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Evaluation of nickel tolerance by identified *Pseudomonas aeruginosa* isolated from Egyptian polluted soils

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In Egypt, heavy metal pollution causes environmental problems to human, animal, plant and ecosystem. This study aims to solve this problem by bacterial isolates. The bacterial isolates were isolated from 12 polluted site soil mainly industrial soils. The total content of Nickel was measured in soil and recorded the dangerous values to environment above safe international ratios. Also, total count of bacterial colonies recorded significant distribution in all soils. Isolation and screening of nickel tolerant bacterial isolates were recorded that some bacterial isolates were tolerate high nickel concentration up to 600 ppm. MTC, MIC and Tolerance index indicated that there was one bacterial isolate were tolerated high concentration of nickel. This bacterial isolate identified as *Pseudomonas aeruginosa* after morphological, biochemical and molecular identification. Also, it included that study the effect of nickel on *Pseudomonas aeruginosa* by using TEM. In finally, it obtained detection of plasmid as indicator for nickel tolerance by *Pseudomonas aeruginosa.*

Keywords: Plasmid, Nickel, *Pseudomonas aeruginosa, Tolerance, Heavy metals and Egyptian soil*

INTRODUCTION

Heavy metal pollution is currently a major environmental problem because metal ions persist in the environment due to their non-degradable nature. The toxicity and bioaccumulation tendency of heavy metals in the environment is a serious threat to the health of living organisms (Ayangbenro and Babalola, 2017). Nickel refer to heavy metallic element which has a relatively high density and toxic or poisonous even at low concentration. Due to various anthropogenic activities, soil and water resources were reported to be polluted by it and have become a serious threat to public health and ecosystems. (Begum and Aundhati, 2016) . Certain types of microbial biomass can keep comparatively high quantities of metal by means of passive processes known as biosorption. Such processes were of industrial interest, since the removal of potentially hazardous heavy metal from industrial effluents by microbial biomass can lead to detoxification, also to recovery of valuable elements. The use of bacterial biomass for the metal removal process of effluents is a viewpoint suggested by many researchers dealing with metal-bacteria interactions(Krishna, 2012). Previous studies have well documented the occurrence and abundance of metal-tolerant microbes in metal- polluted water bodies. Bacterial species such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Proteus mirabilis* and *Staphylococcus* sp. isolated from wastewater samples were found to be tolerate to heavy metals. These microorganisms develop a variety of tolerance mechanisms to survive in different heavy metal concentrations, but the tolerance is often specific to one or few metals (Bhagat et al., 2016). These heavy metal tolerant bacterial species may serve as an important and

cost-effective bioremediation tool for the removal of heavy metals from wastewater and industrial effluents, preventing the pollution of water bodies (Jan et al., 2014). In Egypt, the bacteria displaying a high growth rate were isolated from polluted industrial waste water. The Enterobacteriaceae had variable tolerant to different heavy metals (El-shanshoury et al., 2013).

Accordingly, the present study aims to isolate and identification of tolerant heavy metals bacteria from soil collected from polluted sites, evaluation the tolerance of the most potent bacterial isolate to nickel heavy metal and detection of plasmid before curing and after curing also, study the effect of nickel heavy metal on bacterial cells by using Transmission Electron Microscope (TEM).

MATERIALS AND METHODS

Sampling and experimental localities

36 polluted soil samples were collected from 12 different sources as following in figure (1). According to (Mohammadi and Eslami, 2007), soil samples were collected from the surface of the soil (0 - 30 cm deep) and preserved by using the methods of soil analysis. The soil sampling spots were cleared of debris before sampling. Each composite soil samples were placed in cellophane bags labeled then taken to the laboratory for pre-treatment and analysis. In the laboratory, bulk soil samples were spread on trays and were air dried at ambient conditions for two weeks.

Figure (1): Map of polluted soil samples collection

Determination of total content of nickel in polluted soil samples

According to (Mudhoo et al., 2012), The polluted soil samples were digested to measure it by flame Atomic Absorption Spectrophotometer at *Agriculture Research Center Giza, Egypt*.

Total count of bacteria in soil samples and Colony Forming Units (C.F.U)

According to (Allen, 1959), Serial dilutions were made to cover the range of 10^{-1} to 10^{-7}. 100 µl of each dilution was transferred to petri dishes and pour medium then spreading of dilution and medium. Three plates were prepared for each dilution. Medium used for total count of bacteria is Plate count agar medium. According to (Goldman, 2008), Colony forming unit (CFU) a unit used to estimate the number of viable bacteria or fungal cells in a sample.

Isolation and purification of Ni tolerate bacterial isolates

According to (He et al., 2010), 10 g soil samples were added to Erlenmeyer flasks containing 90 ml of sterile physiological salt solution and shaken at 180 rpm for 30 min. Then, serial dilutions of soil suspensions were plated onto Tris Minimal salt agar medium amended with 25 mg/l (25 ppm), 50 mg/l (50 ppm) and 100 mg/l (100 ppm) of Ni as Nickel Chloride. Bacterial isolates showing different morphological appearance and were selected to and Gram stain to maintain and insure from purified colonies then stored it's on slants contained Nutrient Agar **medium** with low heavy metals concentration.

Screening of bacterial isolates tolerating up to high concentration from nickel

Determination of metal tolerance of the isolated population the metal tolerance range for growth was determined on Tris Minimal salt agar medium. Ni tested using final metal concentrations ranging from 100 to 1200 ppm. Tolerance of the bacterial isolates to varying concentrations of nickel were determined by agar dilution method **(Luli et al., 1983)**.

