Heavy metals resistance pattern of moderately halophytic bacteria

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ABSTRACT

Beside extremely halophilic archaea, moderately halophilic bacteria are considered to be one of the most important groups of microorganisms adapted to live in hypresaline environments. Moderately halophilic bacteria are defined as those microorganisms with optimal growth in media containing 0.5 to 2.5 M NaCl. Twenty two strains of moderately halophilic bacteria were screened for their heavy metal resistant patterns. A strain that showed the highest levels of heavy metal tolerance was selected based on the cumulative levels of sensitivity. Different minimal inhibitory concentrations of metals were determined for this strain and it was shown to be highly resistante to Ni. Cd and Cu ions showed the highest toxic effect on the strain when combined in non-toxic levels with Ni. Analysis of the intercellular metal contents using atomic absorption spectroscopy revealed the ability of the strain to accumulate Ni ions inside the cell. The gene coding for the 16S rDNA was amplified by polymerase chain reaction using gene specific primers and the PCR product was purified and sequenced. Sequence analysis was done using computer based search through Blast program and the database at the National Institute of Health (USA) and the strain was identified as Staphylococcus sp.

Key words: *Heavy metales, resistance, halophilic bacteria.*

INTRODUCTION

Contamination of the environment by heavy metals is a consequence of technological and industrial processes (Nathalie *et al.*, 2002; Nriagu and Pacyan, 1988; Nriagu, 1996). This has led to the increasing concern about the effects of toxic metals as environmental contaminants. The presence of these contaminants in aquatic environments is known to cause severe damage to aquatic life beside the fact that these metals kill microorganisms during biological treatment of wastewater with consequent delay of the process of water purification. Toxicity of heavy metals to microorganisms is well documented (Nies, 1999; Ehrlich, 1997; Lester *et al.*, 1979).

Moderately halophilic bacteria are one of the most important groups of bacteria that could tolerate high salt concentration. Kushner (1985) defined the moderately halophilic bacteria that could grow in optimum growth with 0.5 to 2.5 M NaCl. Very little attention

has been focused on their response to potential inhibitors such as heavy metals. Some of the heavy metals-resistance halophilic bacteria could be used as bioassay indicator organisms in saline aquatic polluted environments (Trevors *et al.*, 1985). Beside the metal tolerance, halophilic bacteria are important to use in biotechnology. For example they are good source for halophilic enzymes (i.e. amylase, proteases and nuclease) as well as compatible solutes which can be used as a stabilising agent for enzymes and whole cells (Galinski, 1993; Ventosa and Nieto 1995). In the present study, the natural metal tolerance level of 22 moderately halophilic eubacteria and detailed physiological analyses for heavy metals resistance of one of these isolates are reported. An additional aim for this work was to study the interaction between different heavy metals in this strain and the effect of other physiological factors on the resistance patterns.

MATERIALS AND METHODS

Microorganisms and growth conditions

A total of 22 moderately halophilic bacterial strains were examined. All the strains were isolated from salterns and salt lakes in Alexandria, Egypt and were characterized as moderately halophilic eubacteria (Abou Kandil, 2001).

All the strains were grown in halophilic media (Ventosa *et al.*, 1982). The medium contained NaCl, 8.1; $MgCl_2$., 0.91; $MgSO_4.7H_2O$, 0.1; $CaCl_2$. $2H_2O$, 0.036; KCl, 0.2; $NaHCO_3$, 0.006; NaBr, 0.023; bacto-peptone, 0.5; yeast extract, 1.0; glucose, 0.1 and agar 2.0 as percentage (W/V). Minimal medium (Ventosa *et al.*, 1982) used for metal starvation experiments composed of NaCl, 10.0; KCl, 0.2; $MgSO_4$ $.7H_2O$, 0.02; KNO_3, 0.1; $(NH_4)_2HPO_4$, 0.1; KH_2PO_4, 0.05 and glucose, 0.2 as percentage (W/V).

Unless otherwise specified cultures were inoculated with 1% inoculum from overnight preculture and the cultures were incubated at 30°C for two days.

