Isolation and characterization of *Pseudomonas* resistant to heavy metals contaminants

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ABSTRACT

In this work, chromium, copper, nickel, cadmium were selected as a model for metal contamination. This selection is based on the fact that these metals are discharged in many of the industries such as electroplating, detergents, oil refining and others. The isolation of bacteria resistant to different metal ions was done by using mixed industrial and domestic wastewater from the western station of sewage treatment plant in Alexandria. Iron limiting Casamino acid media is used in this study, since it can induce the production of fluorescent siderofores of the Pseudomonas species. Eighteen colonies were selected and purified as single colonies. The preliminary observation and the biochemical identification of these isolates indicated that the selected isolates are belonging to Pseudomonas species. Screening of the bacterial isolates for metal resistance against Cr(VI), Cu(II), Cd(II) and Ni(II) was done by the use of MIC and MTC (Maximum tolerable concentrations). Different metal concentrations were used throughout the screening to select bacterial isolates capable to grow and resist the metal toxicity. The optimum pH of metal precipitation was around 6. Whereas the optimum growth pH for Cr and Ni resistant strains was 5.5, while was 6 for Cu and Cd resistant strains.

Keywords: *Heavy metals, wastewater, screening, metal resistance, bioaccumulation.*

INTRODUCTION

The pollution of the environment with toxic heavy metals is spreading throughout the world along with industrial progress. Copper, chromium, cadmium and nickel are known to be the most commonly heavy metals used and the more widespread contaminants of the environment (Patterson, 1977; Aksu, 1998; Doenmez and Aksu, 1999). Traces of these heavy metals are necessary as Co-factors of enzymatic reactions, but high levels of them may cause extreme toxicity to living organisms due to inhibition of metabolic reactions. The microorganisms respond to these heavy metals by several processes; including transport across the cell membrane, biosorption to the cell walls and entrapment in extracellular capsules, precipitation, complexation and oxidation-reduction reactions (Rai *et al.*, 1981; Macaskie and Dean, 1989; Huang *et al.*, 1990; Avery and Tobin, 1993; Brady *et al.*, 1994; Veglio *et al.*, 1997).

The bioremediation of heavy metals using microorganisms has recieved a great deal of attention in recent years, not only as a scientific novelty but also for its potential

application in industry. Metal accumulative bioprocess generally falls into one of two categories, bisorptive (passsive) uptake by nonliving, non growing biomass or biomass products and bioaccumulation by living cells (Macaski and Dean, 1989; Aksu and Kutsal, 1990; Huang *et al.*, 1990; Volesky *et al.*, 1992; Avery and Tobin, 1993; Brady and Duncan, 1994; Aksu, 1998; Doenmez and Aksu, 1999; 2001).

Industrial wastes containing toxic metals were characterized by their differences rather than their similarities. These toxic metals can arise from a wide variety of industrial processes. The quality and the quantity of the wastes containing toxic heavy metals are dependent upon their industrial sources.

Intrinsic bacteria, which are capable of metal accumulation, existing in soil on or near the site of contamination have adapting mechanisms to the contaminant. Naturally occurring bacteria that are capable of metal accumulation, have been extensively studied since it is difficult to imagine that a single bacterium could be capable to remove all heavy metals from its polluted site (Clausen, 2000).

Apparently, the metal, which has been introduced into the bacterial suspension by vigorous mixing, forms complexes with various ligands available (constituents which will complex heavy metal ions) (Hussein *et al.*, 1998; 2001). Consequently, the largest amount of metals will be found as hydroxide or as a stable metal-ligand complex. Under a specific stress conditions, a relatively constant amount of metal reacts to stable and inactive complexes with active cellular components (Hussein, 1999). However, it is very important before the optimization of the bacterial growth process is to study at which pH value will be found as metal ions to study the real interaction between the free metal ions and the bacterial strain.

MATERIALS AND METHODS

Isolation and identification of Pseudomonas bacteria resistant to the four metallic ions under investigation

Samples were taken from sewage treatment plant located in west Alexandria, Egypt. Samples were diluted 10- 10,000 fold in sterile distilled water and plated on casamino acid agar plates containing 1mM Cr(VI), 1mM Cu(II), 1mM Ni (II) and 1mM Cd(II). These plates were incubated for 72 hrs at 30 °C and then a number of morphologically different colonies were randomly picked and isolated after successful purification process on the same medium. In this preliminary screening, colonies showing resistance to Cr(VI), Cu(II), Ni(II) and Cd(II) were selected for further screening processes. Biochemical identification was done according to Burg`s manual.

