ml medium of 25 x ^{5}10 cells / 100 ml medium.

Estimation of Optical Density (OD)

Taking 2 ml of N.B culture medium in sterile tubes and recording optical density and chromatography values at a wavelength of 600 nm using a spectrophotometer. This process was performed after each of the optimal conditions applied to the culture medium.

Identification

Bacterial colonies obtained through laboratory culture results of study samples were diagnosed using the Vitek compact2 System

Results and Discussion

Isolation

Primary Screening

Several local bacterial isolates were isolated from different soils contaminated with lead collected from different areas of the city of Ramadi, as the number of isolates isolated from the soil (10 isolates) was given symbols (A, B, C, D, E, F, G, H, I, J) respectively and planted on the NA medium after a series of decimal dilutions and incubated at a temperature of 30 °C for an incubation period of 24 hours and a vaccine volume of 1 ml (CFU density 25×510) and at pH 7. It was observed that the bacterial colonies grew, and then the isolates were replanted on the same subculture medium to obtainpure single colonies.

The results of the initial screening showed that lead-tolerant bacterial isolates were obtained from different isolation areas. The isolates varied in their tolerance to the heavy element based on the growth intensity in different concentrations of lead.

After transplantation and incubation of bacterial isolates and with an incubation period of 24 hours, the colonies showed a difference in their tolerance to the heavy element (lead). This may be due to the nature of the species in their tolerance to heavy elements.

In this area, (Al-shamary & Taha, 2017) managed to obtain two isolates of bacteria (Bacillus subtilis) from soil contaminated with lead element isolated from the soil of the industrial district. On the other hand, (Shartooh et al., 2021) managed to isolate bacteria from soil contaminated with heavy elements.The removal efficiency of heavy elements from the soil was 98.0 ± 7.8).

Secondary Screening

4-1-2-1 Testing the efficiency of the selected isolates to tolerate lead concentrations

After conducting a series of purification processes for the isolates and obtaining the pure single bacterial colonies, the bacterial isolates were re-cultivated on the mediumN.A containing concentrations of lead (1000, 750, 500, 250) mg/L, for each of the fourth and fifth dilutions respectively. The dishes were incubated for 24 hours at a temperature of (30 °C) at a pH of 7 and an inoculum volume (1 ml) and observing the growth of the colonies to choose the most efficient isolates to bear the lead element by observing the growth density on the culture medium. The growth intensity of the isolates varied according to the different types of isolates.

The most efficient isolation was re-cultured with optimal growth conditions that included the best (pH, vaccine size, incubation time, ventilation) to improve the environmental conditions for the growth of selected bacteria and thus obtaining the highest percentage of lead removal from the soil.

Many studies have indicated the ability of many negative and positive bacterial species of Cram dye to remove heavy metals from soil contaminated with heavy elements in different ways and mechanisms and with different removal ratios.

The Lipopoly saccharide layer plays a role in the metal binding process in the outer membrane of the Cram-negative bacteria. As regards the Cram-positive bacteria, the Peptidoclic layer with Teichuronic and Teichoic acids play the main role in the metal binding process (Deepti *et al*., 2012).

Orji*et al*., (2021) studied Pseudomonas sp. carry heavy elements, including lead, and the results of the study showed that these bacteria have the ability to remove the lead element by 73.33%.

Identification of lead-tolerant isolate

After obtaining the most efficient bacterial isolate, which tolerated the highest concentration of lead added to the culture medium, the isolation was diagnosed using the VITEK2 Compact device.It was confirmed that the isolation was *Pseudomonas oleovoranes.*

Effect of Environmental Factors on Lead-tolerant *Pseudomonas Oleovorans*

pH

Table (1) shows the effect of the pH numbers used in this study on the growth density of *P. Oleovorans*bacteria. the results of the optical density (OD) showed that the highest growth density of bacteria was at pH 6. This is higher than the rest of the other pH numbers after an incubation period of 24 hours, as it had the highest growth in terms of optical density 1.346, followed by pH 5, where the growth density was 1.121.There was a decrease in growth intensity at other pH numbers, as the density of growth at pH 4, 7 and 8 (0.985, 0.962, 0.584) respectively.

The current study agreed with the study of Menhas*et al.*, (2021) on the Microbase-EDTA approach in the plant treatment of the contaminated soil with the use of maize a mays) and in the sakazakii cronobacter bacteria which confirmed that the highest growth was at pH 6. 47 The growth density is 1.150.

