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Bioremoval of Chromium by Local Isolates of *Pseudomonas aeruginosa* in Respect to its Genotype

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Abstract

The current study included bioremoval of chromium metal ions from aqueous solution by using seventeen *Pseudomonas aeruginosa* species isolated from different environments. The experimental results showed that isolates *Pseudomonas aeruginosa* have high efficiency in removal of chromium where the *P. aeruginosa* p.8 was the most efficient ($P \geq 0.001$) in bioremoval of chromium with a removal capacity reached 92.5 mg/L and removal index reached (96.5%). While *P. aeruginosa* p.4 was the least efficient ($P \geq 0.001$) in bioremoval of chromium from aqueous solutions reached 74.6 mg/L and removal index reached (79.8%). The REP-PCR detection using BOX-primer, showed genetic relatedness among the isolates of *P.aeruginosa*. The isolates were grouped according to the REP-PCR to different genotyping, named clusters which included C1, C2, C3 and C4 with relatedness: 2 (88%), 2 (73%), 4 (73%) and 2 (77%), respectively. The REP-PCR analysis showed that the genetic relatedness between isolates regarded to the source of the isolation as well as their efficiency of bioremoval process.

Keywords: *Pseudomonas aeruginosa*, bioremoval, genotyping, chromium

الازالة الحيوية للكروم بوساطة عزلات محلية من بكتريا *Pseudomonas aeruginosa* بالأعتماد على النمط الوراثي

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الخلاصة

تتضمن الدراسة الحالية الأزالة الحيوية لايونات الكروم من المحاليل المائية بأستخدام 17 عزلة بكتيرية من *Pseudomonas aeruginosa*معزولة من مختلف البيئات. أظهرت النتائج التجريبية أن عزلات *P.aeruginosa* كانت تمتلك كفائة عالية في أزالة الكروم أذ كانت العزلة 8 لبكتريا *P.aeruginosa* الأكثر كفاءة ($P \geq 0.001$) في الأزالة الحيوية لايونات الكروم من المحاليل المائية مع كفاءة ازالة وصلت الى 92،5 ملغم/لتر وبنسبة ازالة 96،5%. بينما كانت العزلة 4 لبكتريا *P.aeruginosa* الاقل كفاءة ($P \geq 0.001$) في الأزالة الحيوية لايونات الكروم بلغت 74،6 ملغم/لتر وبنسبة 79،8%. أظهرت عملية تنميط تفاعل REP–PCR بأستخدام BOX–primer، وجود قرابة وراثية بين عزلات *P.aeruginosa* . اذ قسمت العزلات الى مجاميع وفقا لتفاعل البلمرة الى انماط وراثية مختلفة سُميت بالمجموعات وتشمل C1، C2، C3 و C4 مع قرابة او درجة تشابه وصلت: 2(88) %، 2(73) %، 4(73) % و 2(77) % على التتالي. وبين تحليل نتائج سلسلة البوليميريز المتكرر REP–PCR أن هناك قرابة وراثية بين العزلات تبعاً لأماكن العزل فضلاً عن كفاءتها في عملية الأزالة الحيوية.

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Introduction

The presence of inorganic pollutants such as metal ions in the ecosystem causes a major environmental problem. Toxic metal compounds coming to the earth's surface not only contaminate earth's water (seas, lakes, ponds and reservoirs), but can also contaminate underground water in trace amounts by leaking from the soil after rain and snow [1]. The problem associated with metal ions pollution is that they are not biodegradable and are highly persistent in the environment. Thus they can be accumulated in living tissues, causing various diseases and disorders [2]. Chromium is one of the most toxic heavy metals discharge into the environment through various industrial wastewaters, such as leather tanning, electroplating, paints, pigment production, steel manufacture [3] .According to World Health Organization (WHO) the metals of most immediate concern are chromium, lead, zinc, iron, nickel, mercury and copper [4]. The chromium metal ion is considered to be a human carcinogen and mutagen [5].The conventional methods of removal of heavy metals from solutions, such as ion exchange, chemical oxidation/reduction, filtration, electrochemical methods, precipitation and membrane technologies may often be ineffective or uneconomic [6].