Chemicals

The heavy metals tested were all of high grade and as chloride salts. Stock solutions were made in distilled water and were sterilized by filtration through 0.22 (m pore size membrane filters and kept at 4°C).

Response to heavy metals

Inhibition zone assay

Inhibition zone assay was done to screen the heavy metals resistance patterns for the different strains. Five ml of halophilic media (HM) with 0.7% agar were inoculated with 100 l inoculum from preculture then poured into plates with solidified halophilic medium-agar. After solidification, 0.5 cm paper disk with 10 μl of 100 mM metal stock solution was added on the top of the plate, then incubated for 24hr and the diameter of the inhibition zone was measured.

MIC Determination

The minimal inhibitory concentration (MIC) was done by inoculation of a liquid halophilic medium containing different metal concentrations from 0.5 mM to 10 mM with 1% inoculum from preculture, then incubated on a shaker at 30°C with 200 rpm. The optical denisty at 600 nm was recorded.

Bacterial identification

DNA was extracted according to Sambrook *et al.*, (1989). A DNA fragment of 1300 base pair of the 16S-rDNA gene was amplified using the primers 5`-CAGGCCTAACACATGCAAGTC-3' and 5'-GGGCGGWGTGTACAAGGC-3'. The PCR conditions were 95°C for 1 min, 53°C for 1 min and 53°C for 2 min, 72°C for 2minutes. The PCR product was purified using Qiagen

PCR purification kit and sequenced using ABI PrismTM DNA automated sequencer and dye terminator cycle sequencing kit (MWG Germany). The DNA sequence was analyzed for similarities using Blast program. Multiple sequence alignment and phylogenetic tree analysis was done using BioEdit software (Hall, 1999).

Metal accumulation determination

The cells were cultivated in halophilc medium or minimal medium for 24 hr and 84 hr, respectively with different metal concentrations. The biomass was collected by centrifugation using Pekmann centrifuge (Avanti TM J- 25) at 10000 rpm for 20 min at 20°C, washed twice with sterile water, dried at 105°C, then weighed. The dried cells were re-suspended in 8 ml distilled water and digested with addition of 5:1 nitric acid:hydrogen peroxide and heated at 150°C. The samples were analyzed for metal content using atomic absorption spectroscopy (Perkin Elmer, Analyst 300 atomic Absorption spectrophotometer).

In all experiments, the actual metal added was measured in a blank set of media with a different metal concentration that was incubated without inoculation. All data are the average of at least three independent experiments and each sample measurement on the atomic absorption spectroscopy was the average of three readings.

Influence of the binary metal at non-inhibitory concentration on metal toxicity

The effect of non-inhibitory concentrations of different metals on Ni and Cd toxicities was investigated. It was determined by cultivation of bacterial cells in culture containing different concentrations of each metal (Ni or Cd), supplemented with 0.5 mM of different binary metal ions. The selected binary metal ions were Ni (II), Cu (II), Cd(II), and Zn (II). After 24 hr of incubation at 150 rpm and 37°C on shaker, the OD was measured at 600 nm.

Influence of different salt concentrations on Ni resistance

The effect of salt concentration on Ni resistance was determined by cultivation of an overnight culture in halophilic liquid medium with different salt concentrations (0.2, 2, 15 and 20%), and different Ni concentrations from 0.5-8.0 mM. The cultures were incubated for 24 hr and the OD was measured at 600 nm.

RESULTS AND DISCUSSION

Screening of halophilic strains for metal resistance

The 22 isolated moderately halophilic strains showed different patterns of resistance to the 5 different tested metals. These metals have been chosen due to their presence in the environment from the industrial waste disposal. Figure (1a), shows the commulative resistant pattern of the 22 isolated stains to these metals. Strain 90 showed high resistant levels to all metals while strains 85 and 86 were the most sensitive. Thus, strain 90 (Gram-positive strain) was selected for further analysis. The percentages of tolerance for the 22 strains to the tested metals are shown in Figure (1b). The senstivity was determined based on the arbitary assumptions that, if the diameter of the zone is less than 0.5 cm, the strain is considered as resistant, if the diameter is between 0.5-1.5 cm it is slightly sensitive, between 1.5-2.5 cm is moderately sensitive and sensitive if it is more than 2.5cm. The prevalence of Ni-resistant isolates in Egyptian saline environment has been showed before (Sabry *et al.*, 1997). This might be due to the high contamination levels of Ni and Cu resulting from increased industrialization.