Screening of the metal resistant bacteria

Unless otherwise indicated, 50-ml liquid medium was inoculated with 0.5-ml inoculum of overnight culture. For screening of the isolated strains on the different metal ions, the obtained colonies were plated on casamino acid agar plates containing Cu Cl_2, Cd Cl_2, Ni SO_4 and $K_2Cr_2O_7$ as sources of the different metal ions to give final concentration of 1 to 10 mM/l of each metal ion. The colonies were subcultured on casamino acid medium and the culture purity was checked. The level of the metal tolerance for the specific metal isolated strains was tested individually by subculturing on liquid casamino acid medium with different concentrations: from 1 to 10mM/l for Cd(II) and Ni(II) and from 1 to 5mMl/l for Cr(VI) and Cu(II).Growth was quantified with respect to the control which containing no metals. To evaluate the levels of resistance, the following two parameters were used: Minimum inhibition concentration (MIC) and maximum

tolerable concentration (MTC). The MTC is the highest concentration of metal, which has no effect on the growth of the resistant strain. From these, one strain was chosen for its specific metal tolerance ability and then the isolated strains undergo growth optimization process.

Medium

Casamino acid media (Solid)

Casamino acid	5 g/l
$K_2H\ PO_4$	0.9 g/l
$Mg\ SO_4$	0.25 g/l
Agar	15 g/l

Casamino acid agar media (Liquid)

Casamino acid	5 g/l
$K_2H\ PO_4$	0.9 g/l
$Mg\ SO_4$	0.25 g/l

Preparation of metal solutions

Salts of Cr (VI), Cu (II), Ni (II) and Cd (II) were individually added in equivalent weights to certain volume of de-ionized water to reach final concentration of 100 mM/l Solutions were sterilized by filtration through a flow pore filter of 0.22 µm pore size and further used for the preparation of the different metal concentrations in the study.

Effect of the pH change on the metal precipitation

The effect of pH variation on the metal precipitation and the proportion of free metal ions in the solution which undergo accumulation or interaction with the bacterial strains was studied. The pH of medium solution containing 10mM/l of each metal ion was adjusted individually from 2 to 9. The suspension was then centrifuged using Pekmann centrifuge (Avanti ^{TM} J- 25) at 10000 rpm for 20 min at 20 °C and the metal concentration was measured in the supernatant using Perkin Elmer, Analyst 300 atomic Absorption spectrophotometer which then represented the free metal ions in the solution or the metal at equilibrium.

Effect of the pH on the growth of the different isolates

Optimisation of the growth of the four isolates (strain 2, 9, 12 and 16) was done. For each of these bacterial strains, 50 ml of the casamino acid media were adjusted at different pH values varied from 5 to 8 and autoclaved at 120 °C and 105 k Pa for 20 min without metal ion after the preculture process. The growth of the bacteria was tested through optical densit measurement at 600 nm.

RESULTS AND DISCUSSION

Sampling environment that contains elevated concentrations of heavy metals was a potential source for toxic metal-tolerant bacteria. It is likely that such environment foster adaptation and selection for heavy metal resistance (Clausen, 2000). This study resulted in the isolation and purification of 18 bacterial isolates (originated from 50-150 colonies) from heavy metal contaminated wastewater site. These isolates have high ability to resist and accumulate one or more of the metal mixture Cr(VI), Cu(II), Cd(II), and Ni(II). All isolates were highly fluorescent and belong to *Pseudomonas* sp. (Koedam *et al.*, 1994). The eighteen isolates were screened for their metal resistance on casamino acid media containing different metal concentrations of each Cr, Cu, Cd and Ni which ranged between 1 to 10 mM/l, to select bacterial isolates capable to grow and resist high level of metal toxicity. MIC and MTC of the 18 bacterial isolates were also investigated using plate assay presented in Tables 1 and 2. Further investigation of the most resistant strains to each metal ion was done in broth cultures amended with different concentrations of each metal. The obtained results were presented in

figures 1-4. From this screening test, the best *Pseudomonas* isolates were selected based on their ability to resist and accumulate certain metal ion. These four selected strains were used for biochemical identification according to Burgy's manual for systematic microbiology as shown in Table 5. Strain 12, which is specific for Cr accumulation, was identified as *Pseudomonas fluorescens*, while strain 16 specific for Cu accumulation was identified as *Pseudomonas putida*, strain 9 specific for Ni accumulation was identified as *Pseudomonas putida* and strain 2 specific for Cd accumulation was identified as *Pseudomonas putida.*

Table (1): MICs of 4 heavy metals for 18 Pseudomonas isolates.