In recent years, attentions have been focused on possible biological methods for the removal of heavy metals from industrial wastewater [7].Different biological methods, bioaccumulation and biosorption have been demonstrated to possess good potential to replace conventional methods for the removal of metals [8].The advantage of biological methods is the low cost of operation and the high capacity to remove large quantities of heavy metals [9-10]. Bioaccumulation is an active metal uptake into the cell across the cell membrane, dependent on the cell metabolism. It is irreversible reaction, slower than biosorption, and requires expenditure of energy by living microorganisms [11]. Biosorption can be defined as the ability of biological materials to accumulate heavy metals from wastewater through metabolic mediated or physico-chemical pathways of uptake [12]. DNA typing methods have been frequently used to investigate the diversity of collections of *P. aeruginosa* [13]. Repetitive element based PCR (rep-PCR) using BOX primer rep-PCR is a method for fingerprinting bacterial genomes, which examined strain-specific patterns obtained from PCR amplification of repetitive DNA elements present within bacterial genomes [14]. BOX-PCR is a rapid, highly discriminatory and reproducible assay that proved to be powerful surveillance tools for typing as well as characterizing *P. aeuroginosa* isolates [15].

Material and methods

Sampling

The samples were collected for isolate *P. aeruginosa* from industrial waste water of Al-Tabieaa Paints Producing Co. Ltd, Asia Paints Manufacture Co. Ltd and Haditha Paints industries Co.; Tigris River, irrigation drainage water and sewage water in a sterile glass bottles (500 ml capacity). Then the samples were brought to the lab aseptically. The sample was taken from a depth of 20 cm. While in case of environmental hospital samples were collected by using sterile swab. The swabs were taken from wash basins, floor, and medical instruments and cultured in nutrient broth to dilute any disinfectants present and to encourage growth of low organism numbers. After 24 hr. incubation, subcultures were made onto MacConkey agar plates and incubated at 37 °C. Soil sample was collected in a sterile glass container; One gram of the soil sample was added into 9 ml of sterile distilled water and serially diluted. Then 0.1 ml of the serially diluted samples was cultured in nutrient broth and incubated at 37°C for 24 hrs. Subcultures were made onto MacConkey agar plates and incubated at 37 °C.

Isolation of *P. aeruginosa* isolates

The collected samples were inoculated on MacConkey agar then incubated at 37°C for 24 hr., the pale non lactose fermenting colonies were selected to achieve Gram's stain test, catalase test and oxidase test. Then a single colony was inoculated on Blood agar for the activation and detection of bacterial ability to lyses red blood cells (β-hemolysis). The large flat colonies that produced zones of Beta-haemolysis with a grape like odor were inoculated on Cetrimide medium, incubated at 37 °C for 24 hr. and then single colonies were inoculated on Nutrient agar to carry out other biochemical tests that confirmed the identification of bacterial isolation according to Bergey's manual.

Chromium standard solution

In order to prepare chromium standard solution of 10000 mg/L concentration, 51.2502 gm $CrCl_3.6H_2O$ was dissolved in 1000 ml of deionized distilled water. The solution was sterilized by filtration through 0.45 µm membrane filter, and served as stock solution for further preparations.

Chromium estimation in wastewater samples

To estimate total chromium *in situ*,100 ml of wastewater sample was taken and then five ml of concentrate nitric acid were added to the water sample. The sample was put over a hot plate and evaporated until dryness. The sediment was cooled and another (5) ml of nitric acid were added and covered with a glass watch. Then it was evaporated until desiccation as in previous method to obtain the sediment which dissolved by addition 1-2 ml of nitric acid. The sides of beaker and watch glass was washed with a little of sterilized deionized distilled water that absolutely cleared from ions. The chromium in the filtrate solution was estimated [16].

Preparation of Biosorbents

Bacterial isolates were propagated in brain heart infusion broth at pH 7 and incubated at 37 °C for 24 hr. Thereafter, growing cells were harvested by cooled centrifugation 4 °C at 5000 rpm for 30 min. Then washed thoroughly with deionised distilled water three times, placed in sterile test tubes and resuspended in small amount of DDW. One millilitre of suspension was dried in the oven at 100 °C in order to estimate the dry weight [17].

Biosorption experiment procedure

Twenty ml of metal solution (100 mg/L) chromium ions were placed into 100 ml volumetric flasks and the pH was adjusted to 6. Biosorbents-bacterial cells- were added to each chromium solution at a final concentration of almost 1 mg dry weight biosorbent / ml metal solution as triplicates. All samples were left for 1 hr. at 50°C. Control samples, free of biosorbents, were also carried out. All samples were centrifuged at 5000 rpm for 30 min. at 4°C. Thereafter each sample was passed through 0.45µm filter paper. Metal concentration was determined by flame atomic absorption instrument [18].

