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Selective Isolation of Heavy Metals Resistant Bacteria From Waste Water

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ABSTRACT

The current study was conducted to isolate bacteria resistant to heavy metals lead, chromium and cadmium from waste water. The concentration of lead, chromium and cadmium was measured in the waste water samples collected from different sites in Baghdad city using a flame atomic spectrometry. The results showed a significant increase in metals concentration in some sites compared to the standard determinants, as well as a significant variation between the concentration of the three metals, and nil values were recorded in some sites.

The primary isolation of lead, chromium and cadmium tolerant bacteria from five sites which recorded the highest concentration of these metals showed 25 bacterial isolates distributed between 9 bacterial isolates tolerating 300 mg/l lead, 9 isolates tolerant to 100 mg/l chromium and 7 isolates tolerant to 100 mg/l of cadmium. Well diffusion method was used to choose resistant bacterial isolates, and the results showed a difference in the resistance of bacterial isolates to metals, and the bacterial isolates Pb1, Pb4, Cr8, Cr9, Cd5 were chosen which recorded resistance to high concentrations of heavy metals and more than one metal at the same time. The maximum tolerance concentration of the bacterial isolates was determined using liquid medium, and the bacterial isolate Pb1 recorded a maximum tolerance concentration of 1300, 700, 350 mg/l for lead, chromium and cadmium respectively, while the isolate Pb4 recorded the maximum tolerance concentration of 2300 mg/l for lead, and 700 mg/l for chromium, 300 mg/l for cadmium. The isolate Cr8 showed a maximum tolerance concentration of 700, 650, and 450 mg/l for lead, chromium and cadmium respectively, while the bacterial isolate Cr9 showed a maximum tolerance concentration of 700 mg/l for both lead and chromium and 500 mg/l for cadmium, while the bacterial isolate Cd5 recorded the highest maximum cadmium tolerance 650 mg/l among bacterial isolates, with a maximum tolerance concentration 800 mg/l for lead and 550 mg/l for chromium. Primary identification showed that all five bacterial isolates were Gram negative bacteria.

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1. Introduction

The problem of heavy metal pollution has increased rapidly in recent years as a result of various anthropogenic activities, including mining, chemical, agricultural and electronic industries, to meet the needs of the evolution, which have negative impact on the health of human, terrestrial, aquatic communities and ecosystems (Vareda *et al*., 2019). Heavy metals characterized by their inability to biodegrade and possibility of their transformation from toxic to more toxic form and their ability to accumulate and remain within the tissues of living organisms and thus their transfer through the food chain (Igiri *et al*., 2018). So the presence of heavy metals in the environment must be within specific concentrations for some metals, especially the essential ones which become toxic at high concentrations such as cobalt, zinc, copper, nickel, and chromium. As for nonessential metals, they are very toxic even in low concentrations and have no significance for the organisms, such as lead, cadmium, mercury, and silver (Jaishankar *et al*., 2014; Abo Gabal *et al*., 2018). Heavy metals toxicity results from their competition and substitution for functional metals, which leads to distorting enzymes and obstructing their work. Heavy metals also interfere with some proteins by binding with their active groups containing thiols causing proteins to be deformed. Moreover, heavy metals are concentrated on the cell membrane, which changes their structural composition, thereby impeding the exchange of ions and essential organic materials (Cervantes *et al*.,2001; Abou Zeid *et al*., 2009). One of the most dangerous sources of heavy metal pollution is the frequent drainage of many industries that use heavy metals in their production processes, including battery factories, tanning, textile, electrical materials, cleaning materials, dyes, electroplating, plastics, pesticides, and fertilizers (Papfilippaki *et al*., 2008). Metal pressure leads to establishment of metal resistant microorganisms which have intrinsic mechanisms and developed new resistance mechanisms for heavy metals in response to increased pollution (Hafeburg and Kothe, 2007; Johancy-Rani *et al*., 2010). Among microorganisms bacteria developed multiple mechanisms to resist heavy metals and these mechanisms can be employed as a technique for removing heavy metals from wastewater and the aquatic environment. Bacteria can be tolerant or resistant to heavy metals, and the tolerance described as the ability of bacteria to survive in a polluted environment through intrinsic properties, while resistance is the ability of bacteria to remain in a high concentration of the metals through detoxification mechanisms as a direct response to the presence of similar pollutants as well as intrinsic characteristics (Dixit *et al*., 2015). So the objectives of our study were estimation of heavy metals in waste water and selective isolation of heavy metals resistant bacterial isolates.

