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Microbial Bioremediation of Lead by Lead-Resistant Pseudomonas chlororaphis Strain Hel-KE`-14 Isolated from Industrial Wastewater

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ABSTRACT

Along with industrial progress, environmental pollutants like toxic heavy metals are widely spreading throughout the world. Bioremediation of metal pollutants from industrial wastewater using metal resistant bacteria is a very important aspect of environmental biotechnology. During our screening program for heavy metal resistant bacteria a bacterial strain coded Hel-KE`-14 was isolated from industrial wastewater sample collected from Helwan governorate, Egypt was able to grow on nutrient agar media supplemented with individual concentrations; 50, 200 & 200 ppm of the heavy metals; Hg^{2+}, Cd^{2+} & Pb^{2+} respectively. The maximum tolerable concentrations (MTC) of the tested heavy metals for this isolate were detected as 70, 1100 & 2800 ppm for Hg^{2+}, Cd^{2+} & Pb^{2+} respectively. The heavy metal removing capacity of this isolate was determined by estimation the metal content in the final solution of industrial wastewater sample and it was found that this isolate exhibited ability to accumulate the heavy metals in the tested sample where the concentrations were 0.072 ppm compared with control one. Examination the cells of this isolate growing in wastewater by transmission electron microscope showed thickness in the cell wall of some cells and rupture or malformation in another cells. Identification of this isolate was performed based on morphological, physiological and biochemical characteristics in addition to phylogenetic analysis of 16S rRNA gene which indicated belonging of this isolate to the genus Pseudomonas with high similarity to 99% to Pseudomonas chlororaphis. Based on the data obtained in this study, it can be concluded that biomass of this bacterium can be used for bioremediation of lead from industrial waste processing plants with high efficiency.

Keywords: *Bioremediation, Lead, Wastewater, Pseudomonas chlororaphis, 16S rRNA gene.*

INTRODUCTION

Living system requires special transport and handling mechanisms to keep them from toxic metals [1]. The toxicity occurs in humans due to environmental pollution via soil or water contamination or due to occupational exposure. Some of these metals are useful to us in low concentrations but are highly toxic in higher concentrations [2].

Global pollution is increasing due to the variations in natural and anthropogenic activities, leading to contaminations in various aquatic and terrestrial ecosystems with heavy metals, organic and inorganic chemical compounds. Among various pollutants, heavy metals are released into soils [3]. Presence of heavy metals such as Cu^{2+} and Cd^{2+}, in fly ash causes metal toxicity in plants [4,5].

Heavy metals are natural constituents of the environment, but indiscriminate use for human purposes has altered their geochemical cycles and biochemical balance. This results in excess release of heavy metals such as cadmium, copper, lead, zinc etc. into natural resources like the soil and aquatic environments. Prolonged exposure and higher

accumulation of such heavy metals can have deleterious health effects on human life and aquatic biota. The role of microorganisms and plants in biotransformation of heavy metals into nontoxic forms is well-documented, and understanding the molecular mechanism of metal accumulation has numerous biotechnological implications for bioremediation of metal-contaminated sites [6].

Even at low concentrations, heavy metals like mercury (Hg), cadmium (Cd), and lead (Pb) directly cause oxidative stress, lipid peroxidation, carcinogenesis, mutagenesis, and neurotoxicity in humans, animals, and plants [7-9].

Lead is commonly found in industrial settings and lead exposure has the tendency to cause adverse health effects. The adverse health effects induced by lead exposure are dependent on two important components: dose (how much of a contaminant) and duration (how long there has been contact with the contaminant). Exposure to lead can occur from eating and drinking contaminated water or breathing in high levels of lead [10].

Bioremediation processes are very attractive in comparison with physicochemical methods such as electrochemical treatment, ion exchange, precipitation, reverse osmosis, evaporation, and sorption for heavy metal removal techniques because they can have lower cost and higher efficiency at low metal concentrations [11,12]. There are a number of bio materials that can be use to remove metal from waste water, such molds, yeasts, bacteria, and seaweeds [13,14].