Determination of Maximum Tolerance Concentration (MTC) and Minimum Inhibitory Concentration (MIC) for more nickel tolerant bacterial isolates

After preliminary selection of nickel tolerant isolates, the MTC and MIC of nickel was determined. For the quantitative determination of heavy metal tolerance, the isolated strains were inoculated in Tris Minimal salt broth supplemented with increasing concentrations of nickel (100 to 1000 ppm). The highest concentration of metal ions at which bacterial growth was observed and defined as the maximum tolerance concentration (MTC) of the respective heavy metal for the isolated strain while the lowest concentration

defined as Minimum Inhibitory Concentration (MIC). Tris Minimal salt broth without the metal ions was also inoculated with the isolated strains to serve as controls. All experiments were performed in triplicates (Schmidt and Schlegel, 1994).

Tolerance Index (TI)%

A 1 mL pre-inoculum of purified isolates grown for 24 hours in nutrient broth at 37 ^{0}C was inoculated (three replicates and a control) aseptically in 50 mL of Tris Minimal salt broth supplemented with concentration of nickel (200 ppm). The broth flasks were incubated at 37°C for 48 hours in an incubator shaker (150 rpm). Bacterial growth was observed (as indicated by optical density values at 600 nm). The tolerance index is defined as the ratio of the optical density values (O.D.) in the Ni treated medium and in the untreated medium (Muñoz et al., 2012). The tolerance index (TI) was calculated by the following formula**:**

$$\text{Tolerance index}(\%) = \frac{\text{growth in solution} + \text{metal}}{\text{growth in solution} - \text{metal}} \times 100\%$$

Identification of the most potent Ni tolerant bacterial isolate

According to (Benson, 1990), (Cowan, 1993), (Collins et al., 1984), (Farrow et al., 1989) and (Krieg and Holt, 1984), morphological, biochemical and molecular identification of the most potent bacterial isolate were done.

Morphological and biochemical identification

Colony morphology on agar medium and bacterial cell morphology by Gram stain. Also, Automated biochemical Identification by using the Biomerieux Vitek 2 System.

Molecular identification

DNA extraction by use protocol of Gene Jet genomic DNA purification Kit (Thermo K0721). Then PCR amplification was made by using Maxima Hot Start PCR Master Mix (Thermo K1051) and the following primers:

F:- AGA GTT TGA TCC TGG CTC AG

R:- GGT TAC CTT GTT ACG ACT T

Then we made PCR clean up to the PCR product using GeneJET™ PCR Purification Kit (Thermo K0701). Finally, sequencing was made to the PCR product on GATC Company by use ABI 3730xl DNA sequencer by using forward and reverse primers. Only by combining the traditional Sanger technology with the new (454) technology. A phylogenetic tree was constructed using the neighbour-joining distance method with the MEGA4 software and the reliability of the bootstrap consensus inferred from 1000 replicates. Some reference sequence from the Gene Bank of most closely related to that of bacteria were used in generating phylogenetic tree (Tamura et al., 2007).

Plasmid Extraction

The plasmids were extracted from the *P. aeruginosa* by the modified miniprep method (Birnboim and Doly, 1979). The isolated plasmids were characterized by agarose gel electrophoresis according to the standard procedure of (Sambrook et al.,1989).

Plasmid Curing

The plasmids were cured by treatment with Chemical agents as Ethidium Bromide, Acridine Orange and SDS according to the method of (Brown, 2000).

Study the effect of heavy metals on bacterial cells by using Transmission Electron Microscopy (TEM)

A 1 mL pre-inoculum of purified isolates grown for 24 hours in nutrient broth at 37 ^{0}C was inoculated (three replicates and a control) aseptically in 50 mL of Tris Minimal salt broth supplemented with concentration of nickel (200 ppm). The broth flasks were incubated at 37°C for 48 hours in an incubator shaker (150 rpm). Specimens of each species were prepared for TEM using the procedures described below according to (Fultz and Howe, 2007) at the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University, Nasr City, Cairo, Egypt.

Data analysis

All experiments were carried out by triplicate sample. Values reported in this study were the means ± Standard Deviation and ± Standard Error using Sigma Plot software program (SigmaPlot 12.5).

RESULTS And Discussion

Determination of total content of nickel in polluted soil samples

Data in figure (2) showed measurement concentration of nickel in polluted soil samples. Results recorded high Ni concentration above safe international ratios in soil as table (1). The most polluted soil with high concentration of Ni was collected from fertilizers industry field. It was

known that long term exposure of soil to heavy metals can considerably modify their microbial populations, reducing their activities and their numbers (Abou-Shanab et al., 2007). In addition, heavy metal tolerant microorganisms were playing an important role in the bioremediation of heavy metal polluted soils (Ray and Ray, 2009)

Table (1): International standard Ni content in soil

International standard	Maximum allowed Nickel concentration (ppm)
Normal in soil	2.7
USEPA	40
GLC	20
WHO	68

USEPA: United State Environmental Protection Agency standard. GLC: Great London Council standard. WHO: World Health Organization Standard Normal concentration (ppm): standard rate in normal agriculture soil according to (Kabata Pendias and Pendias, 1992).

Total count of bacterial colonies in polluted soil samples

Data in figure (2) showed the total count of bacterial colonies in 36 polluted soil samples. The polluted soil with pesticides were recorded the highest number while the cement industry polluted soil recorded low CFU numbers. These values were expected and reported that heavy metals prolong bacterial growth rate, exponential phase of growth, lag period and generation time, leading to decreased bacterial counts (Mahapatra and Banerjee, 1996).