Cd was shown to have the highest

toxicity, almost 50% of the tested isolates were sensitive to cadmium. Nieto *et al.* (1989) showed the same results with 238 moderately halophilic stains, only 7 of them were considered to be tolerant to cadmium. This might be due to the fact that Cd is toxic per se and not essential for biological functions in bacteria.

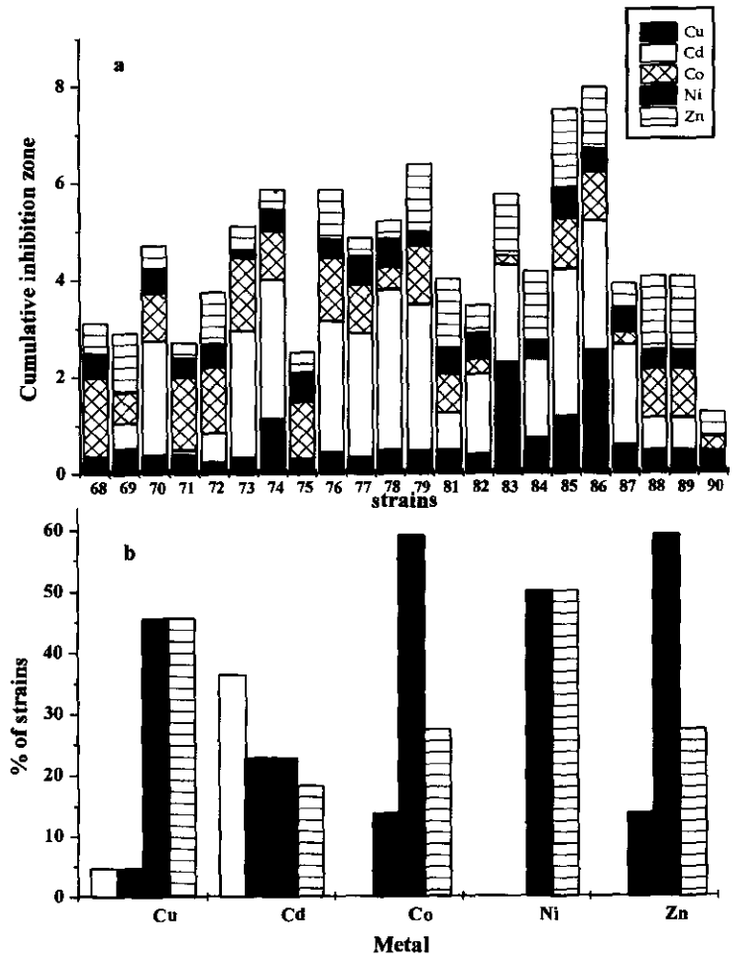
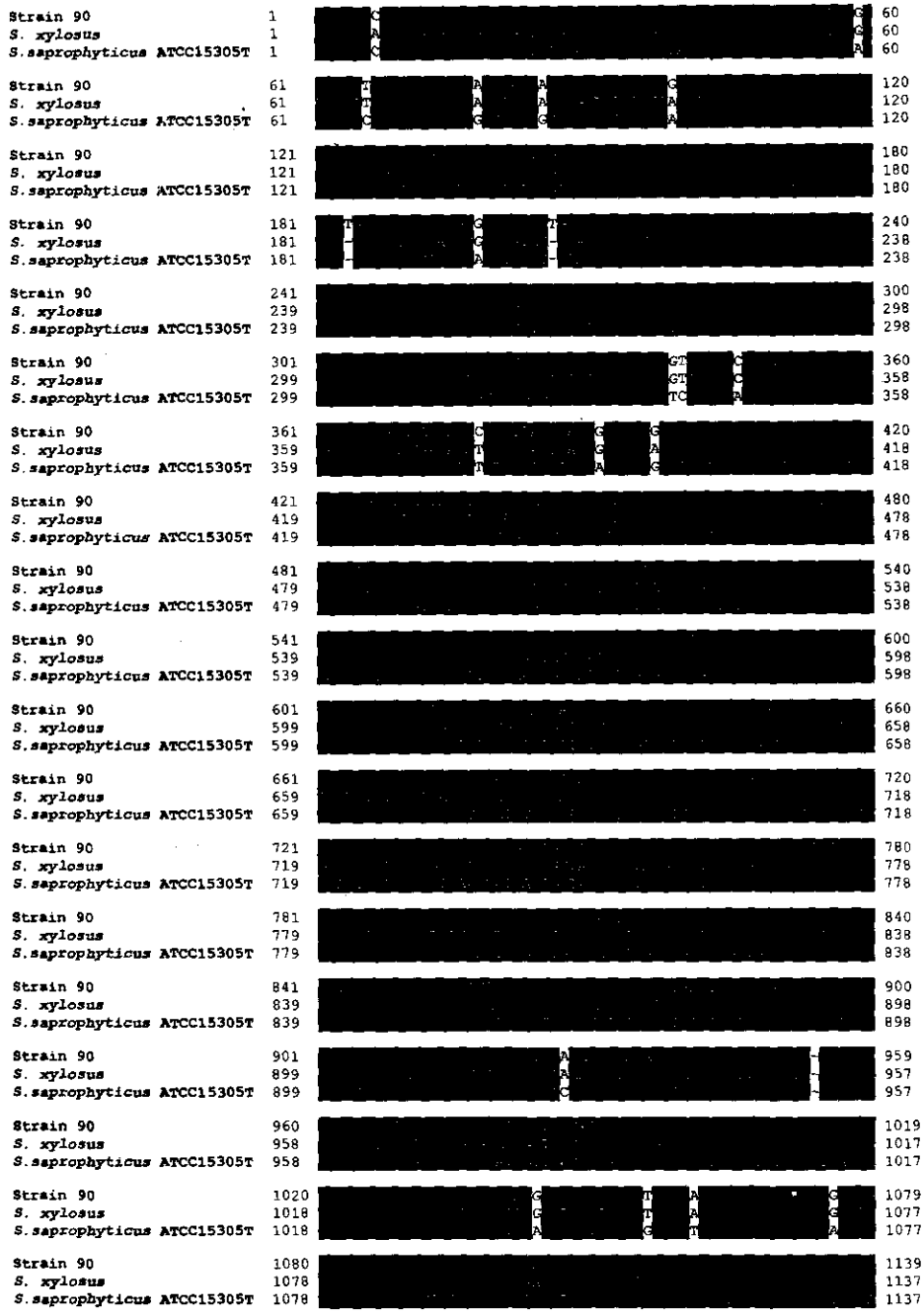