Materials and Methods

1.1. Sampling

Ten samples included five soils and five water samples were collected from four different sites (Helwan, Shubra Al Khaimah, Abu Zaabal and Mahalla Al-Kubra) contaminated with different heavy metals. Approximate 100 g of each sample below 10 cm from surface were collected into sterile plastic bags and labeled. Besides soil samples, 250 ml of each of liquid samples were also collected from discharging points of wastewater. Samples of wastewater were collected in sterile plastic bottles and used within 24 h of collection for bacteriological analysis.

1.2. Isolation of Bacterial Strains

For the isolation and enumeration of the bacterial populations each 10 g soil or sediment sample was added to 90 ml sterilized water and mixed on the magnetic blender for 30 min to separate bacteria from the soil completely. After being deposited for 20 min 10 ml suspension was added to 90 ml of sterile nutrient broth medium while in case of wastewater samples only 10 ml of agitated water sample were added to 90 ml of sterile nutrient broth medium for enrichment of all bacterial isolates which found in the collected samples, all flasks incubated at 30°C for 48 h. For serial dilutions 1 ml of the enriched samples was added to 9 ml of sterile distilled water to make a one in 10 dilutions (10^{-1}) then one ml of this dilution was added to 9 ml of sterile distilled water to make a one in 100 dilution (10^{-2}). This procedure was repeated until 10^{-8} dilution was reached in the presence trace salt solution 20 ml/l [15].

1.3. Screening for Heavy Metal Resistant Bacteria

For the selection of heavy metal resistant isolates among the isolates obtained from the purification, concentration of each heavy metal at 200 ppm for lead (Pb^{2+}), 200 ppm of cadmium (Cd^{2+}) and 25 & 50 ppm mercury (Hg^{2+}) were incorporated separately into NA medium and the purified isolates were streaked on the surface of this media, the inoculated plates were incubated at 30°C for 48 h, after that, the most resistant isolates were selected according to their tolerance to the all metals under studying at highest concentrations [16].

1.4. Determination of Maximum Tolerable Concentrations (MTCs) of Heavy Metal Resistant Bacteria

Several concentration of Pb^{2+} up to 3000 ppm were cultivation in broth media for 48 h on shacking incubator at 30°C & 150 rpm until it stop the growth of resistant organisms then read the result in spectrophotometer at 540 nm. All experiments were performed in triplicates and the average values were calculated [17].

1.5. Determination of the heavy metals removal capacity by free living bacterial cells of the most resistant isolates

The heavy metals removing capacity were determined by estimation of metal content in the final solution of wastewater samples and treated organisms by using atomic absorption spectrophotometry. One ml bacterial suspension (OD at 620 nm was 0.8-0.9) was transferred in to 50 ml Nutrient broth supplemented with wastewater samples from two painting companies, Mido company (sample no. 1) and Italian company (sample no. 2) values of each isolate in 250 ml Erlenmeyer shake flasks which designed as (B). Cultures were incubated at 30°C on an environmental rotary shaker incubator (New Brunswick, New Jersey) at 150 rpm for 48 h. also, the control of wastewater samples which designed as (A) transferred into 50 ml Nutrient broth supplemented with wastewater samples instead of distilled water and organisms which designed as (C) transferred into 50 ml nutrient broth supplemented with distilled water were done. After incubation time the samples were centrifuged for 10 min at 6000 rpm using centrifuge (sigma) and the supernatants filtered through sterilized 0.22 µm Millipore syringe filter [18].

Filtrates were diluted 10-fold with 10% HNO_3 for estimation of residual metal by using a (Analyst 400 Perkin Elmer) atomic absorption spectrophotometer, all the experiments were performed in triplicates and the average values were determined. The metal removing efficiency can be described in terms of percentage as the following

Percentage of heavy metal removing = I-F/I × 100

Where, F is the final reading after removing and I is the initial reading before removing. The initial reading is the amount of metal concentration in the basic solution before being removing by cells and the final reading is the of concentration metal in the solution after removing. The subtraction of the final reading from the initial reading results in the amount of metal concentration removed by the cell pellets [19].

Electron microscopy

The control and treated cultures were examined by transmission electron microscopy (TEM) in order to identify the location of accumulation particles within the cells [16]. Cells of control and treated cultures were prepared for TEM [20].