However, this current finding differs from previous studies conducted by Li et al.(2016) onthe biological treatment of lead-contaminated soil using *Rhodobacter spiroids*, where the results confirmed that the highest biological density of bacteria was obtained at pH 7. This corresponds to a study byKalita & Joshi (2017) on the biological treatment of lead by exogenous polysaccharides producing metal-loving bacteria isolated from extreme habitats. It proved that the best molecular Pseudomonas sp was at pH 7.

The results of the statistical analysis also showed that there is no significant difference for pH 6 for the rest of the pH and below the level of significance 0.05P< despite the increase in growth intensity at some pH figures.

Table (1): Effect of pH on bacterial growth density of lead-tolerant *Pseudomonas oleovorans* at 30°C, 24-hour incubation period and 1ml vaccine volume of 1 ml (bacterial density CFU 25×10^5cell /100 ml medium)

pH	optical density (O.D)
4	**0.985**
5	**1,121**
6	**1,346**
7	**0.962**
8	**0.584**
	LSD P<0.05=0.1812

Inoculate size effect

The liquid media were inoculated after setting the optimum pH = 6 ,pH with different volumes of bacterial inoculum (1, 2, 3, 4,5)ml and incubated for 24 hours.Then, the growth density was measured, and it was highest at a vaccine volume of 3 ml reaching1.843. This was followed by the inoculum size of 4 ml, where the growth density was 1.276, while the other inoculum volumes showed a decrease in the growth density as shown in Table (2).

The low intensity of growth in other sizes can be attributed to the specific environmental conditions of growth such as the pH number and the length of incubation that acts as a inhibitor to the growth of bacteria, or the lack of high intensity of the growth of bacteria with the use of larger vaccine volume can discourage the growth of bacteria by the same type i.e. competing between the two types on the source of food when the food source is low or drained from the center by rapidly growing bacteria so that the low-growth colonies do not reach a noticeable or discoverable volume (Casida, 1968).

The results of the statistical analysis showed that there was a significant difference in the size of the vaccine (3 ml) compared to the rest of inoculum volumes and below the significancelevel of ($P<0.05$).

Table 2: the effect of vaccine volume on the growth intensity of *P. oleovorans* at 30 °C, 24-hour lap length and 6 hydrogen number (bacterial density CFU 25×10^5cell /100 ml medium)

inoculum size	optical density (O.D)
1 ml	0.753
2 ml	0.927
3 ml	**1,843**
4 ml	1,276
5 ml	0.937
LSD P<0.05=0.245	

Effect of incubation time on bacterial growth density

Table 3 shows the effect of incubation time on the intensity of the bacterial growth of the bacterium*P.oleovorans* as the pH set on 6 and the quantity of a 3 ml vaccine and the use of different lap extended (24,48 72,96,120) hour was the highest density of growth at 96 hours of incubator reaching 1. 767 with optical density function. This was followed by the incubation time of 120hours at 1.337 and it was observed that there was a reduction in density for the duration of 24,48,72 hours.

The low intensity of growth in the 5-day incubation period can be attributed to the competition for the medium and the increase of toxins that will change the nature of the medium pH which affects the efficiency of bacteria. The results showed a significant difference between the extended incubation time in the study and at a significance level ($P<0.05$).

Table (3): the effect of the incubation time on the intensity of the growth of *P. oleovorans* at 30 °C and at the pH 6 vaccine volume 3 ml (bacterial density CFU 25×105cell /100 ml medium).

Incubation time (hour)	growth intensity
24	0.321
48	0.842
72	1,214
96	**.7671**
120	1,337
LSD P<0.05=0.138	

Applying optimal conditions for ventilation

The optimal conditions for growth were applied from pH, vaccine size andincubation time of the bacterial isolation, once without ventilation and another by using ventilation using the vibrating incubator with a speed of 150 cycles. /Minute. To compare and see the effect of the ventilation factor on the intensity of the bacterial growth (Table 4). The results showed that the intensity of the bacterial growth with the use of the shaking speed was better than not using it. The growth density was 1,997 compared to the non-use of the shake which recorded a growth density of 1.