2. Material and methods :

2.1 Sample collection

Waste water samples were collected from water basins of the treatment unit for wastewater and industrial wastewater discharged from many companies, factories and power stations in Baghdad city, with three replicates per sample. Samples were collected from a depth of approximately 10-15 cm in two stages, the first using polyethylene bottles to measure the concentration of dissolved heavy metals, and the second using sterile glass bottles for bacterial culture. In the first stage, the bottles were sealed after sample collection and transferred to the laboratory to prepare the sample to measure the concentration of dissolved heavy metals. As for the second stage, samples were collected only from sites where high concentrations of dissolved heavy metals were recorded, then samples were collected in a cool container and transferred to the laboratory for bacterial culture.

2.2 Estimation of heavy metal concentration by Flame Atomic Absorption

100 ml of the sample was taken and 5 ml of concentrated nitric acid was added to it, and allowed to evaporate on the hot plate at 80 ° C until dehydration, then 5 ml of concentrated nitric acid $HNO3$ was added to it and heated to the point of dryness, after that 3 ml of concentrated hydrochloric acid HCL was added and the volume was completed to 10 ml with the addition of distilled deionized water. The concentration of lead, cadmium, and chromium was measured using a Flame Atomic Absorption Spectrometer (Abbawi *et al*., 1990).

2.3 Primary isolation of heavy metals tolerant bacterial isolates

The samples were mixed well and dilution series was made using sterile distilled water, then 0.1 ml of the third dilution was spread on nutrient agar supplemented with lead at a concentration of 300 mg/l, and 0.1 ml was also spread on nutrient agar supplemented with chromium at concentration of 100 mg/l, in addition 0.1 ml was spread on nutrient agar with cadmium at concentration of 100 mg/l. The plates then incubated at 37 °C, and the developing bacterial isolates were observed after 48 hours (Marazan *et al*., 2016).

2.4 Selection of heavy metals resistant bacterial isolates

Well diffusion method was used to select bacterial isolates which are resistant to toxic metals (Hassan *et al*., 1998; Neethu *et al*., 2015). Increasing concentrations of lead, chromium, and cadmium were prepared separately beginning from 100 mg/l. The bacterial isolates were activated on the Luria Bertani Broth and incubated for 24 hours at 37 °C, then the bacterial suspension was neutralized to obtain turbidity equal to the turbidity of the standard McFarland tube 0.5 using normal saline. The nutrient agar was prepared and poured into petri plates, then five holes were made in each plate with a size of 10 mm and a width of 4 mm. 0.1 ml of bacterial suspension was spread on the agar plates, After this left for 30 minutes to dry, then placing 25 µl from each concentration of prepared heavy metal in each hole with 25 µl of deionized water as a control in the middle hole. The plates were incubated at 37 °C for 2 days, after that the diameter of inhibition zone was measured, and the zone of inhibition less than 1 mm was regarded as a resistant bacterial isolate.

2.5 Determination of maximum tolerance concentration (MTC)

Maximum tolerance concentration was identified as highest concentration of heavy metals that allowed growth in a liquid medium after 48 hours (Moghannem *et al*., 2015). Nutrient broth contain increasing concentrations (100 , 200, 300, 400, 500-2500) mg/l of lead and (50, 100, 150, 200, 250, 300, 350, 400-1000) mg/l of chromium, and cadmium separately were prepared. Microplates composed of 96 well have been used and 150 microliters of nutrient broth contain concentration of metals were put in each well, and added to it 10 µl of bacterial suspension with 3 replicates for each concentration, noninoculated nutrient broth free of heavy metals and noninoculated nutrient broth containing the same concentration of heavy metal were used as control. By using ELISA microplate reader (USA) optical density was measured at a wavelength of 630 nm after 48 hours of incubation at 37 ° C.

2.6 Primary identification of resistant bacterial isolates

Five resistant bacterial isolates were purified on HiCrome™ UTI agar medium, and Gram stain was used to differentiate between gram positive and gram negative bacterial species.

2.7 Statistical Assessment

The statistical program SPSS version 25 was used to analyze the results which expressed as a mean ± standard deviation using one way ANOVA, two way ANOVA (AL-Ukaelii and AL-Shaeb, 1998).