Isolation of nickel tolerant bacterial isolates from polluted soil samples

Data in figure (4) showed number of bacterial isolates can be grown at zero, 25 ppm, 50 ppm, 75 ppm and 100 ppm concentration from nickel chloride for each polluted soil sample. The total bacterial isolates can be grown up to 100 ppm concentration from nickel chloride recorded **63** bacterial isolates. As obtained results, it found that the strains isolated from polluted soil were more tolerant to heavy metals stress according to (Malik and Aleem, 2011).

Screening of nickel tolerant bacterial isolates concentration up to high concentration from nickel chloride

Data in figure (5) showed the ability of **63** nickel tolerant bacterial isolates can grow up to 100 ppm concentration to grow up high concentration (200 ppm, 300 ppm, 400 ppm, 500 ppm, 600 ppm, 700 ppm, 800 ppm and 900 ppm) from nickel chloride. 21 tolerant bacterial isolates were tolerated up to 600 ppm concentration from nickel chloride. Indeed, microbial communities were often found to recover after an initial inhibition by high metal inputs (Holtan-Hartwik et al., 2002).

Determination of Maximum Tolerance Concentration (MTC) and Minimum Inhibitory Concentration (MIC) for more nickel tolerant bacterial isolates

Data in figure (6) showed Maximum Tolerance Concentration (MTC) and Minimum Inhibitory Concentration (MIC) for more nickel tolerant bacterial isolates that tolerate nickel chloride concentration at 600 ppm. Maximum Tolerance Concentration (MTC) determined as maximum concentration from nickel chloride which more nickel tolerant bacterial isolates can be grown. Minimum Inhibitory Concentration (MIC) determined as lower concentration of nickel chloride which more nickel tolerant bacterial isolates were inhibited. The same inoculum size, tris minimal salt medium preparation and incubation conditions were applied. Number of nickel tolerant bacterial isolates tolerating up to 600 ppm 21 isolates and after MTC and MIC number of nickel tolerant bacterial isolates became 7 isolates. As obtained results, heavy metal tolerance was limited according growth inhibition and recorded MTC concentration (Nakahara et al., 1978).

Determination of Tolerance Index % (TI%) for more nickel tolerant bacterial isolates

Data in figure (7a) and (7b) showed that determination of Tolerance Index % (TI%) for (21) more nickel tolerant bacterial isolates. Tolerance Index % (TI%) determined about Optical densities of (21) more nickel tolerant bacterial isolates in broth tris minimal salt medium treated with nickel chloride (600 ppm) and Optical densities of (21) more nickel tolerant bacterial isolates in broth tris minimal salt medium untreated with nickel chloride for each isolate.