Table (4): the effect of ventilation factor with the application of optimal conditionson the bacterial growth rate of *P. oleophorans*at a temperature of 30 °C and at a pH of 6, the inoculum volume was 3 ml (bacterial density 105×25 CFUcell/100 ml medium)

Applying optimal conditions for growthwithout shaking	**optical density**
pH6	1,911
Vaccine volume of 3 ml	
Lap duration96 hour	
Apply optimal conditions with shaking	1,997
pH6	
The vaccine volume is 3 ml	
Incubation time of 96 hours	

Percentage of lead removal in the soil

Through tests on the soil-contaminated soil, the results showed (table 5) that the concentration rate of the total lead element in the control sample was 566 mg/L and the remaining lead element was 325.1mg/L whilethe percentage of the removal was 42. 56% in the control sample.The treatment S (sterile soil with vaccine and concentration of bullets 750 mg/L) the total lead element concentration rate was 1316 mg/L and the remaining lead element concentration rate was 756. 53 mg/L and percentage of removals 42.53%.

The overall lead factor concentration in the NS sample (natural soil with lead element concentration of 750 mg/L) was 1316mg/L and the remaining lead element concentration rate 632.13 mg/L was 51. 96% and treatment (SP Sterile soil sterile with lead element 750mg/L) concentration rate of total lead element was 1316 mg/L.The remaining lead concentration rate 788.53 mg/L and the rate of displacement was 40.08%.

The results of the statistical analysis showed that there were significant differences between the rates of residual lead concentrations in the soil and at the significance level($P<0.05$) for all treatments.

Table (5): the total and residual lead concentration rate and the percentage of removal in the contaminated soil.

Removal percentage(%)	Average total of lead concentration (mg/L)	Residual Lead Concentration (mg/L)	Type of treatments
42.56	566	325.1	C control sample
42.53	1316	756.53	S Sterile soil with inoculum and lead concentration of 750 mg/l
N.S Normal soil with a lead concentration of 750 mg/l	1316	632.13	51.96
40.08	1316	788.53	SP Sterile soil with a lead concentration of 750 mg/L
LSD P<0.05=59.124			

Defining surface functions

Infrared spectroscopy analysis (FT-IR) was used to determine the functions of the chemical surface.

Infrared spectroscopy(FT- IR)

To determine the surface functions, Infrared spectroscopy Analysis (FT-IR) was used within the range (cm-1 4000-400) to determine effective functional aggregates of P. The sample control *oleovorans* and the bacterial sampling of the lead element after the shake has emerged as the shake packets of the described ingredient appear in Figures (1) & (2).

Figure (1): Spectrogram of the FT-IR control sample control for the bacteria *P. oleovorans*

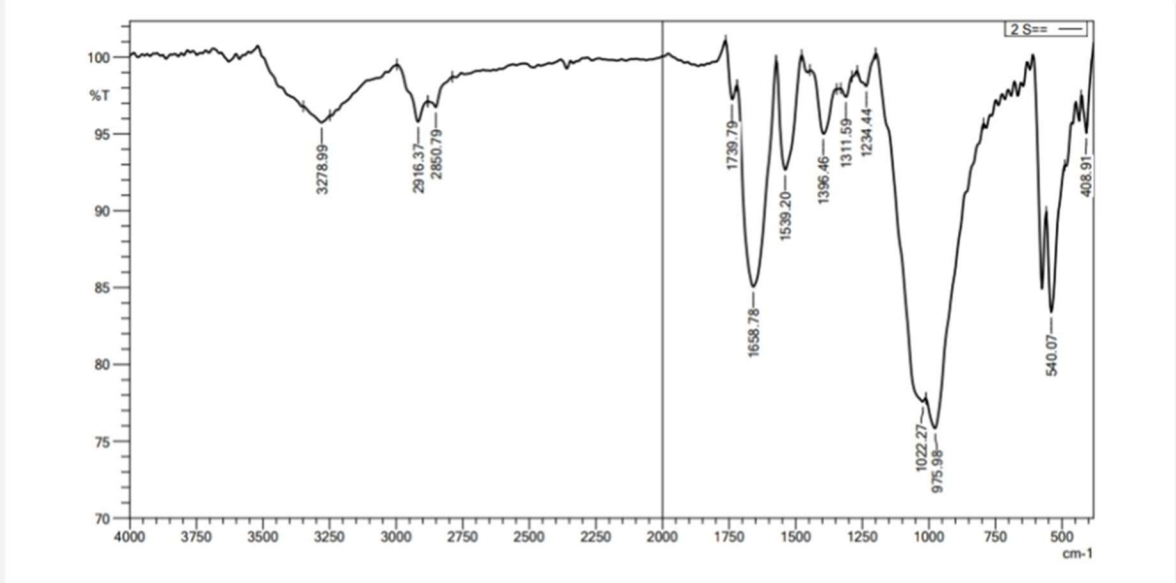

Figure 2 Absorbance spectrogramFT-IRfor a control sample for bacteria*P. oleophorans* after adding lead

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