3. Results

3.1 Concentration of lead, chromium and cadmium (ppm) in waste water samples

The results of the current study showed a significant increase in the concentration of lead, chromium, and cadmium in some study sites. The results also showed a clear significant difference between the concentration of lead, chromium, and cadmium as shown in table (1), where lead recorded the highest concentration compared with the concentration of chromium and cadmium. The first site showed the highest lead concentration of 5 ppm, while the fourth site showed the lowest lead concentration at 0.2 ppm. As for chromium, it recorded lowest concentration than lead, where the highest concentration of chromium was 0.6 ppm in the fourth site and the lowest concentration was 0.002 ppm in the second and nine sites. As for cadmium, it recorded the lowest concentration among heavy metals. Where the highest cadmium concentration 0.12 ppm was observed in the fourth site, while the lowest cadmium concentration 0.001ppm was recorded at the tenth site. In addition, nil values were recorded in some sites.

Table. 1: Concentration of lead, chromium and cadmium (ppm) in waste water of the study sites

No.	Name of site	Heavy metal concentration Mean± standard deviation		
		Lead	Chromium	Cadmium
1	Babel Battery Factories	5.0±0.08Aa	Nil	Nil
2	State Company for Vegetable Oil Industry / Al-Rasheed Factory	Nil	0.002±0.0001Aa	0.01±0.005Aa
3	State Company for Vegetable Oil Industry / Al-Maamoun Factory	1.0±0.002Ca	0.1±0.003Bb	0.05±0.004Ac
4	The State Company for Electrical and Electronic Industries 1	0.2±0.001Da	0.6±0.01Cb	0.12±0.001Bc
5	The State Company for Electrical and Electronic Industries 2	0.35±0.002Ea	0.07±0.002Ab	0.02±0.004Ab
6	The State Company for Textile and Leather Industries	0.23±0.004Da	0.01±0.0005Ab	Nil
7	State Company for Construction Industries Cement Factory	0.98±0.008Ca	0.003±0.0004Ab	Nil
8	The State Company for Construction Industries, production plant of plastic tubes	0.52±0.02Fa	Nil	Nil
9	Aldora thermal power station	1.0±0.002Ca	0.002±0.0001Ab	Nil
10	Aldora thermal power station2	0.3±0.003Ea	0.04±0.004Ab	0.001±0.0002Ac

3.2 Primary isolation of heavy metals tolerant bacterial isolates

The first five sites in which the highest concentration of heavy metals was recorded were chosen for collecting samples to isolate the resistant bacteria. Twenty five pure bacterial isolates were obtained from the primary isolation of heavy metal tolerant bacteria as shown in table (2), distributed among nine bacterial isolates tolerant to 300 mg\1 lead, nine isolates tolerated 100 mg/l chromium and seven isolates tolerated 100 mg/l cadmium.

Table. 2: Number and percentage of bacterial isolates tolerating heavy metals isolated from waste water

Type of isolates	Number	Percentage (%)
Lead tolerant isolates of 300 mg/l	9	36
Chromium tolerant isolates of 100 mg/l	9	36
Cadmium tolerant isolates of 100 mg/l	7	28
Total	**25**	**100**

3.3 Selection of heavy metal resistant bacterial isolates

Five bacterial isolates were selected using well diffusion method as heavy metal resistant bacteria. Lead tolerant bacterial isolates Pb1 and Pb4 showed resistance to high lead concentrations of 1000 and 1900 mg/l respectively and the inhibition zone diameter was 18 and 3 mm at concentrations 1100 and 2000 mg/l respectively. The cadmium tolerant isolate Cd5 showed resistance to lead at a concentration of 600 mg /l and the inhibition zone diameter was recorded as 3 mm at a concentration of 700 mg/l. The chromium tolerant isolates Cr8 and Cr9, showed resistance to 500 mg/l of lead, and the inhibition zone diameter was recorded 5 and 3 mm respectively at the concentration 600 mg/l as shown in the figure (1). It is also noted that the diameter of the inhibition zone increases with increasing the concentration of lead.

Resistance results of the bacterial isolates to chromium showed that the bacterial isolates Pb1, Pb4, Cr8, Cr9 recorded highest resistance to concentration 500 mg /l of chromium, and the inhibition zone diameter was 3, 3, 10, 2 mm respectively at 600 mg/l. The cadmium tolerant isolate Cd5 showed resistance to 400 mg/l, and inhibition zone diameter was 2 mm at the 500 mg/l. It is also noticed from the figure (2) that the diameter of the inhibition zone increases with increasing the concentration of chromium.