Fig. (1): Screening of the isolated strains for their resistant patterns for different metals, (a) The commulative resistant pattern of the stains to Ni, Co, Cd, Cu and Zn (b) The percentages of tolerance for the 22 strains to the 5 tested metals. (white = very sensitive, grey = slightly sensitive, black = slightly sensitive, striped = resistant).

Bacterial identification

The DNA of the Gram-positive strain (No. 90) was isolated and the 16SrDNA gene was amplified by PCR using specific primers. The amplified product was purified and sequenced using chain terminator method. Computer assisted DNA similarity search, showed 99% similarity to the 16SrDNA gene of two *Staphylococcus sp.*, *S. saprophyticus* and *S. xylosus*. Multiple sequence alignmenet and phylogenetic analyses showed high degree of similarities to those strains (Figures 2 and

3). The 16SrDNA sequence analysis did not give final identity to a specific species, thus called *Staphylococcus sp.* RA90. Further molecular techniques such as 23SrDNA sequencing and DNA hybridization will be required to identify the strain on the species level.



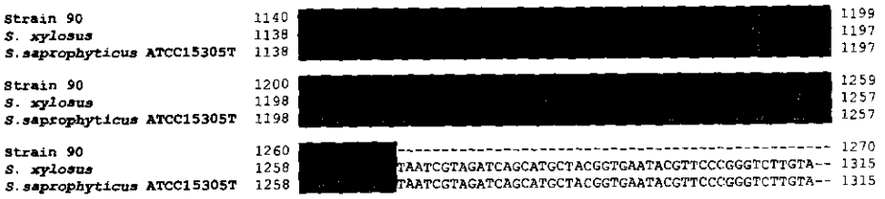


Fig. (2): Multiple sequence alignment of the 16S rDNA sequence of the strain 90, S. Saprophyticus and S. xylosus.

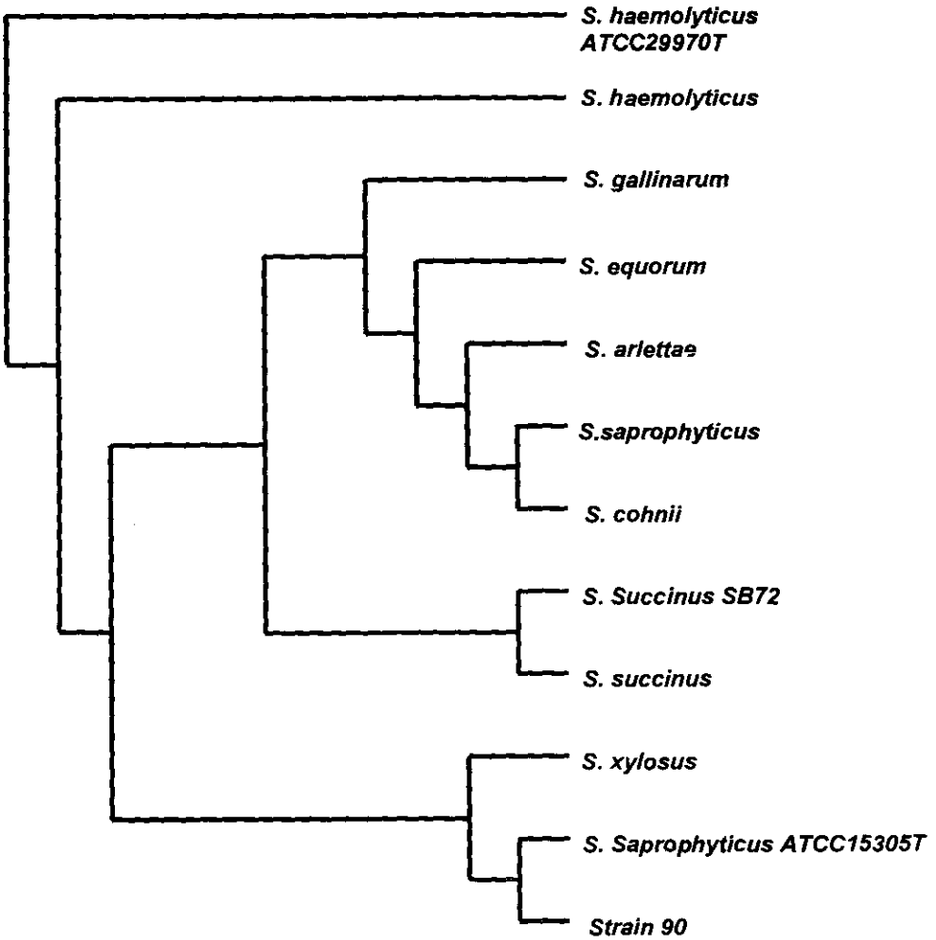


Fig. (3): Phylogentic tree based on the sequence of the 16S rDNA of 90 and other staphylococcus species..