Calculations

The amount of biosorbent that was added to chromium solution to achieve 1 mg/ml as a final concentration was calculated as the following equation: [17].

$$\textbf{Required volume (ml)} = \frac{\mathbf{20}}{\textbf{Biosorbent concentration (mg dry wt./ml)}}$$

While the following equation was followed to estimate the initial chromium concentration after the addition of the biosorbent [17].

$$\textbf{Initial chromium concentration} = \frac{\textbf{20 – The added biosorbent volume}}{\textbf{20}} \times \textbf{Control concentration (µg/ml)}$$

In order to calculate the bioremoved chromium concentration, the following equation was used [17].

bioremoved chromium concentration (µg/ml) =Initial chromium concentration (µg/ml) - residual chromium concentration.

In order to calculate the bioremoval efficincy, the following equation was used.

bioremoval index (%) = [Initial chromium concentration (µg/ml) - residual chromium concentration / Initial chromium concentration (µg/ml)] X 100%

Genotyping detection

DNA was extracted from all 17 *P. aeruginisa* isolates using a commercial purification system (Reagent Genomic DNA Kit, Geneaid, Taiwan). Rep-PCR fingerprinting was carried out using one BOX primer of sequence 5' - CTA CGG CAA GGC GAC GCT GAC G - 3' [19]. PCR mixture was set up in a total volume of 25µl included 12.5µl of Master Mix, 2.5 µl of BOX primer (10 picomole /µl) and 2.5 µl of template DNA have been used. The rest volume was completed with sterile deionized distilled water. Negative control contained all material except DNA, that D.W. was added instead of template DNA. PCR reaction tubes were vortexes and finally placed into thermocycler PCR instrument. DNA was amplified according to [19]. According to the following procedure. Initial denaturation at 94°C for 5 min followed by 35 cycles of PCR consisting of denaturation at 94°C for 1 min, annealing at 48°C for 2 min, and extension at 72°C for 1 min; in the last cycle, the extension time was 5 min. The PCR products (7 µl) was analyzed using a 2% agarose gel with Ethidium bromide dye in the (1x) TBE buffer. Thereafter the electrophoresis was in fixed electrical current 75 volt with 1 hour. And photographed under the UV light. The size of the products was analyzed in comparison to a M100-2000 bp ladder M.W. size marker (Bioneer, Korea).

Result and discussion

The isolation and identification of *Pseudomonas aeruginosa* isolates

Seventeen species of *P. aeruginosa* isolates were obtained from different environments. Bacterial identification was done according to the characteristics of bacteria and the biochemical tests. Table-2 showed the results of morphological characteristics and biochemical test were used in their identification.

Table 1- The source and number of the *P. aeruginosa* isolates

Isolation source	No. of the *P. aeruginosa* isolates
Tigris river	p.2, p.15 and p.17
sewage wastewater	p.7, p.9 and p.10
industrial wastewater	p.3, p.6, p.8, p.11 and p.12
hospital environment	p.1, p.14 and p.16
Irrigation drainage water	p.4 and p.13
Soil	p.5

Table 2- Morphological and biochemical characterization of *P. aeruginosa*

Tests	Result
Gram stain	- ve
Cell shape	Bacilli
Colony shape	Smooth convex
Odor	Sweat grape
Lactose fermentation on MacConkey	- (pale color)
Oxidase test	+
Catalase test	+
Hemolysin production test	+
Selective media cetrimide agar	+
Growth at 42ºC	+
Growth at 4ºC	-
Indol production test	-
Methyl red	-
Voges proskauer	-
Citrate (simmons)	+
Urase test	-

Concentrations of chromium in the samples

Concentrations of chromium in seventeen samples were taken from different environments showed in Table-3.

Table 3- Mean ± SD of chromium concentration in all examined samples.