Strains	MIC (mM of metal ions)			
	Cr (VI)	Cu (II)	Ni (II)	Cd(II)
1	3	6	3	5
2	3	6	3	10
3	3	4	3	3
4	3	4	3	3
5	3	5	9	5
6	3	6	10	5
7	3	5	2	1
8	2	5	8	6
9	3	5	11	5
10	4	4	3	1
11	4	4	2	2
12	4	5	4	1
13	3	5	3	5
14	2	5	3	6
15	3	5	10	5
16	3	7	6	7
17	3	4	4	2
18	3	6	4	4

Table (2): MTCs of 4 heavy metals for 18 Pseudomonas isolates.

Strains	MTC (mM of metal ions)			
	Cr (VI)	Cu (II)	Ni (II)	Cd(II)
1	2	5	2	4
2	2	5	2	9
3	2	3	2	2
4	2	3	2	2
5	2	4	8	4
6	2	5	9	4
7	2	4	1	-
8	1	4	7	5
9	2	4	10	4
10	3	3	2	-
11	3	3	1	1
12	3	4	3	-
13	2	4	2	4
14	1	4	2	5
15	2	4	9	4
16	2	6	5	6
17	2	3	3	1
18	2	5	3	3

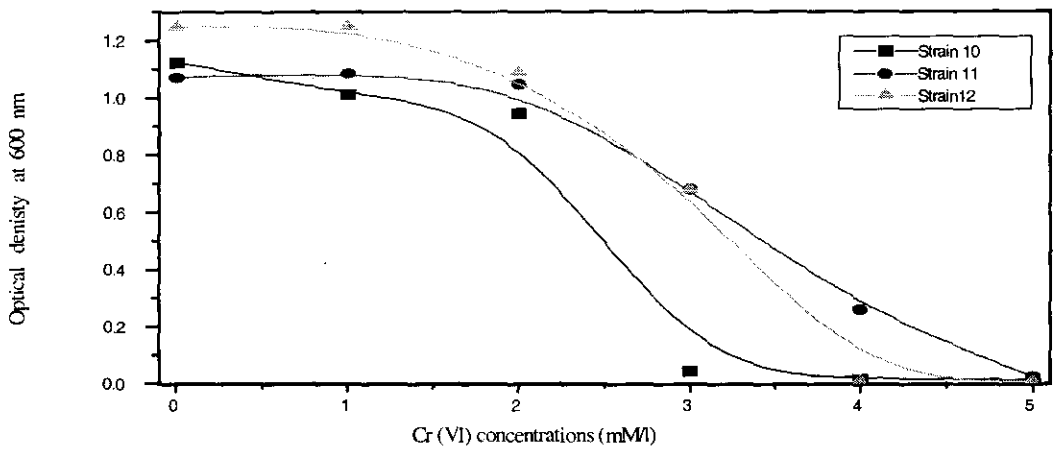

Fig. (1): Comparison between the growth of the three bacterial strains using liquid casamino acid medium in presence of different Cr (VI) concentrations.