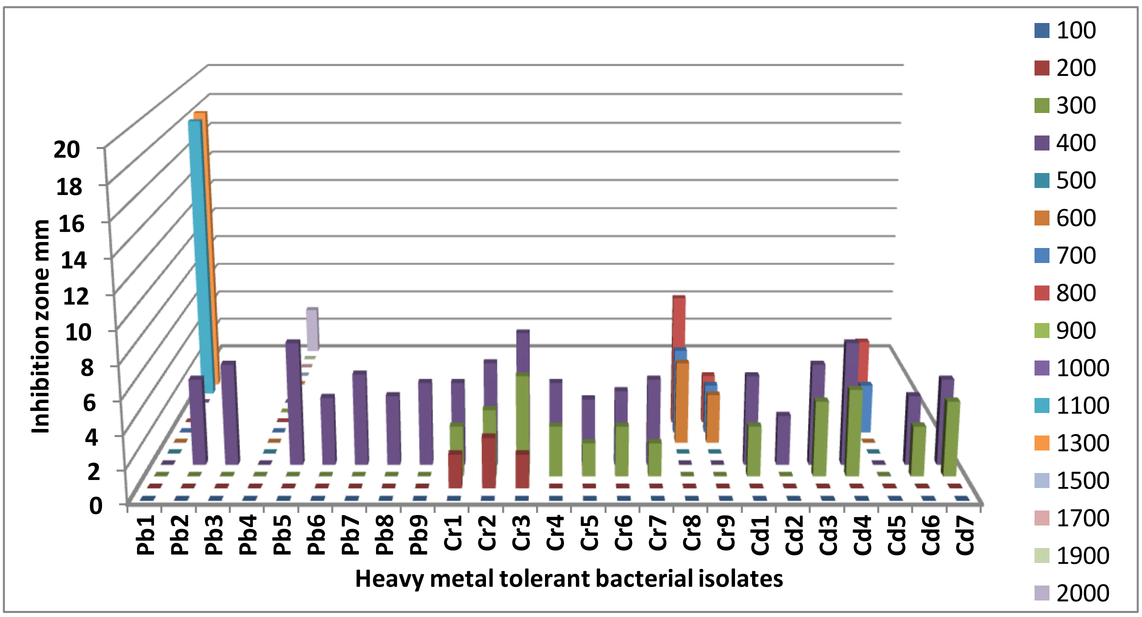

Figure. 1: Lead resistant of bacterial isolates by well diffusion method

Resistance results of the bacterial isolates to cadmium, Cd5 bacterial isolate showed the highest resistance for 500 mg/l, and the inhibition diameter zone was 5 mm at 600 mg/l, while the bacterial isolates Cr8, Cr9 showed resistance at 400 mg/l and recorded diameter of inhibition 7, 2 mm respectively at 500 mg/l. Pb1 bacterial isolate recorded resistance at 200 mg/l, and the inhibition diameter zone was 13 mm at 300 mg/l, while the isolate Pb4 showed resistance to 100 mg/l, and the inhibition diameter zone was 12 mm at 200 mg/l as shown in the figure (3). It is also observed that the diameter of the inhibition zone increases with increasing cadmium concentration.

3.4 Determination of maximum tolerance concentration (MTC)

The bacterial isolates Pb1, Pb4, Cr8, Cr9, Cd5 were selected as heavy metal resistant isolates and their maximum tolerance concentrations (MTCs) were determined as the highest concentration allowed for growth after 48 hours incubation in liquid medium. The results showed a difference in MTCs of the bacterial isolates to heavy metals. Figure (4) showed the maximum tolerance concentration for lead, where the isolate Pb4 showed the highest tolerance concentration of lead and reached 2300 mg /l, followed by the isolate Pb1 with the tolerance concentration 1300 mg/ l, and the isolate Cd5 showed a maximum tolerance concentration of lead 800 mg/l, then the two isolates Cr8, Cr9 with a maximum tolerance concentration of 700 mg/l.