No. of isolate	Chromium concentration (mg/l)	No. of isolate	Chromium concentration (mg/l)
p.1	Nil	**p.10**	0.14±0.02
p.2	0.01±0.01	**p.11**	2.3±0.6
p.3	4±0.86	**p.12**	2±0.5
p.4	0.9±0.1	**p.13**	1.3±0.4
p.5	11±1.2	**p.14**	Nil
p.6	3±0.72	**p.15**	0.12±0.0
p.7	0.05±0.002	**p.16**	Nil
p.8	2.7±0.4	**p.17**	0.025±0.0
p.9	0.07±0.0		

The ability of *P. aeruginosa* isolates to bioremove of chromium ions

The mean of chromium ions bioremoved by seventeen bacterial isolates are given in Table-4. The highest mean value 92.5± 1.23 mg/L and removal index reached (96.5%) was recorded by bacterial isolate number p.8 while the lowest mean value 74.6 ± 1.64 mg/L and removal index reached (79.8%) was found in isolate number p.4. In general, there are certain bacterial isolates had better for chromium bioremoval capacities while others showed significantly less removal capability. Analysis of variance of these data shows very high differences (P≥0.001) between the ability of examined bacterial isolates.

Table 4- chromium bioremoved by different bacterial isolates with initial chromium concentrations

Isolate No.	Initial Cr concentration	Bioremoved chromium concentration(mg/L)±SD	Bioremoval index %
p.1	93.8	82.8 ± 1.28	88.2
p.2	93.9	77.6 ± 1.02	82.6
p.3	94.7	87.3 ± 1.61	92.1
p.4	93.4	74.6 ± 1.64	79.8
p.5	93.2	75.3 ± 1.59	80.7
p.6	95.0	91.6 ± 1.77	96.4
p.7	93.8	87.4 ± 0.94	93.1
p.8	95.8	92.5 ± 1.23	96.5
p.9	94.3	85.7 ± 0.99	90.8
p.10	92.7	82.7 ± 1.25	89.2
p.11	95.1	89.3 ± 0.25	93.9
p.12	94.6	84.2 ± 1.22	89
p.13	93.4	80.5 ± 1.08	86.1
p.14	94.0	83.6 ± 0.85	88.9
p.15	94.6	85.4 ± 131	90.2
p.16	93.9	77.2 ± 0.42	82.2
p.17	94.1	81.7 ±0.99	86.8

In the present study, *P. aeruginosa* were used because of *Pseudomonas* genus has efficiency for metal uptake and plays an important role in biosorption studies. The genus includes members with well-characterized biochemical and genetic characteristics, and for which a considerable range of genetic tools are available [20]. Several investigations have reported that *P. aeruginosa* displays efficiency in bioremoval of the heavy metal. Noaman [21]. Found that *P. aeruginosa* can remove 90.5% of chromium found in a standard solution 145.8 mg/L.

The results of present work showed that there are significant differences among the isolates in their ability to bioremoval chromium ions, despite these isolates belong to the same species. This may attributed to the variation in the chemical composition of the cells microbial which is the main reason for the difference in removal capacity among different types and even among different cells within the same species [22]. [23] concluded that the removal capacity does not depend on genus and species bacterial but depends on other factors such as the number of active sites, easy access to these sites, chemical status of the site and the affinity between the active site and the metal. Present results were agreed with wahbi [24]. Who used *P. aeruginosa* to remove chromium with percentage reached to 98.1%. While [25] used *Bacillus pumilus* in chromium removal and the percentage reached to 99.5% removal.

Genotyping

BOX-PCR DNA fingerprinting was carried out to differentiate precisely among the seventeen *P. aeruginosa* isolates collected from several sources environmental. The results of BOX-PCR fingerprinting revealed 4 main clusters at 73% similarity level as it is shown in Figure-2.Our results showed according to genotyping relatedness with sources isolation in C1, C4 and most C3 that there was a similarity in sources isolation for each cluster, while in C2 there was a different in sources isolation may be due to transmission of bacteria from one place to another in the environment. Nassir [26] and Alsaadi [27] found that the clinical and environmental isolates of the *p. aeruginosa* belong to

the same cluster. The results of the current study regarding to genotyping relatedness with removal of chromium ions in C1 there was a convergence in the efficiency of the removal of chromium ions. While in C2 and C4 there was a different in removal efficiency of chromium ions. regarding C3 was contain the isolates that have high efficiency in process removal the chromium ions this case due to the fact that *P. aeruginosa* possess a large and diverse genome [28]. Genotyping for discrimination of bacterial strains based on their genetic content has recently become widely used for bacterial strain typing. The methods already used in genotyping of bacteria are quite different from each other [29]. Syrmis [15] represented that BOX-PCR has shown high discriminatory power with reproducibility, stability, fast turnaround times, and cost effective alternatives for typing bacteria. BOX-PCR is the most robust of the three rep-PCR methods. BOX-PCR patterns are not affected by the culture age of the strain to be analyzed [30] and fingerprinting output can be easily analyzed by computer assisted methods [31]. In a study carried out by Rao [32] indicated that genotyping of *P.aeruginosa* strains could not be associated to the phenotypic characteristics studies.