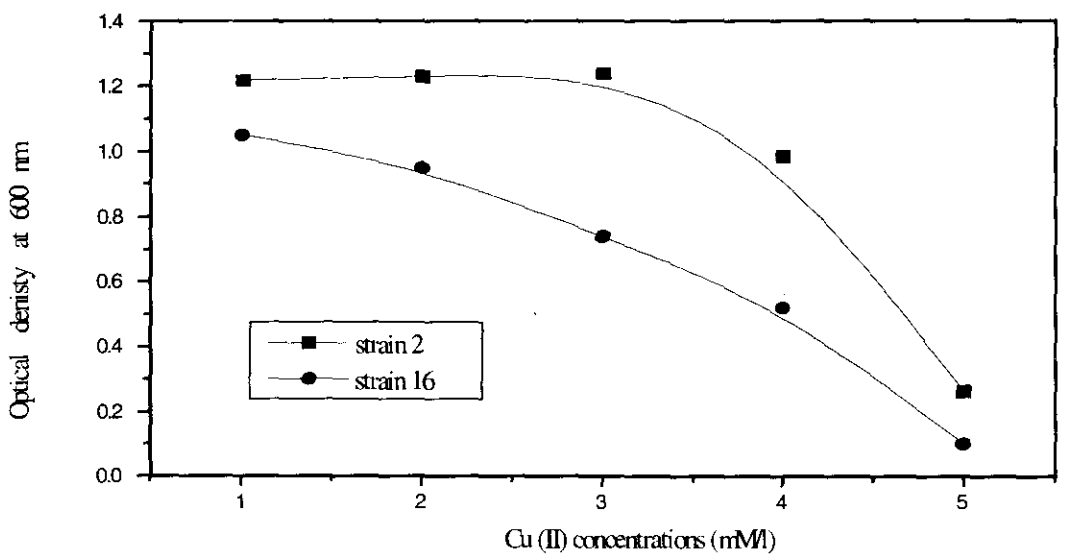

Fig. (2): Comparison between the growth of the two bacterial strains using liquid casamino acid medium in presence of different Cu (II) concentrations.

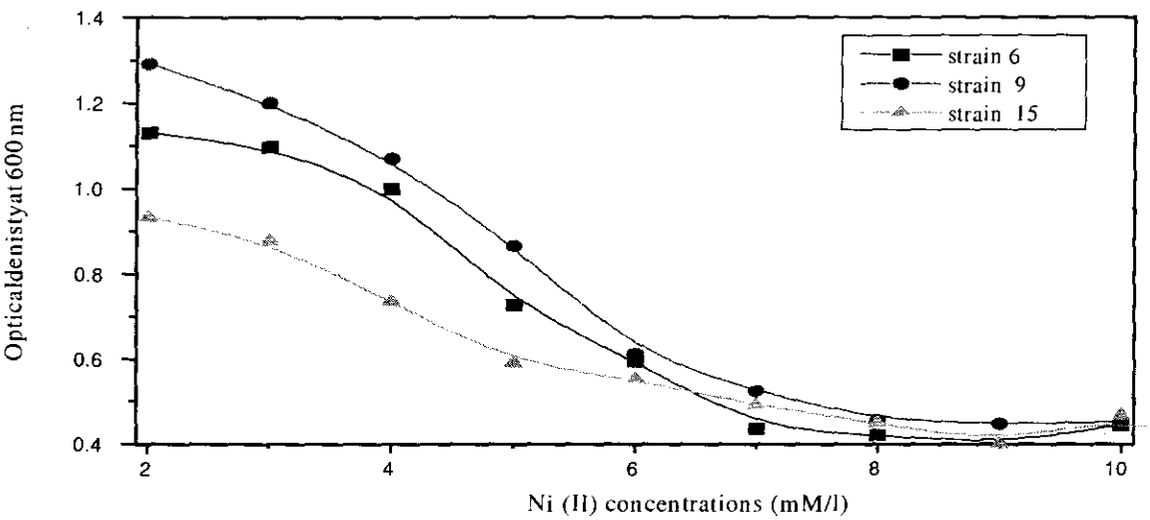

Fig. (3): Comparison between the growth of the three bacterial strains using liquid casamino acid medium in presence of different Ni (II) concentrations.

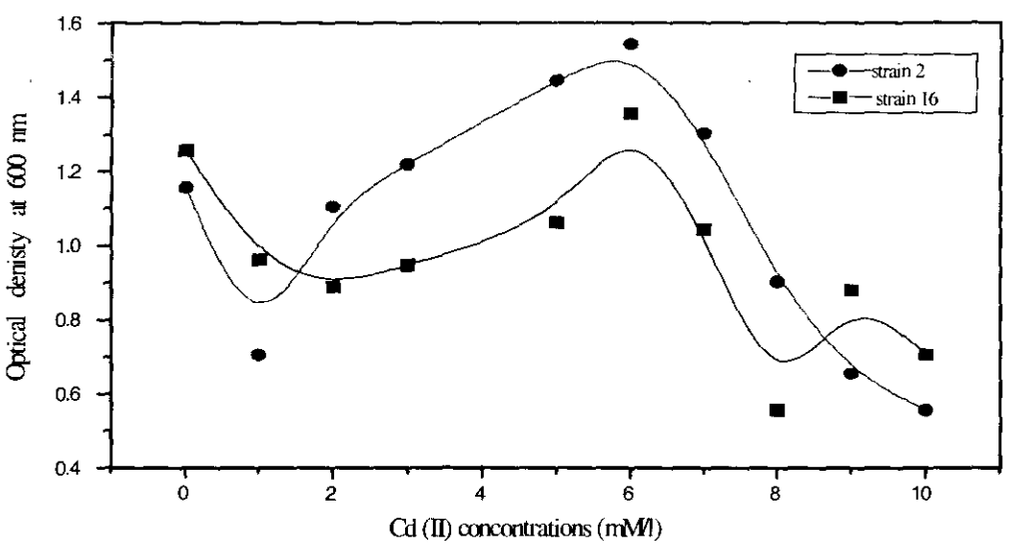

Fig. (4): Comparison between the growth of the two bacterial strains using liquid casamino acid medium in presence of different Cd (II) concentrations.