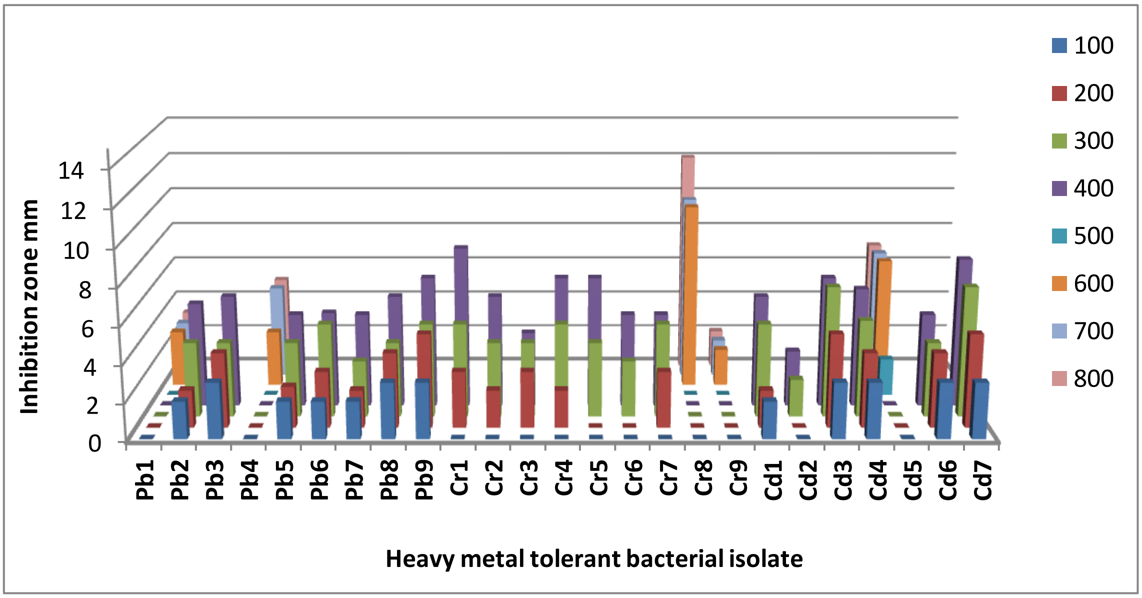

Figure. 2: Chromium resistant of bacterial isolates by well diffusion method

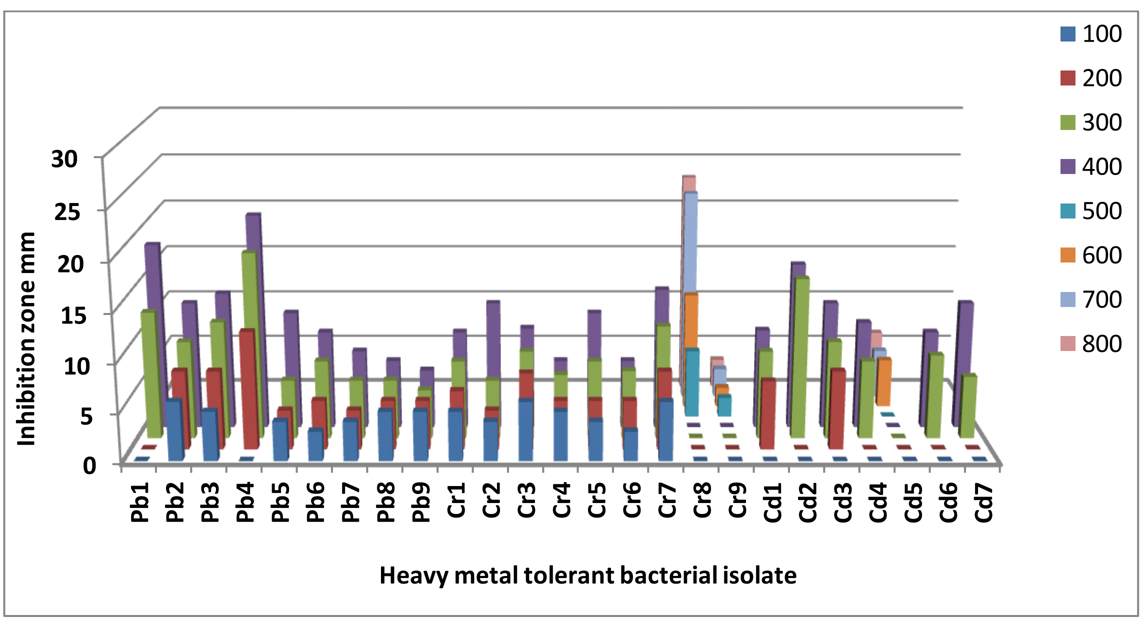

Figure. 3: Cadmium resistant of bacterial isolates by well diffusion method

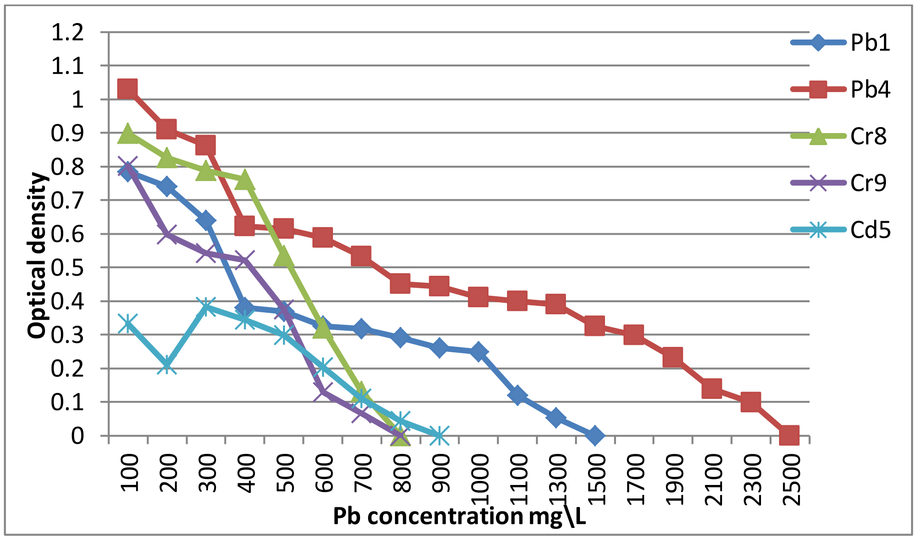

Figure. 4: Lead maximum tolerance concentration

Figure (5) shows the maximum tolerance concentration to chromium, where the bacterial isolates Pb1, Pb4, and Cr9 recorded a maximum tolerance concentration of chromium 700 mg/l, while the bacterial isolate Cr8 recorded maximum tolerance concentration of chromium 650 mg/l, while the isolate Cd5 recorded a maximum tolerance concentration reaching 550 mg/l.

Figure (6) shows the maximum tolerance concentration of cadmium, where the bacterial isolate Cd5 recorded the highest maximum tolerance concentration of cadmium 650 mg/l, while the bacterial isolates Cr9, Cr8 recorded a maximum tolerance concentration of 500, 450 mg/l, and the bacterial isolates Pb1, Pb4 recorded the lowest concentration 350 and 300 mg/l respectively.

3.5 Primary identification of resistant bacterial isolates

All five bacterial isolates were negative to Gram stain.