1.6. Identification of the Selected Resistant Isolate

1.6.1. Phenotypic Identification

1.6.1.1. Morphological Characteristics

Morphological characteristics namely, colony morphology (color and shape) cell morphology (shape and gram reaction) of the selected isolate were studied.

1.6.1.2. Physiological and Biochemical Characterization

The biochemical characteristics of the selected isolate were identified using Biolog system (Biolog, Hayward, Calif.) [21] at laboratory of Microbiology, Egyptian Company for Biological Products, Drugs and Vaccines (Egy-Vac/VACSERA), Cairo, Egypt. The biochemical and physiological tests used to identify the target isolate were compared to Bergey's Manual of Determinative Bacteriology [22].

1.6.2. Molecular and Phylogenetic Identification

1.6.2.1. Isolation of Genomic DNA

Total DNA was extracted by a modified method of Moore et al. [23]. In brief, bacterial cells were collected by centrifugation at 13,000 rpm for 2 min followed by suspension in 564µl Tris-HCl-EDTA buffer and incubation with 10µg lysozyme (50 mg/ml) at 37 °C for 30 mins. 6µl proteinase K (20 mg/ml) and 30µl of 10% SDS were added, mixed and incubated for 1 hour at 37 °C. To the lysis solution, 100µl of 5M NaCl was added followed by incubation for 2 min at 65 °C. This was followed by an addition of 80µl CTAB/NaCl and a further incubation for 10 min at 65 °C. The mixture was treated with phenol/chloroform/isoamyl alcohol (25:24:1). The supernatant was collected and precipitated with isopropanol by keeping at – 20 °C overnight. Genomic DNA was washed in 70% ethanol and dissolved in 100µl TE buffer. RNase treatment was carried out to remove traces of RNA from the sample.

1.6.2.2. Amplification of 16S rDNA Genes

The 16S rDNA genes were amplified with bacterial universal primers specific for eubacterial 16S rDNA gene (Forward, AGTTTGATCATGGCTCAG) and (Reverse, TTACCGCGGCTGGCA) according to the method described by Hookoom and Puchooa [24]. The PCR (50µl) contained 0.5µl of each forward and reverse primer, 1.5mM of 10X Taq buffer (stock 20mM), 0.125 mM (2.5µl) of each deoxynucleotide (ddATP, ddGTP, ddCTP and ddTTP), 1.25 units of Taq DNA polymerase (5units/µl) and 5µl DNA. PCR conditions were as follows: denaturation at 94 °C for 3 min, 30 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, extension at 72°C for 1 min, and a final extension step at 72 °C for 10 min. The PCR products obtained from DNA extracted from the samples were first analyzed by electrophoresis in 1.5% agarose gel and was stained with ethidium bromide and visualized under short-wavelength UV light.

1.6.2.3. Nucleotide Sequencing and Alignment

A DNA fragment was eluted by using QIAgen Gel Extraction Kit. PCR product was sequenced by 3730x1 DNA synthesizer (Applied Biosystems, California, USA). The part of DNA isolation and purification, 16S rRNA gene amplification and sequencing was carried out at Sigma Scientific Services Co, Lebanon Square, El Giza, Egypt. Sequences were matched with previously published bacterial 16S rDNA sequences in the National Center for Biotechnology Information (NCBI) database using the GenBank BLAST search available through the center's website (http://www.ncbi.nlm.nih.gov/BLAST). The 16S rDNA sequences were then submitted to the Gene Bank using the Sequin service. Further phylogenetic tree, similarity index was generated and compared with known sequences using MEGA 4 software [25].

Results and Discussion

3.1.Isolation of Bacterial Isolates from Different Samples

Twenty nine bacterial isolates were obtained from the collected samples, these isolates are distributed as follows: Eleven isolates (37.9%) were obtained from Shubra Al Khaimah region (painting company); the isolates from this region were symbolized as (SH KH –1 to SH KH –11). Five isolates (17.2%) were obtained from Kafr El–Ealw (petroleum land), the isolates from this region were symbolized as (KE PL`-12 to KE PL`-16). Five isolates (17.2%) were obtained from Kafr El-Ealw (Agriculture soil behind to the wall of cement plant), the isolates from this region were symbolized as (KE CP-17 to KE CP-21). Seven isolates (24.1%) were obtained from Abu Zaabal (factory for fertilizers and chemicals), the isolates from this region were symbolized as (AB ZA-22 to AB ZA-28). One isolates (3.4%) were obtained from Mahalla Al-Kubra (company for spinning weaving and dyeing), the isolates from this region were symbolized as (MA KU-29). Many studies reported isolation of heavy metal resistant bacteria from these regions [26,27].