Table (3): Biochemical characterization of the four bacterial isolates.

Character	Isolate number			
	Isolate 2	**Isolate 9**	**Isolate 12**	**Isolate 16**
Cell shape	Rods	Rods	Rods	Rods
Gram reaction	-	-	-	-
KOH (3%) reaction	+	+	+	+
Motility	+	+	+	+
Spore formation	-	-	-	-
Oxidase	+	+	+	+
Catalase	+	+	+	+
Glucose fermentation	+AG	+A	+A	+A
Sucrose	+AG	-	-	-
D (-) Galactose	+AG	+A	+A	+A
Raffinose	+AG	-	-	-
L (-) Rhamnose	+AG	-	-	-
D (-) Mannose	+AG	+A	+A	+A
Maltose	+AG	-	-	-
D (-) Mannitol	+AG	-	-	-
Lactose	-	-	-	-
D (-) Fructose	+AG	-	-	-
Sucrose	+AG	-	-	-
Ribose	+AG	-	± A	±A
Arabinose	+AG	+A	+A	+A
Trehalose	+AG	-	-	-
Aesculin	-	-	+	-
Enzyme production				
Cellulase	-	-	-	-
Lipase	-	-	+	-
Urease	+	±	-	-
Protease	-	-	-	-
Amylase	+	-	-	-
Pectinase	+	+	+	+
Blood Haemolysis	+G	+G	+G	+G
MacConkey agar	+	+	+	+
KCN	+	+	+	+
Levan formation	+	+	-	+
(O/F) test	+/+	+/-	+/+	+/+
Growth on:				
King Ward & Raney's agar.	-	-	-	-
* Pyocianin	-	-	-	-
* Florescent	-	-	-	-
H_2S production	-	-	-	-
Citrate utilization	+	+	+	+
Identified Species	*Pseudomonas putida*	*Pseudomonas putida*	*Pseudomonas fluorescens*	*Pseudomonas putida*

+: Positive. -: Negative ±: Doubtful .A: Acid production. AG: Acid and gas production. P.R.: Partial reduction. C.R.: Complete reduction. O/F: Oxidation /Fermentation. S.S. agar: *Salmonella* and *Shigella* agar. DCC agar: Desoxchsy cholate citrate agar. TSB agar: Trypticase soy broth.

Effect of the pH change on the metal precipitation

The effect of the pH variation on the metal precipitation was examined in a solution containing 10 mM/l of each metal ion. The metal state was judged by both precipitation of this metal and the residual metal content in the supernatant (Hussein, 1999). It was found that more than 50% of the metal ions were precipitated as hydroxy complex at pH more than 6 as shown in Fig. (5).

Effect of the pH change on the bacterial growth

pH plays a major role for the growth and metal bioaccumulation properties of the bacterial strains (Doenmez and Aksu, 2001). Accordingly, to study the actual interaction of the metal ions and the biomass accumulation it is a must to determine the optimum pH for the bacterial growth. This was examined using the isolated four strains selected from the previous experiments. Figure (6) shows that strains 9 and 12, resistant to Ni and Cr respectively, have optimum growth rate pH of 5.5. Whereas, strains 2 and 16 have corresponding optimum growth pH close to 6. From these results, it is clearly shown that the optimum microbial growth pH lies in the range between 5.5 to 6 which is nearly the same range of pH at which the different metals under investigation exist in free ion state.