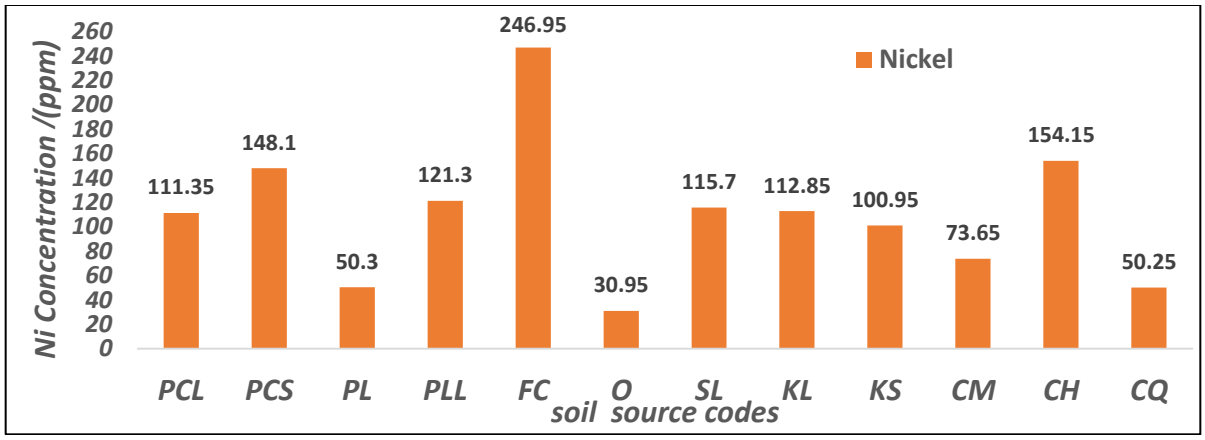


Fig. (2): Determination of total content of nickel

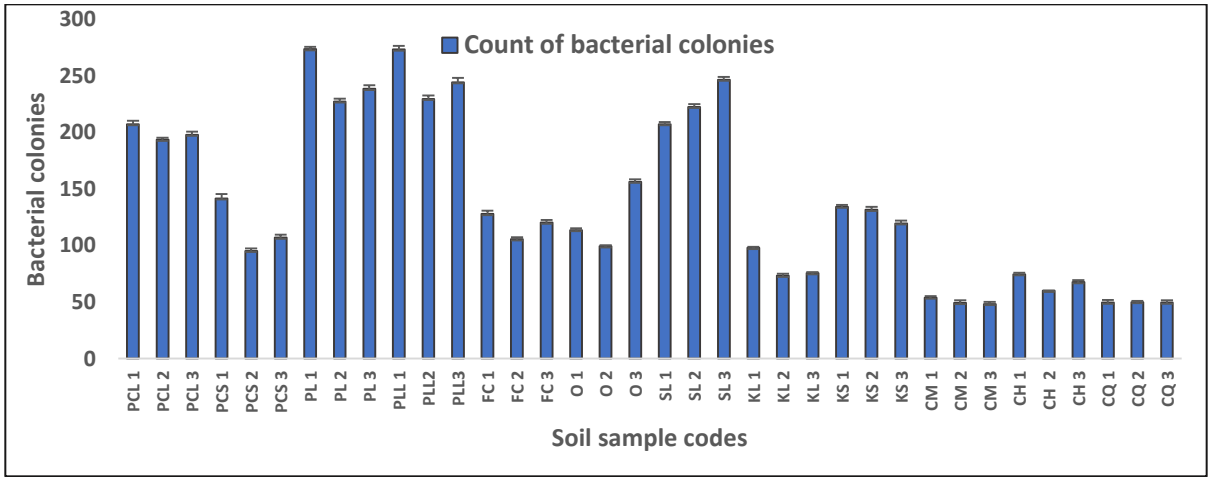


Figure (3): Total count of bacterial colonies in polluted soil samples

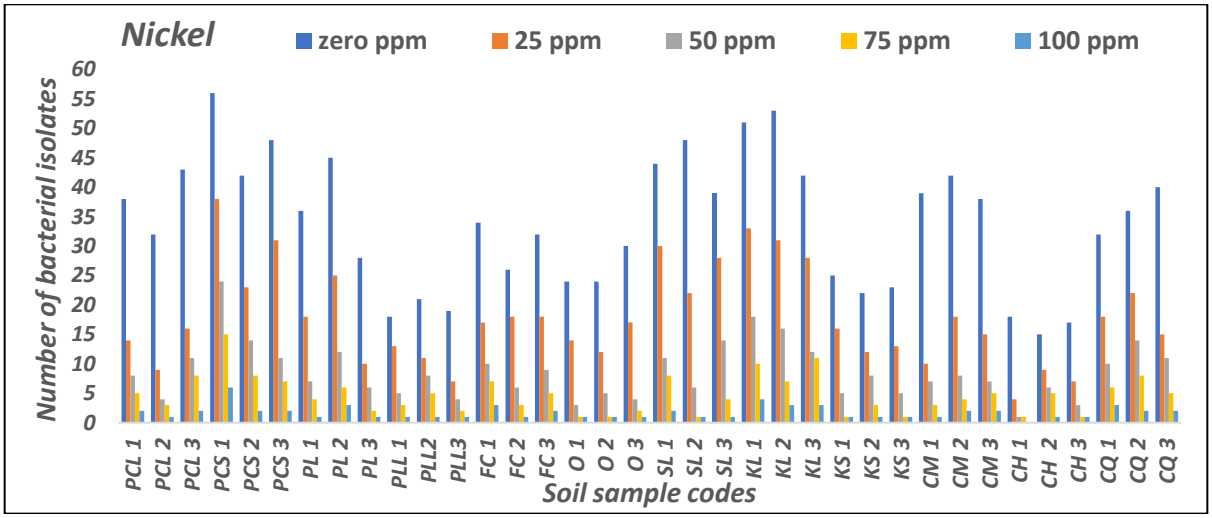


Figure (4): Isolation of nickel tolerant bacterial isolates from polluted soil samples

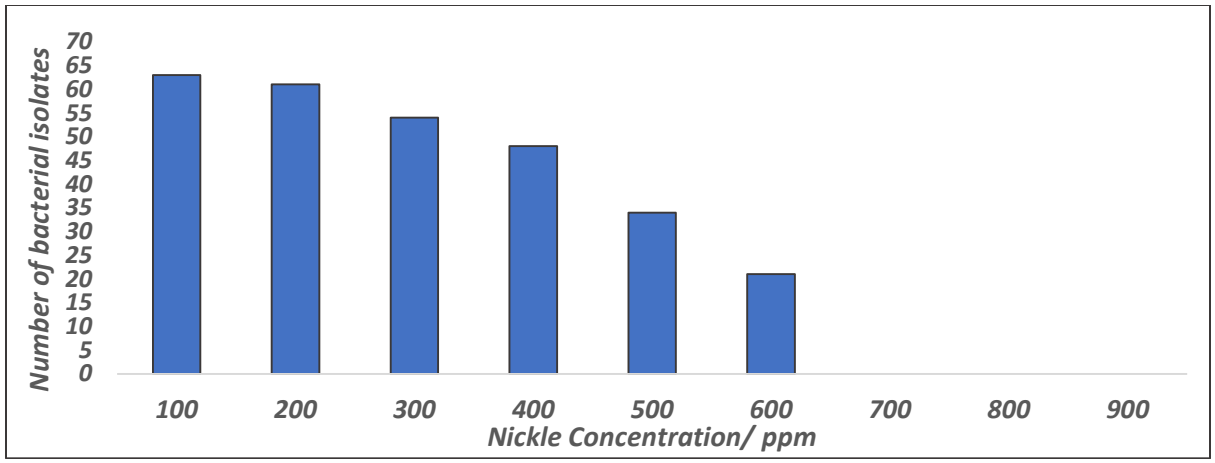


Figure (5): Screening of nickel tolerant bacterial isolates up to high concentration from nickel