3.2.Screening for Heavy Metal Resistant Bacteria

High levels of tolerance to the tested heavy metals were detected in isolated bacterial strains through screening on solid media. The ability of the obtained twenty nine isolates to grow on individual concentrations; 50, 200 & 200 ppm for $HgCl_2$ & $CdNO_3$ & $Pb(CH_3COO)_2$ respectively have been recorded in table (1). From this table, it is clear that isolates (SH-KH-1& SH-KH'7& Hel-KE`-12& Hel-KE`-13 & Hel-KE`-14 & AB-ZA-1& MA-KU-1) were the most resistant to these concentrations of heavy metals.

3.3.Screening for lead (Pb2+) resistant bacteria using microdilution technique

The most resistant isolates Hel-KE`-12, Hel-KE`-13 & Hel-KE`-14 which showed a high tolerance to each concentration of the tested metals after growth in solid media were selected and tested for lead resistant types (Table 2).

3.4.Determination of maximum tolerable concentrations of Pb2+

The selected bacterial isolates; Hel-KE`-12, Hel-KE`-13 & Hel-KE`-14were allowed to grow on different concentrations; 1200, 1400, 1600, 1800, 2000, 2200, 2400, 2600, 2800 & 3000 ppm of lead acetate and it was found that MTC of $Pb^{2+}$ was at 2000, 2600 & 2800 ppm for the isolates Hel-KE`-12, Hel-KE`-13 & Hel-KE`-14 respectively, data are recorded in table (3) and represented in figure (1). Gupta et al. [28] reported that, the maximum tolerance of some bacterial isolates to lead was at 50 mg/l 33.3 ppm. While Kaur et al. [29] showed resistant to lead up to 186.9 ppm. Also Durve et al. [30] recorded some bacterial species could tolerate 625.80 ppm of lead. Based on the above results the isolate Hel-KE`-14 was found to be the most resistant isolate and selected for identification.

3.5.Identification the selected isolate Hel-KE`-14

The identification of the isolate Hel-KE`-14 was done on the basis of morphological, physiological and biochemical characteristics as well as phylogenetic analysis of 16S rRNA gene.

3.5.1.Morphological characteristics

3.5.2.Morphological characteristics of Hel-KE`-14 isolate was studied through microscopic examination of the stained cells showed Gram-negative, bacilli cells (Figure 2).

3.5.2.Biochemical and physiological characteristics

Biochemical characteristics of Hel-KE`-14 isolate were identified using Biolog system (Biolog, Hayward, Calif.) which indicated that the isolate under study is closely related to *Pseudomonas chlororaphis*. Morphological, physiological and biochemical characteristics of the isolate Hel-KE`-14 were compared with data of the genus *Pseudomonas* in Bergey's Manual of Determinative Bacteriology [22] and indicated that the isolate Hel-KE`-14 is closely related to *Pseudomonas chlororaphis.*

3.5.3.Molecular and phylogenetic identification

The morphological and biochemical identification of isolate Hel-KE`-14 was confirmed by 16S rRNA gene sequencing. The genomic DNA was extracted and 16S rRNA gene was amplified by PCR then examined by agarose gel electrophoresis (Figure 3). The nucleotide sequence of 16S rRNA gene was obtained as a partial sequence (883-bp) (Fig. 4). The sequence was submitted to the GenBank database and deposited under GenBank accession number KM007105.1.