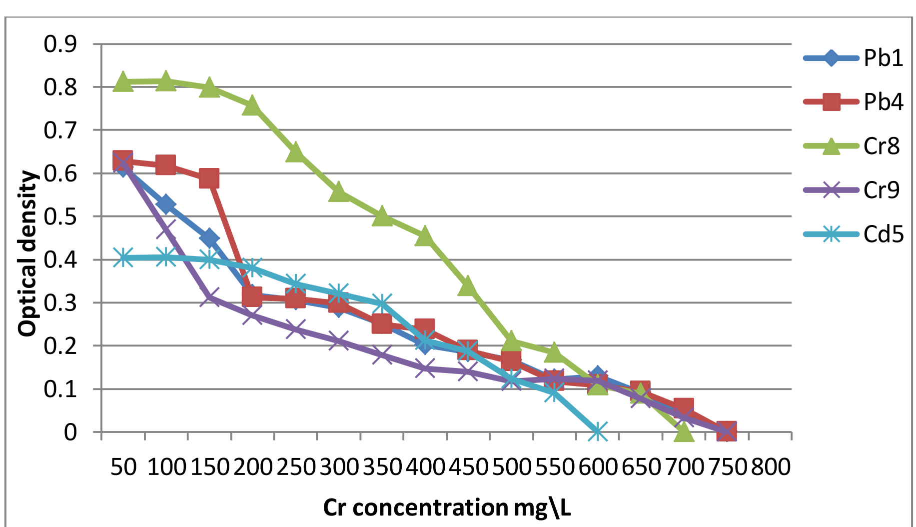

Figure. 5: Chromium maximum tolerance concentration

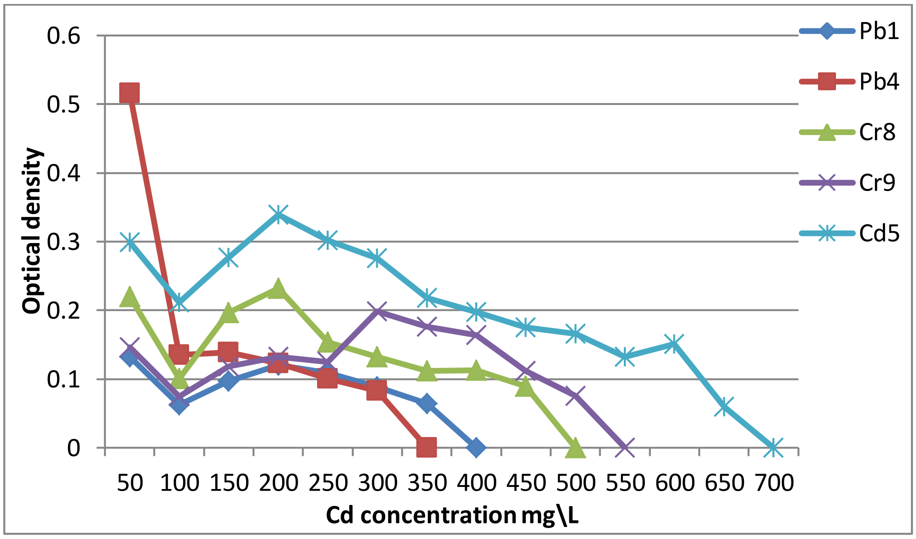

Figure. 6: Cadmium maximum tolerance concentration

4.Discussion

The waste water networks in Iraq suffer from neglect and most of the methods used and treatment units are ineffective, which causes the disposal of polluted water that does not meet to some local and international standards (Kinuthia *et al*., 2020), especially in recent years as a result of destruction during wars and continuation of the situation even after 2003 due to the lack of effective plans, the failure to implement construction projects, the length of the implementation period and the lack of rehabilitation, accompanied by an increase in the quantities of the offering and the difference of the materials offered from the previous as a result of the development of life and the entry of various chemicals as raw materials in various industries, and the failure to benefit from the experiences of countries in this field (Nasuri and Jaafar, 2016). The mismanagement of industrial effluent is the most important cause of heavy metals entering to the waste water networks and their accumulation in the environment (Velma *et al*., 2009; Khan *et al*., 2015,; Fatima and Ahmed, 2018).

The difference in the concentration of lead, chromium, and cadmium in different sites may be due to the different industries that using heavy metals in their production, and the high concentration of heavy metals in some locations compared with the standard determinants may be attributed to the lack of effective treatment units for the disposal of toxic heavy metals in most industries. The high concentration of lead may due to its frequent use in various industries because of its properties, the most important of which is the production of electric batteries. The production of plastic tubes for transporting water, as this industry consumes large quantities of water and mixes with production materials and uses a small portion of the water put out for reuse and the bulk of it is discharged without treatment for the plant's, lack of treatment units, as is the case in cement factories and power stations (Redfern, 2006). Through the results, it is clear that cadmium concentration exceeds the standard determinants in several of locations, including the General Company for Electrical and Electronic Industries and the Al Rasheed and Al Mamoun Factory. Cadmium has good electrical conductivity, light metal, high melting point, corrosion resistance and is used as a stabilizing agent in various industries (Yazdankhah *et al*., 2010). Also, all its compounds are soluble in water and this is the most important reason for its liquidity in industrial waste (Xu *et al*., 2014; Aksoy *et al*.,2014).

Chromium is one of the metals that are frequently thrown into wastewater, especially industrial wastewater, high concentrations of it have been recorded in the General Company for Electrical and Electronic Industries (Viti *et al*., 2014) because using in painting (Cervantes *et al*., 2001; Saha and Orvig, 2010). It was also noted that its concentration increased in the State Company for textile industries and tanning factories because it is included in the composition of the used dyes (Sivakumar, 2016). Lead, chromium and cadmium are among the most dangerous components of industrial effects, and the use of excessive quantities of these chemicals in industrial processes leads to high concentrations in liquid wastes with no effective treatment, which causes environmental pollution and leads to the emergence of microorganisms resistant to these metals (Moghannem *et al*.,2015; Marazan *et al*., 2017).