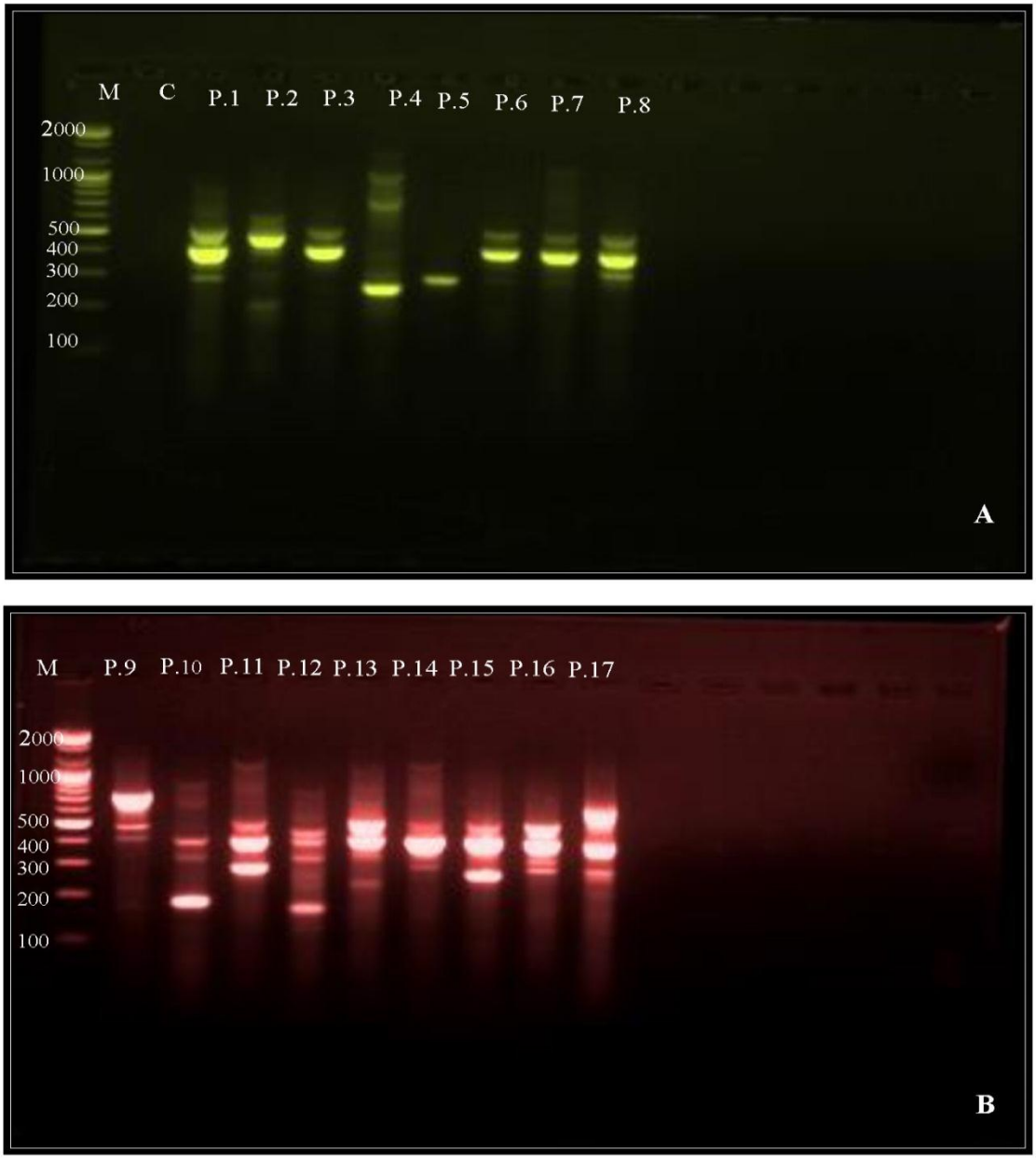
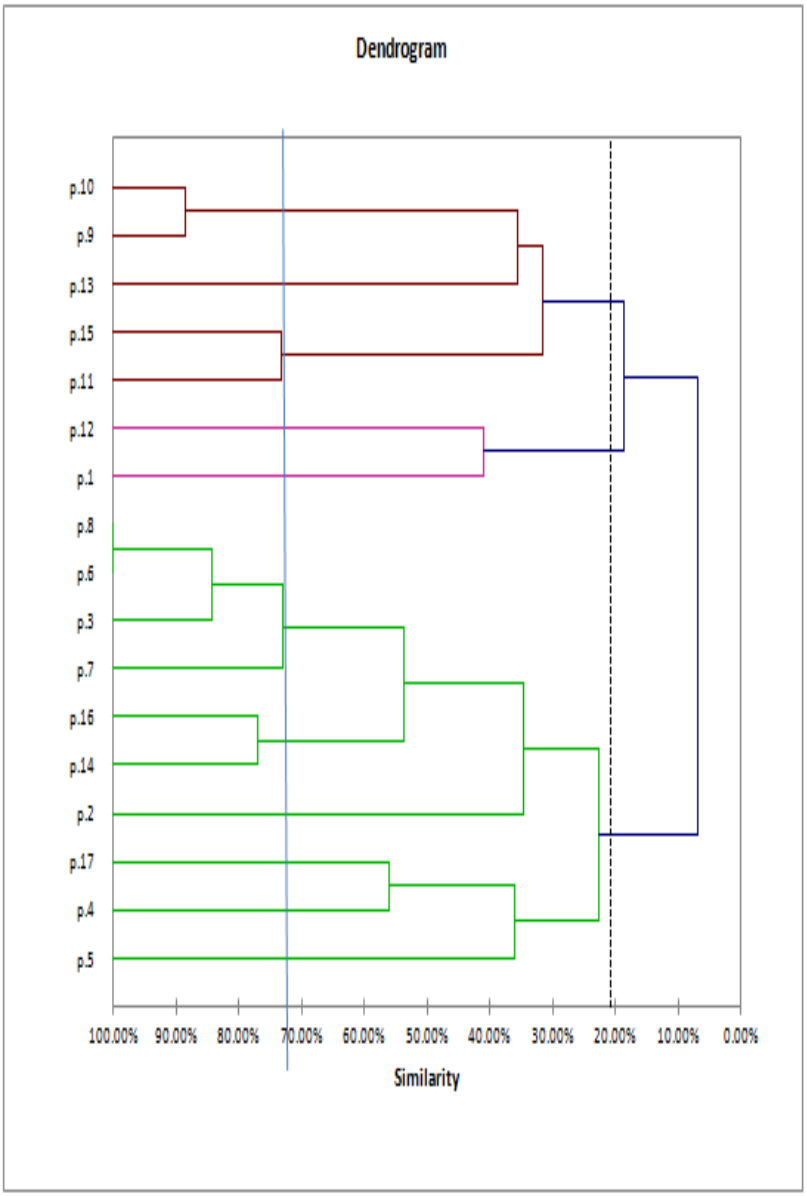


Figure 1- BOX-PCR fingerprinting of *P. aeruginosa* isolates. The circumstances of electrophoresis (2% agarose gels in 1X TBE buffer, 75 volt for 1 h at room temperature and stained with Ethidium bromide), A- Lines 1-9 *P.aeruginosa* isolates. B - Lines 10 to 17 *P. aeruginosa* isolates. Lane M: Molecular weight marker (MW100-2000bp). Lane C: Control



Dendogram

Figure 2- Dendrogram (cluster analysis) using BOX-PCR fingerprint patterns of 17 *P.aeruginosa* isolates from different environment, where showing the relatedness of studied *P. aeruginosa* isolates

Conclusion

According to the results of this experiment, *P. aeruginosa* has high efficiency in bioremoval of chromium ions from aqueous solution. REP-PCR using (BOX primer) showed that seventeen isolates were clustered into four different genotypes and the genetic relatedness between isolates regarded to the source of the isolation as well as their efficiency of bioremoval process.

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