According to a sequence homology analysis of GenBank by BLASTN, the highest score was found with Acinetobacter species. Among them strain of *P. chlororaphis* strain TU04 have the highest identities 99%. In order to understand the phylogenetic position of the strain Hel-KE`-14, we constructed a phylogenetic tree based on comparison of 16S rRNA sequences of the isolate and those of reference *Pseudomonas* strains (Fig. 5). These results confirmed that the isolate Hel-KE`-14 is a strain of *Pseudomonas chlororaphis* and designated as *P. chlororaphis* strain Hel-KE`-14. These results are in accordance with results of several studies which established the dominance of *P. chlororaphis* in heavy metal polluted areas [30-32].

3.6.Determination of the heavy metals removal capacity of Pseudomonas chlororaphis Hel-KE`-14 In this experiment, *Pseudomonas chlororaphis* Hel-KE`-14 exhibited ability to accumulate the heavy metals in the two tested samples with varied degrees comparing with non-treated (control samples). Other studies showed that *Pseudomonas aeruginosa* had high bioaccumulation capacities in respect to mercury, lead, arsenic and cadmium even at high salt concentrations [30].

Growing of *Pseudomonas chlororaphis* Hel-KE`-14 on the wastewater sample no. 1 exhibited ability of this organism to remove only two metals; Mg^{2+} & Mn^{2+} from this sample where concentration of the two metals was 12.16 & 0.047 ppm comparing with concentration of control sample 14.26 & 0.055 ppm respectively. While it was recorded increasing in concentration of the metals; Pb^{2+}, Cd^{2+}, Fe^{2+}, Cu^{2+} & Zn^{2+} where the concentration was 0.072, 0.019, 0.919, 0.079 & 1.319 ppm comparing with control sample 0.0, 0.005, 0.552, 0.076 & 0.633 ppm of these metals respectively [30].

The obtained results of the heavy metals estimation in wastewater treated with cells of *Pseudomonas chlororaphis* Hel-KE`-14 were compared with that of growing of this organism on distilled water. Our findings indicate that the organism was able to accumulate these heavy metals. Data of the obtained results in this experiment are recorded in table (4) and represented in figure (6).

3.7.Transmission electron microscopy (TEM) examination of Pseudomonas chlororaphis Hel-KE`-14 under The cells of *Pseudomonas chlororaphis* Hel-KE`-14 treated with wastewater sample (no. 1) compared with control cells growing on distilled water were examined by transmission electron microscopy (TEM) in order to identify the effect of metal on the cell. After examination it was found that, the treated cells appeared with rupture and malformation in the cell shape (Figure 7 a&b).

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Table (1): Heavy metal assay on solid media

Isolates	Heavy metal concentrations (ppm)		
	$Pb(CH3COO)_2$ (200)	$CdNO_3$ (200)	$HgCl_2$ (50)
SH-KH-1	++++	++	-
SH-KH-2	+++	-	++
SH-KH-3	++++	-	++
SH-KH-4	++++	+	++
SH-KH`-5	++++	-	++++
SH-KH`-6	++++	-	-
SH-KH`-7	++++	-	-
SH-KH`-8	++	-	-
SH-KH`-9	++++	+	++++
SH-KH`-10	-	-	+
SH-KH`-11	+++	+++	+++
Hel-KE`-12	++++	++++	++
Hel-KE`-13	+++	++	-
Hel-KE`-14	++++	++++	++
Hel-KE`-15	++	-	-
Hel-KE`-16	+++	+	-
Hel-CP-17	+++	-	-
Hel-CP-18	++++	-	++
Hel-CP-19	++++	-	-
Hel-CP-20	+++	-	-
Hel-CP`-21	+	-	+
AB-ZA-22	++++	++++	-
AB-ZA-23	++++	-	-
AB-ZA`-24	++++	+	-
AB-ZA`-25	+++	-	-
AB-ZA`-26	+++	-	-
AB-ZA`-27	++	-	-
AB-ZA`-28	+++	-	-
MA-KU-29	++++	+	+

(-)= no growth, (+)=weak growth, (++) Moderate growth, (+++)=Very good growth & (++++)=Excellent grow

Table (2): Screening for lead (Pb^{2+}) resistant bacteria using microdilution technique