Addition of heavy metal ions to culture media provides a selective medium for the growth of heavy metal tolerant bacterial communities, but with less diversity (Pal *et al*.,2005; Tsai *et al*., 2005; Rosewarne *et al*., 2010). Contamination of environment with heavy metals also causes a decrease in microbial diversity, with survival of isolates that are more tolerant to heavy metals (El Baz *et al*.,2015). The primary isolation showed ability of 25 bacterial isolates from the waste water samples of the five different sites that recorded the highest concentrations of heavy metals to withstand the concentrations used in the culture medium of lead, chromium and cadmium separately. Sites contaminated with heavy metals contain indigenous microorganisms that have the capacity to tolerate different concentrations of heavy metals (Muneer *et al*.,2016).

The results of resistance of the bacterial isolates to lead, chromium and cadmium using well diffusion method, which is characterized by being an easy and less expensive and effortless method for identifying bacterial isolates resistant to heavy metals (Hassan *et al*.,1998; Neethu *et al*.,2015), showed a variation in the resistance of different bacterial isolates to lead, chromium and cadmium, and the reason may be due to the difference in cell wall composition between different bacterial isolates, as well as different mechanisms of resistance to heavy metals (Zeid *et al*., 2009). Only five bacterial isolates showed highest resistance to heavy metals were selected. The maximum tolerance of bacterial isolates for heavy metals in a liquid medium is an assessment of the toxicity of heavy metals in polluted liquid environments such as waste water, where the toxicity of heavy metals differs in a solid medium from a liquid medium due to the difference in metal abundance and diffusion and the ability of metal to form complexes with the components of the solid medium (Hassan *et al*.,1998; Kumar *et al*., 2013). The low tolerance of bacteria to heavy metals in solid media may be due to the lack of oxygen diffusion that affects cell metabolism, as different bacterial isolates show different degrees of tolerance to heavy metals in different media (Zakaria *et al*., 2007; Kang *et al*., 2014).

The maximum tolerance of five bacterial isolates to lead was highest ranging from 700-2300 mg/l. The discharge of lead in high concentrations in industrial liquid waste compared to other heavy metals, in addition to the many sources of lead pollution that contribute to its high concentration, may be the reason for the increase in resistance and tolerance of bacterial isolates (Niveshika *et al*., 2016). As for chromium, the maximum tolerance of the bacterial isolates was lower than lead, ranging between 550-700 mg/l, while cadmium was the most toxic metal, where the bacterial isolates maximum tolerance ranged between 300-650 mg/l. These results are in agreement with many studies that have concluded that lead is more tolerant compared with chromium and cadmium (Raja *et al*., 2009; Marazan *et al*.,2017), and also agreed with (Needy Aka and Babalola, 2017) that showed cadmium is the metal that is more toxic than chromium, and it also agreed with the results of a study (Moghannem *et al*., 2015) which concluded that lead is the most tolerable and cadmium is the most toxic.

The resistance of bacterial isolates to multiple and high concentrations of heavy metals is attributed to the fact that the bacteria used in this study were isolated from environments containing high concentrations of heavy metals, and they have physiologically and genetically adapted to heavy metal stress by developing many mechanisms to counter the toxic effects of metal ions such as accumulation and complexity of metal ions inside the cell, efflux pump, metal deposition, metal binding on the cell surface, and can also reduce the toxicity of heavy metal ions such as hexavalent chromium by converting it to a less toxic form through enzymatic reduction (Needy Aka and Babalola, 2017; Benmalek and Fardeau , 2016; Klaimurrgan *et al*., 2020). Gram negative bacteria are more tolerant to heavy metals than positive bacteria because of the intrinsic mechanisms that are due to the different components of the cell wall and its containment of the outer membrane consisting of phospholipid, lipoproteins, lipopolysaccharide, as well as outer membrane proteins, that interfere with heavy metals and eliminate its toxicity (Mounaouer *et al*., 2014; Ibal *et al*., 2019). The results of the current study were in agreement with many studies that indicated the isolation of negative bacteria from industrial wastewater (Mounaouer *et al*., 2014; Moghannem *et al*., 2015; Irawati *et al*., 2017; Abo Gabal *et al*., 2018).

Conflict of Interest

The authors declare that they have no conflict of interest.

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