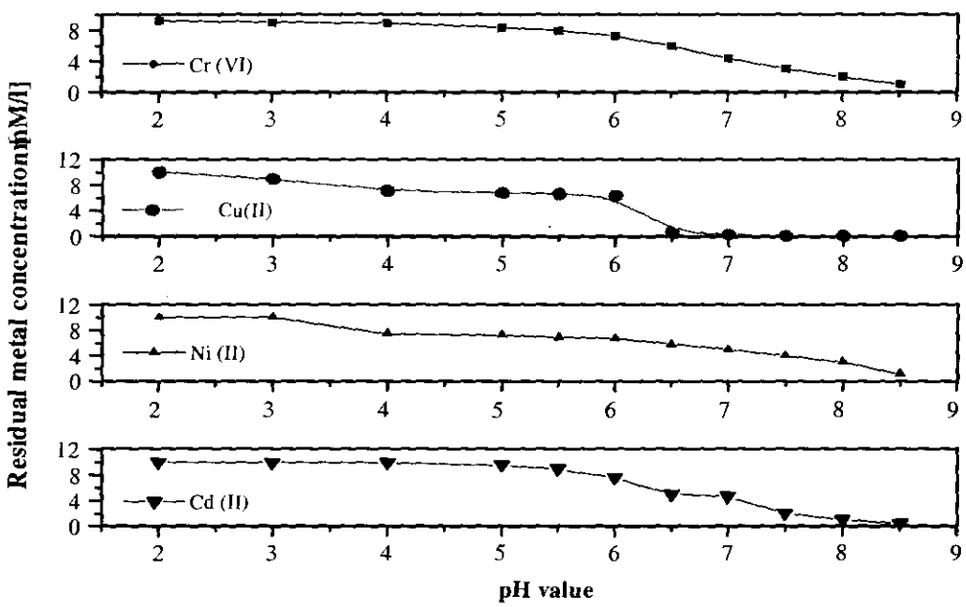

Fig. (5): Effect of pH variation on the precipitation of different metal ions.

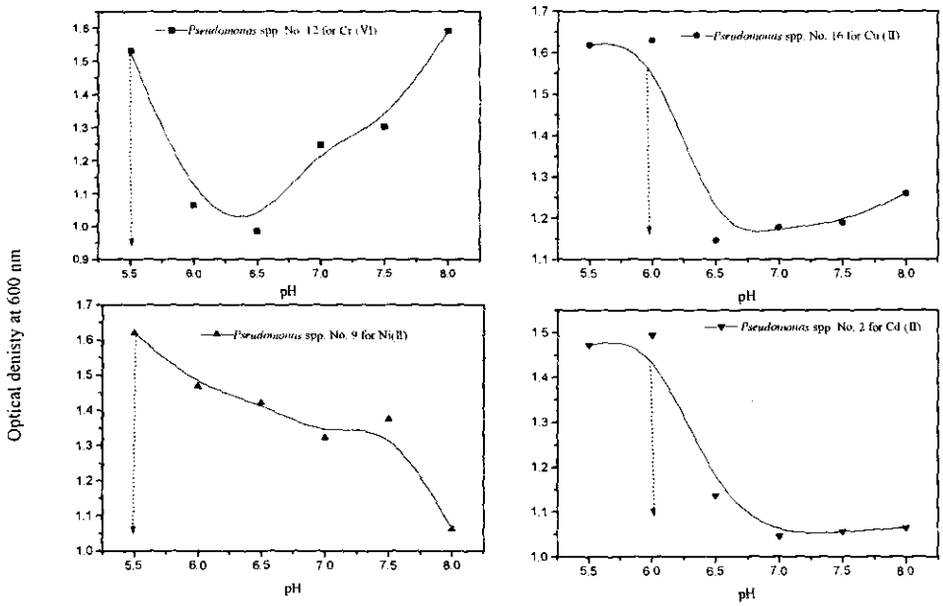

Fig. (6): Effect of pH variation on the growth of the different Pseudomonas species.

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الملخص العربي

عزل وتوصيف بكتيريا السيدوموناس المقاومه للعناصر الثقيله في المخلفات السائله

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في الأونة الأخيرة أثبتت الدراسات أن الكائنات الحية الدقيقة لديها قدرة فائقة على إزالة المعادن الثقيلة من المخلفات السائلة وبالتالي يكون لها دور فعال في تقليل سميتها على النظم البيولوجية . في هذه الدراسة تم عزل ١٨ سلالة بكتيرية من النوع *Pseudomonas sp.* من محطة معالجة صرف صحي بغرب الإسكندرية باستخدام وسط غذائي من النوع Casamino acid medium وتم عمل مسح لهذه العزلات على تركيزات مختلفة من أملاح معادن النحاس الثنائي، الكادميوم الثنائي، النيكل الثنائي، وكذلك الكروم السداسي، وتم اختيار عزلة بكتيرية واحدة لكل معدن لها القدرة على مقاومة تأثيره في الوسط الغذائي. وتم عمل تعظيم لظروف نموها في pH يتراوح بين ٥،٥ و ٦ .