Conc. / Isolates	Screening for lead (Pb^{2+}) resistant bacteria using microdilution technique (ppm)										
	- Ve Control	+Ve Control	200	300	400	500	600	700	800	900	1000
Hel-KE`-12	0.049A	1.049A	0.985	0.987	0.987	0.998	1.003	1.042	1.022	1.003	0.797
Hel-KE`-13	0.044A	0.926A	1.063	1.07	1.061	1.095	0.973	1.156	1.183	0.983	0.973
Hel-KE`-14	0.046A	0.941A	1.024	1.052	1.001	1.017	1.024	1.097	1.058	1.031	1.041
SH-KH`-11	0.053A	0.955A	0.158	0.159	0.132	0.152	0.185	0.262	0.194	0.23	0.208
MA-KU-29	0.056A	0.884A	0.265	0.286	0.267	0.387	0.28	0.268	0.234	0.231	0.351
AB-ZA- 22	0.058A	1.031A	1.038	0.633	0.052	0.066	0.073	0.066	0.066	0.075	0.065
SH-KH-1	0.047A	0.99A	1.029	0.049	0.051	0.052	0.05	0.048	0.043	0.045	0.048

Table (3): Maximum tolerable concentrations of Pb^{2+} for the most resistant bacterial isolates

Conc. / Isolates	**Screening for lead (Pb^{2+}) resistant bacteria using microdilution technique (ppm)**											
	- Ve Control	**+Ve Control**	**1200**	**1400**	**1600**	**1800**	**2000**	**2200**	**2400**	**2600**	**2800**	**3000**
Hel-KE`-12	0.753A	0.839A	0.934	0.923	0.911	0.873	0.842	0.796	0.765	0.741	0.696	0.651
Hel-KE`-13	0.699A	1.743A	0.992	1.023	1.04	0.883	0.875	0.745	0.732	0.711	0.695	0.647
Hel-KE`-14	0.721A	1.833A	1.011	0.998	0.987	0.985	0.977	0.954	0.895	0.877	0.752	0.716

Table (4): Estimation of heavy metals content of wastewater sample for *P. chlororaphis* Hel-KE`-14

Metals / Samples	**Mg^{2+}**	**Mn^{2+}**	**Fe^{2+}**	**Zn^{2+}**	**Cu^{2+}**	**Cd^{2+}**	**Pb^{2+}**
A	14.26	0.055	0.552	0.633	0.076	0.005	0.0
B	12.16	0.047	0.919	1.319	0.079	0.019	0.072
C	7.836	0.812	40.90	2.184	0.038	0.019	0.0

A: Control (non-treated sample), **B**: Treated sample (after growing the organism on wastewater), **C**: After growing the organism on distilled water.