Metal resistance pattern for the selected strain

Staphylococcus sp. RA90 was cultivated in liquid halophilic media with different Ni concentrations to determine its MIC to different metals (Figure 4). The strain showed high resistant levels to Ni and Cd. The MIC of Ni was 750 ppm, which is equivalent to 10 mM NiCl2 and MIC of Cd was 400 ppm equivalent to 4mM $CdCl_2$. On the other hand, the strain could only tolerate up to 250 ppm of the other three metals, Zn, Co and Cu. It was noticed that the metals resistance patterns in the liquid media differ drastically compared to solid media. This could be explained by the different cell physiological status in liquid media, the availability of metal ions and the modification of some metals such as Cu in the presence of agar (Bird *et al.*, 1985). Accordingly, this strain was studied extensively for Ni and Cd resistance and accumulation.

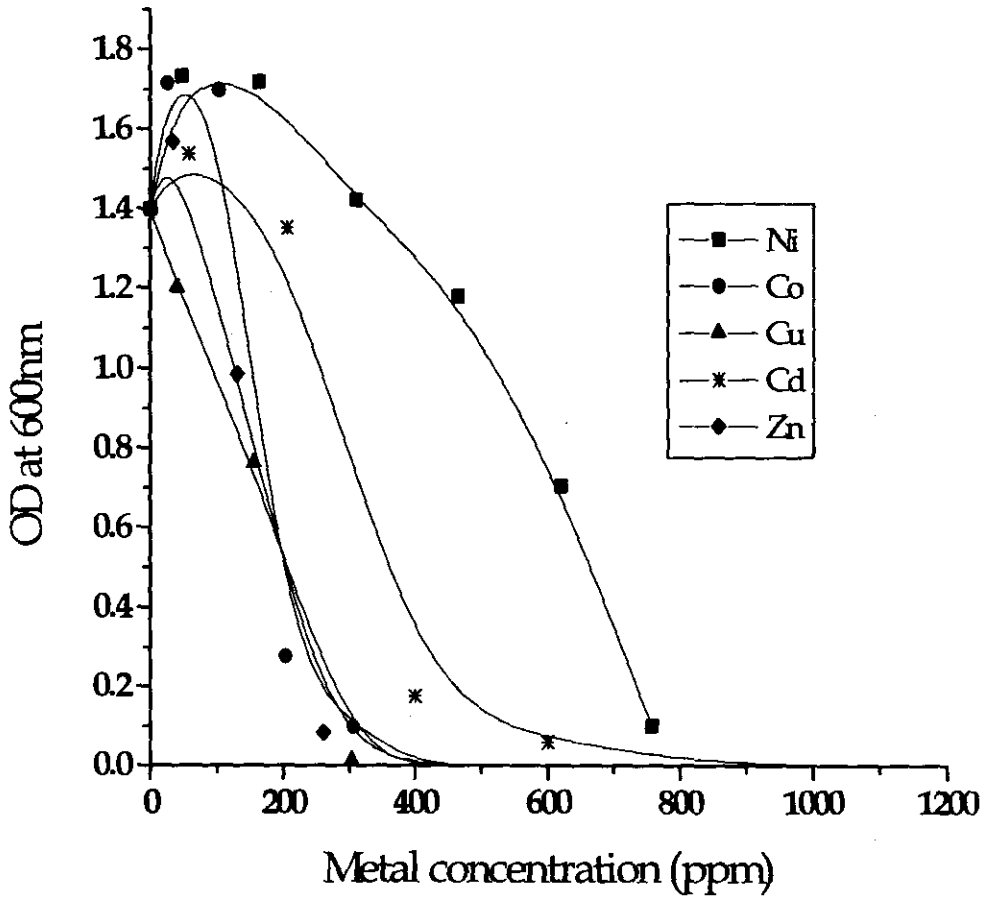


Fig. (4): Multiple sequence alignment of the 16S rDNA sequence of the strain 90, S. saprophyticus and S. xylosus.

Effect of salinity on Ni resistance

With respect to the effect of salinity on bacterial growth and the metal resistance, Figure (5) shows that varying the salinity of the medium affected clearly the sensitivity of bacteria to Ni. The optimium bacterial growth was found at salt concentration about 2% in absence of Ni, but the presence of high salt up to 15% increased the resistance levels of the strain to Ni. Similar to Ni, Onishi *et al.* (1984) observed a reduction in the toxicity of cadmium in moderately halophilic Pseudomonas sp. when the NaCl concentration was increased from 1 to 3M. On the other hand, increased salinity had no effect on Zn toxcicity in several bacteri and fungi (Babich and Stozky, 1978). The reduced Ni toxcicity in high salt might be due the formation of the less toxic forms of the metal and/or the change in the membrane of the cell in such a way that causes higher level of metal tolerence.