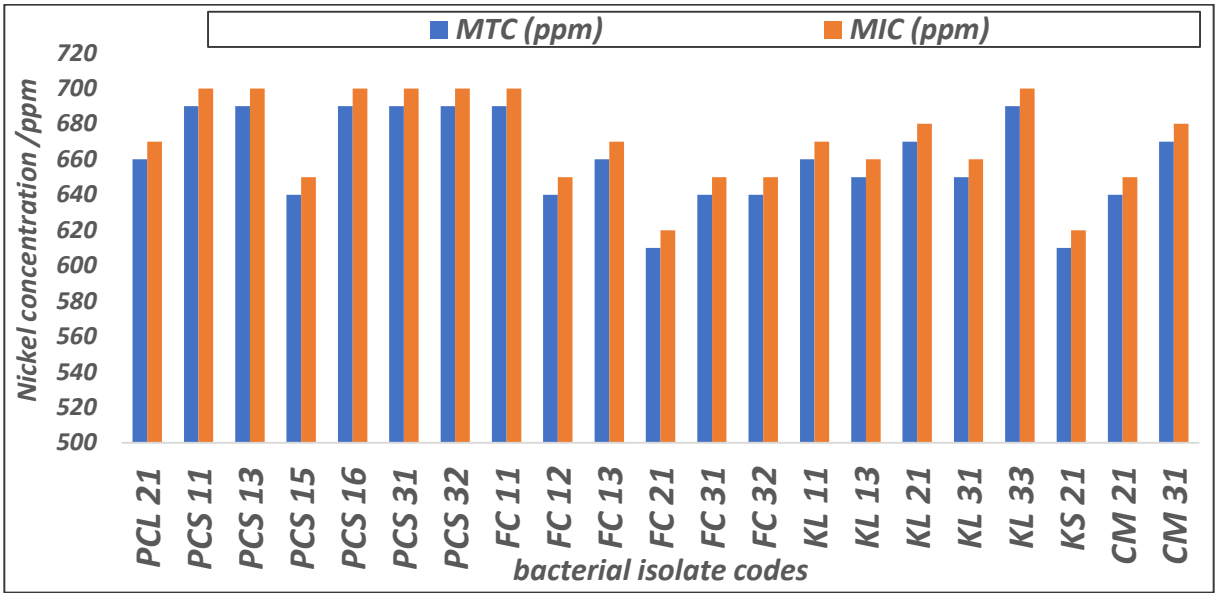


Figure (6): Determination of Maximum Tolerance Concentration (MTC) and Minimum Inhibitory Concentration (MIC) for nickel tolerant bacterial isolates

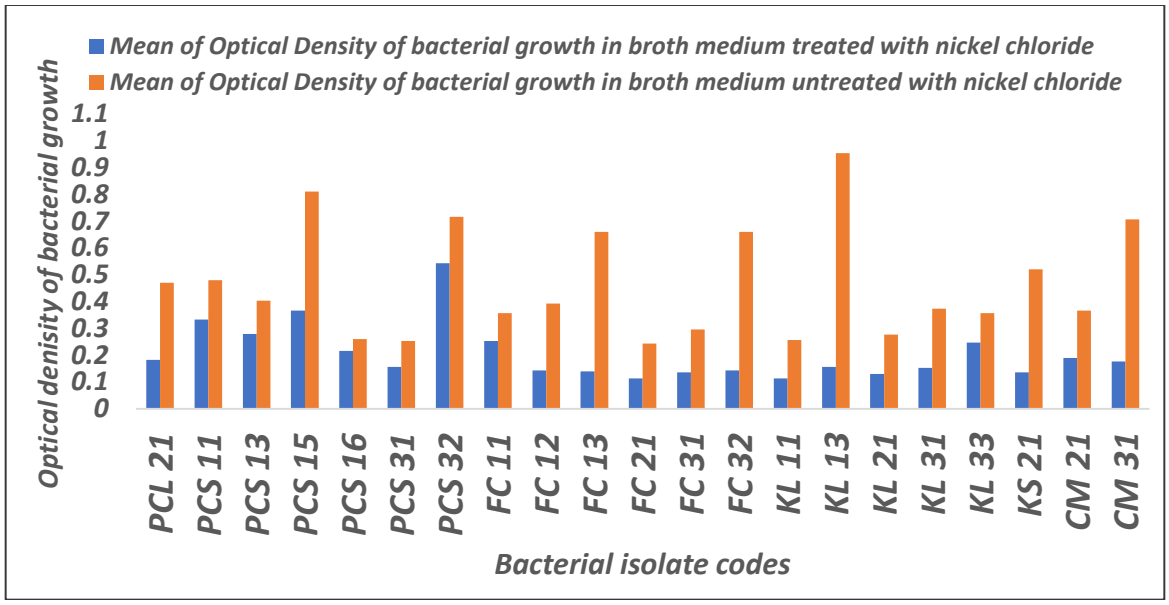


Figure (7a): Optical Density values of bacterial growth in case of treated and untreated by nickel chloride in broth medium