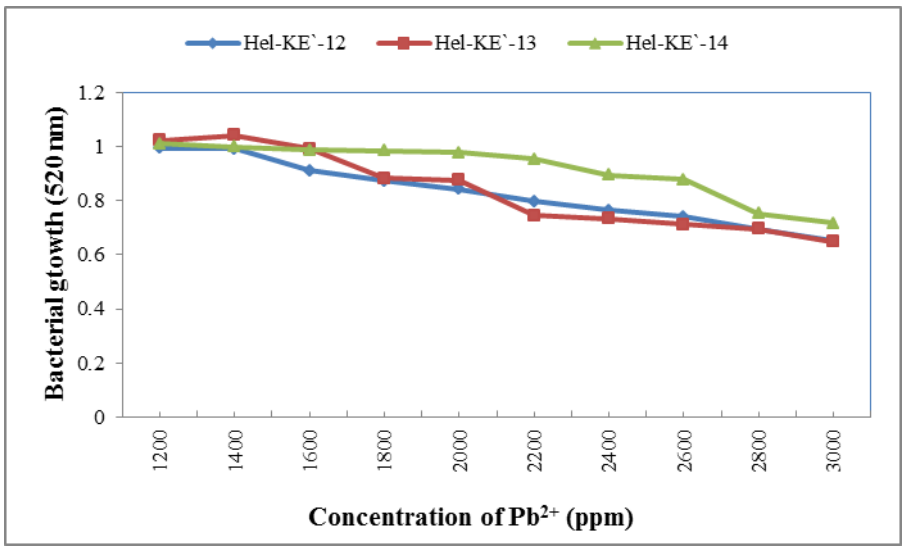


Figure (1): Maximum tolerable concentrations of Pb^{2+} for bacterial isolates

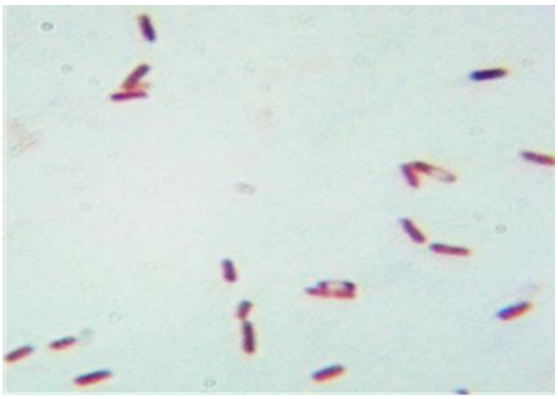


Figure (2): Gram stain for Hel-KE`-14 isolates

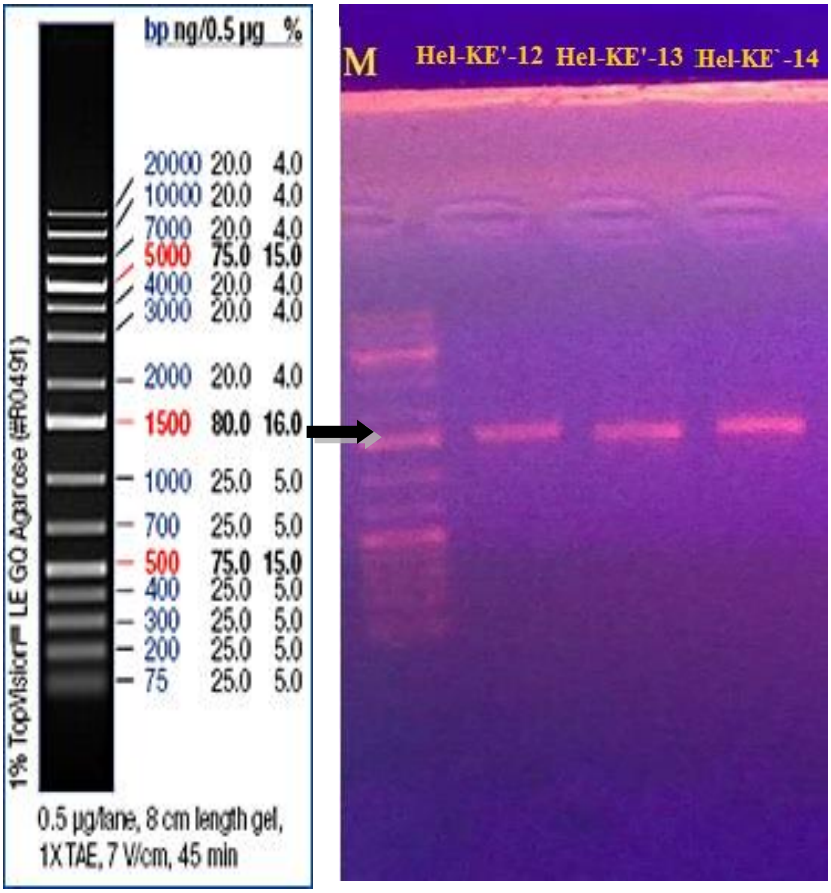


Figure (3): Amplified fragment of 16s rRNA gene of bacterial isolates Hel-KE`-14. M: Marker DNA

Figure (4): Nucleotide sequence of partial 16S rRNA gene (883-bp) of isolate Hel-KE'-14