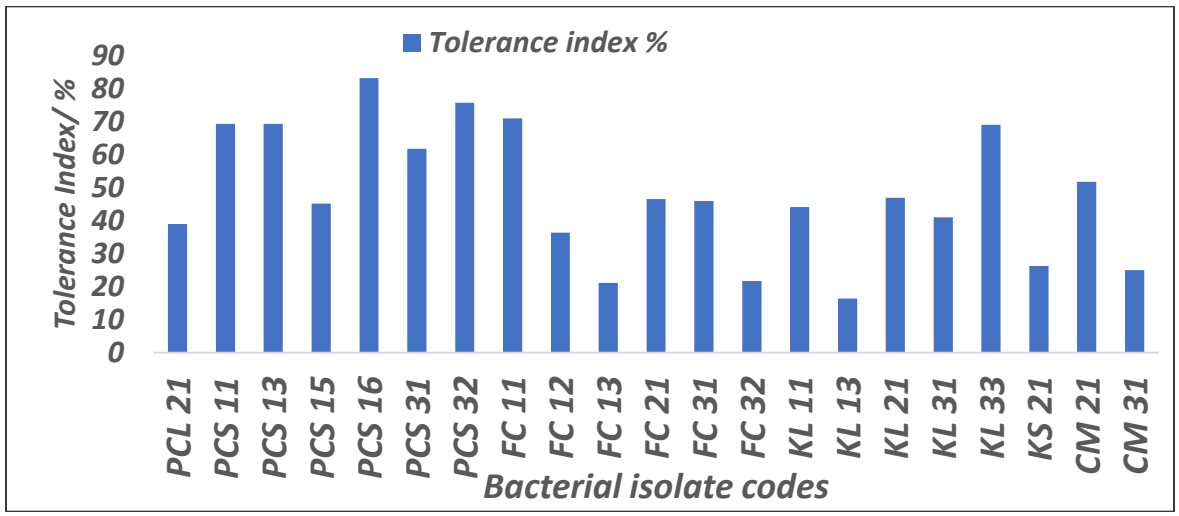


Figure (7b): Calculation of Tolerance Index % (TI%) for Ni tolerant bacterial isolates

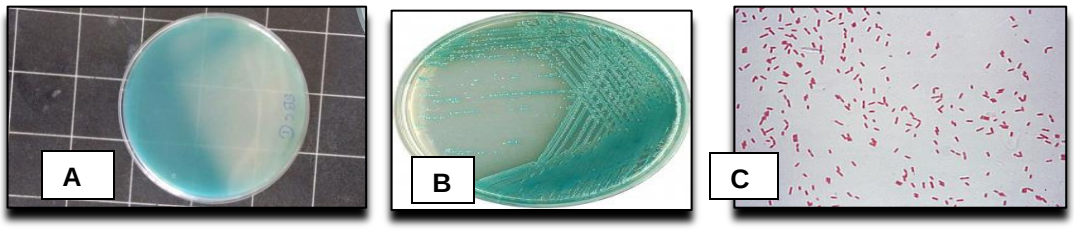


Figure (8): Morphological Identification of the most potent bacterial isolate

After Tolerance Index % (TI%) determinations, number of 21 more nickel tolerant bacterial isolates became 7 isolates. The difference in toxicity toward bacterial isolates could be explained by the conditions of bacterial isolation and the nature and physiological characteristics of each bacterial isolate (Hassan et al., 2008). Also, Ni-tolerant *Micrococcus* sp. was studied for its applicability in bioremediation of industrial waste water (Congeevarama et al., 2007).

Identification of the most potent Ni tolerant bacterial isolate

Photos in figures (8, 9 and 10) showed the morphological, biochemical and molecular identification of the most potent bacterial isolate that tolerate high concentration of nickel. This identification was agreeing with [(Balcht and Smith, 1994), (Allesen-Holm, 2006) and (Shovarani, 2008). The final identification after morphological, biochemical and molecular identification recorded *Pseudomonas aeruginosa* bacterial strain.

Morphological Identification of the most potent bacterial isolate

Photos in figure (8) showed the colony and cell morphology of the most potent bacterial isolate which photos A and B showed the shape of bacteria on solid medium while photos C showed bacterial cell shape, size and arrangement under light microscope examination after Gram stain work.

Biochemical identification of the most potent bacterial isolate by Vitec 2 system

Data in figure (9) showed the report of Vitec (2) system which recorded the result of 64 biochemical tests, the result recorded 94% probability and very good identification for bacterial isolate. Bacterial isolate was identified as *Pseudomonas aeruginosa.*

Molecular Identification of the most potent bacterial isolate

DNA of the most potent nickel tolerant bacterial isolate was amplified with primers. The PCR amplified products were detected by 0.8% agarose gel electrophoresis with ultraviolet (UV) which showed in figure (10-A). The length of object fragment is about ~1, 500 bp. Sequence analysis of the 16S rRNA gene has been considered a fast and accurate method to identify the phylogenic position of bacteria. Partial 16S rRNA of *Pseudomonas* sp were sequenced and used to construct phylogenetic development trees which showed at figure (10-C). Comparative analysis of the sequences with already available database showed that the strains were closed to the members of genus and it was classified in the branch *Pseudomonas aeruginosa*. The blast of sequenced nucleotides was assigned by NCBI Genbank.

Plasmid Extraction and curing to detect their potentiality in Ni tolerance

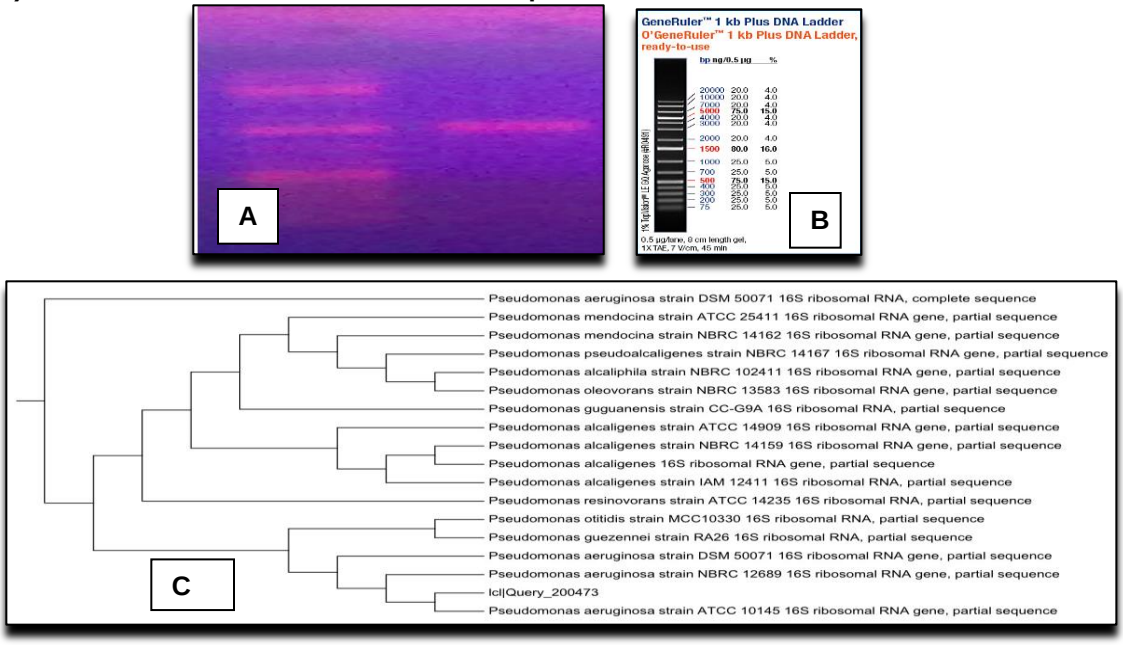
Photos in figure (11-A) showed that isolation of plasmid DNA from bacterial isolates revealed the presences of single plasmid. The size of the plasmid was approximately more than 21 kb. the *Pseudomonas* species have tolerance to a variety of heavy metals; hence they have generated a high degree of interest in environmental bioremediation according to (Bruins, 2003). Plasmids in *Pseudomonas* species were known to code for heavy metal tolerance, resistance and degradation of organic compounds (Chakrabarty, 1976). In the present study, the plasmid DNA was isolated from *P. aeruginosa* which revealed the presence of single plasmid. Moreover, loss of certain genetic markers because of treatment of bacterial cell to plasmid curing agents. Some heavy metal resistance determinants move from plasmid to chromosome (or in the reverse direction). This makes plasmid encoding heavy metal tolerance an important aspect of environmental research (Mesas et al., 2004). The cured colonies were analyzed for the presence of plasmid DNA on agarose gels (Figure 11-B). There was difference of plasmid profile between cured strain and wild strain. plasmid curing by appropriate concentrations of curing agent resulted in colony inhibition so that there was no growth of colony on medium containing nickel. The curing treatment was successfully done when cured cells which still allowed growth in medium containing the highest concentration of curing agent could not grow in medium supplemented with nickel varied among plasmids of bacterial isolates (Irc et al., 2016).

Identification Information	Card:	GN	Lot Number:	2410052103	Expires:	Jan 17, 2018 12:00 GMT+02:00
	Completed:	Aug 14, 2017 16:21 GMT+02:00	Status:	Final	Analysis Time:	8.00 hours
Selected Organism	94% Probability		Pseudomonas aeruginosa			
	Bionumber:	0007451143540?52			Confidence:	Very good identification
SF Organism						
Analysis Organisms and Tests to Separate:						
Analysis Messages:						
Contraindicating Typical Biopattern(s)						
Pseudomonas aeruginosa	dTRE(8),PHOS(5).					

Biochemical Details

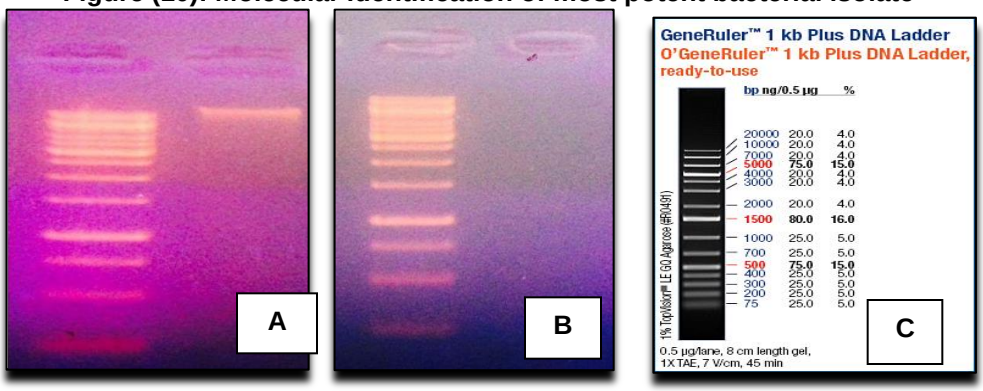
2	APPA	-	3	AlDO	-	4	PyrA	-	5	IARL	-	7	dCEL	-	9	BGAL	-
10	H2S	-	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GGT	+	15	OFF	(+)
17	BGLU	-	18	dMAL	-	19	dMAN	+	20	dMNE	+	21	BXYL	-	22	BAlap	+
23	ProA	+	26	LIP	-	27	PLE	-	29	TyrA	+	31	URE	-	32	dSOR	-
33	SAC	-	34	dTAG	-	35	dTRE	+	36	CIT	+	37	MNT	+	39	5KG	-
40	LATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	-	45	PHOS	+
46	GlyA	(-)	47	OIDC	-	48	LDC	-	53	IHISa	+	56	CMT	+	57	BGUR	-
58	O129R	+	59	G3AA	-	61	IMLTa	+	62	ELLM	-	64	ILATa	+			