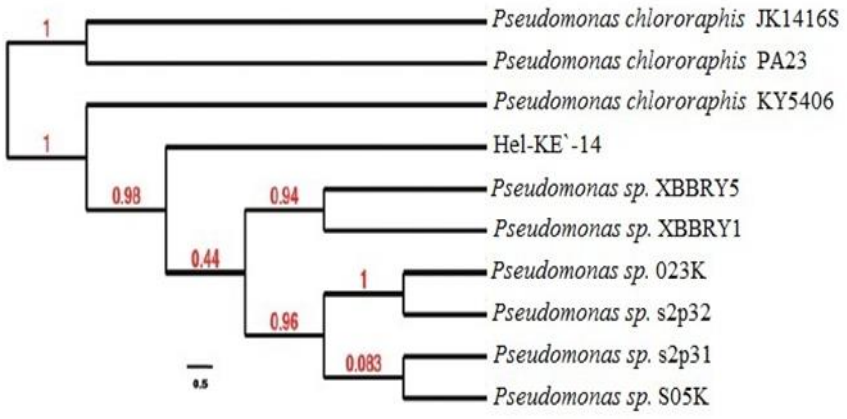


Figure (5): Phylogenetic tree showing genetic relationship of Pseudomonas chlororaphis Hel-KE`-14 with taxonomically similar species based on 16S rRNA sequences using MEGA 4 software

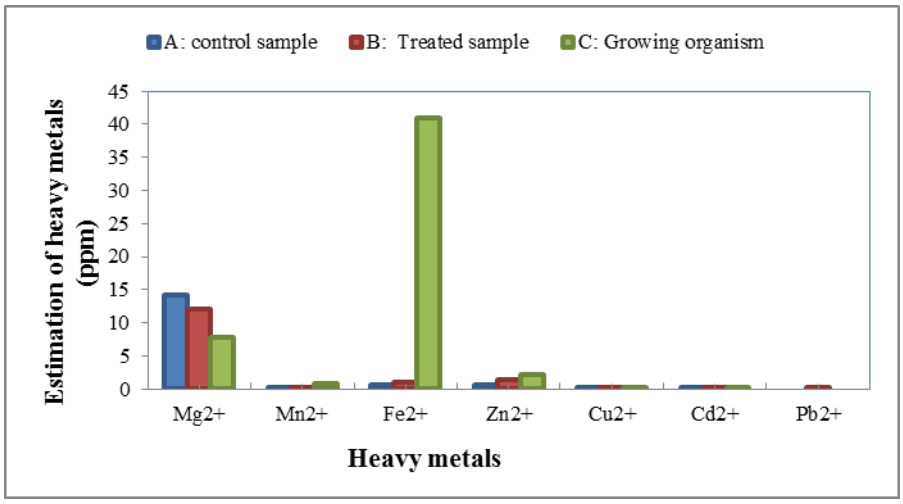
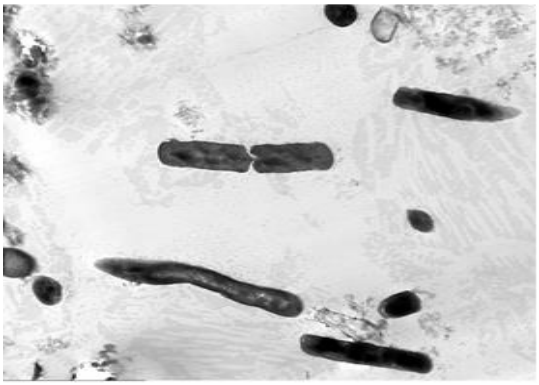
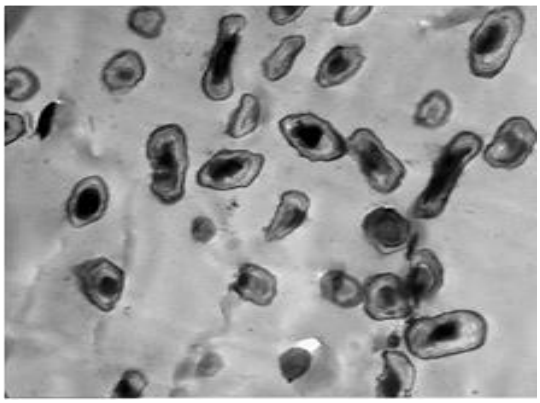


Figure (6): Estimation of heavy metals content of wastewater for *P. chlororaphis* Hel-KE`-14





Transmission electron microscopy of *P. chlororaphis* Hel-KE`-14 (A): Control cells (x15000), (B): treated cells with wastewater (x20000).