Figure (9): Biochemical identification of the most potent Ni tolerant bacterial isolate



A: PCR product, **B:** DNA ladder standard, **C:** Phylogenetic Tree after sequencing and blast on NCBI

Figure (10): Molecular identification of most potent bacterial isolate



A: Plasmid before curing **B:** Plasmid after curing **C:** DNA ladder

Figure (11): Plasmid Extraction before and after curing to detect their potentiality in Ni tolerance

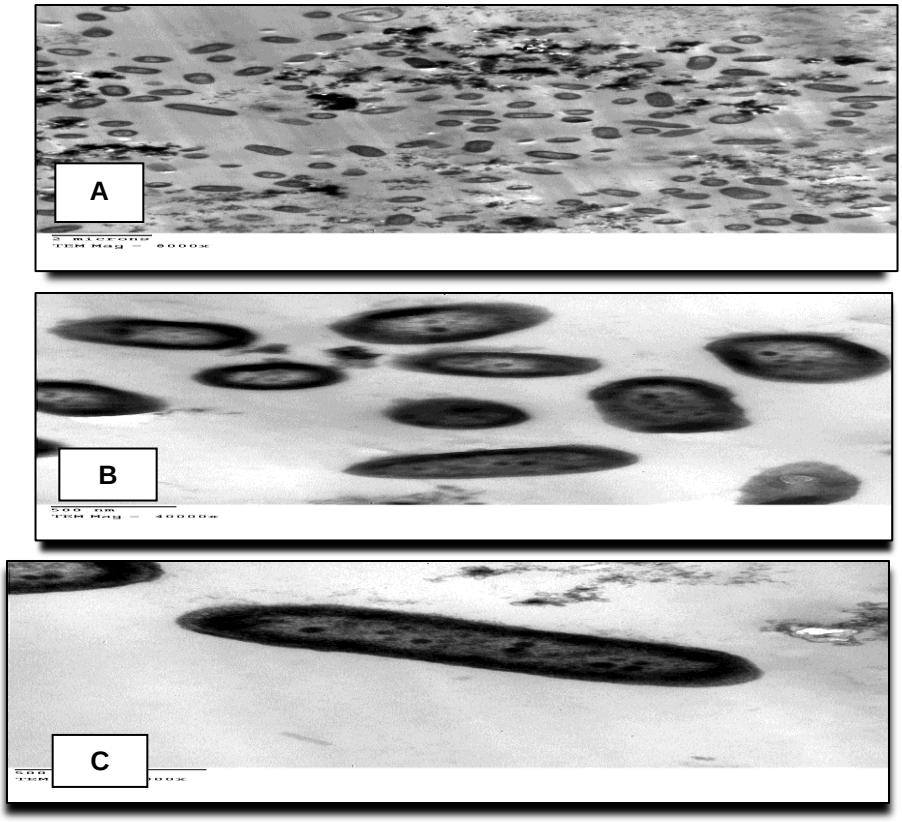


Figure (12): Showing the effect of nickel on bacterial cells by using Transmission Electron Microscopy (TEM)

Study the effect of nickel on bacterial cells and examination by using Transmission Electron Microscopy (TEM)

Photos in figure (12) showed the effect of nickel on *pseudomonas* sp., this effect was examined under TEM as photo A, B and C. In images, there were difference in the uptake may be due to the difference in mechanisms by which the bacteria can tolerate nickel heavy metal and effect of Ni on cellular structures and the cell wall uptake. There are at least three types of microbial processes that can influence toxicity and transport of metals: biotransformation, bioaccumulation and biodegradation. However, microorganisms can inter act with these contaminants and transform them from one chemical form to another by changing their oxidation state through the addition of (reduction) or removing of (oxidation) electrons (Choi et al., 2009). Because of the above mentioned molecular events various cellular metabolic processes were inhibited which were detrimental to the cell. The cellular processes which get inhibited due to metal toxicity. The goal of microbial remediation of heavy metal contaminated soils and sediments were to immobilize the metal in situ to reduce metal bioavailability and mobility or to remove the metal from the soil. Metals are bio- precipitated or undergo biotransformation via various metabolic pathways. Biotransformation may result in a less toxic form of the metal reducing the risk of metal pollution (Maier et al. 2009).

CONCLUSION

In view of the results of heavy metal tolerance and accumulation experiments, it was concluded that *Pseudomonas aeruginosa* bacterial strain has the tendency for tolerate the nickel heavy metals due to it has plasmid that carry genes and play important role in tolerance of heavy metals, so it will be promising for new trends in heavy metals bioremediation and bioaccumulation in the future. Advances in the understanding of metabolic pathways of bacteria were responsible for metal sequestration, improving bacteria survival rates, and their stability has led to the manipulation of metal adsorption. The bacterial cellular structure can trap heavy metal ions and subsequently sorb them onto the binding sites of the cell wall. The amount of metal sorbet depends on the kinetic

equilibrium and composition of the metal at the cellular surface. The current study recommended use of *Pseudomonas aeruginosa* as bio sorbents is eco-friendly and cost effective; hence, it is an efficient alternative for the bioremediation of nickel heavy metal from polluted environments.

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CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest".

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AUTHOR CONTRIBUTIONS

All authors contributed equally in all parts of this study

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