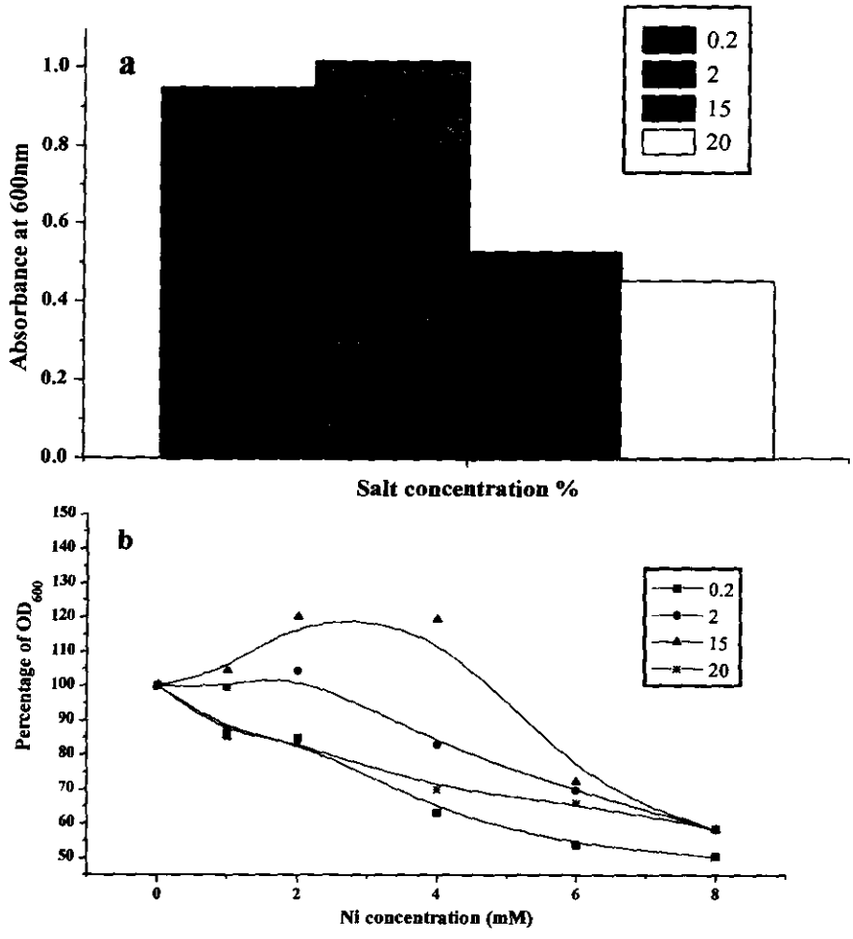


Fig. (5): Effect of salt concentration on Ni resistance by Staphylococcus sp. RA90 (a) growth on different salt concentrations, (b) growth at different salt concentrations with different Ni concentrations.

Effect of binary metals on Cd and Ni toxicity

Figure (6) shows the effect of a non-inhibitory concentrations of different binary metals on the Ni and Cd toxicity of Staphylococcus sp. RA90. Figure (6a) shows the effect of the addition of 0.5 mM of either Cd, Co, Cu, or Zn on Ni toxcity, while Figure (5b) shows the effect of the addition of 0.5 mM of either Ni, Co, Cu, or Zn on Cd toxcity. Zn and Cu proved to increase the toxicity level of Ni on the Staphylococcus sp., while other metals slightly decreased the resistance level of the strain against Ni. The increase of Ni toxicity by Zn and Cu may be due to either increasing Ni uptake by the cell or acting synergistically with Ni to inhibit susceptible cellular fractions (Collins and Stotzky, 1992). In case of Cd there was no drastic effect of added metals on Cd toxicity.

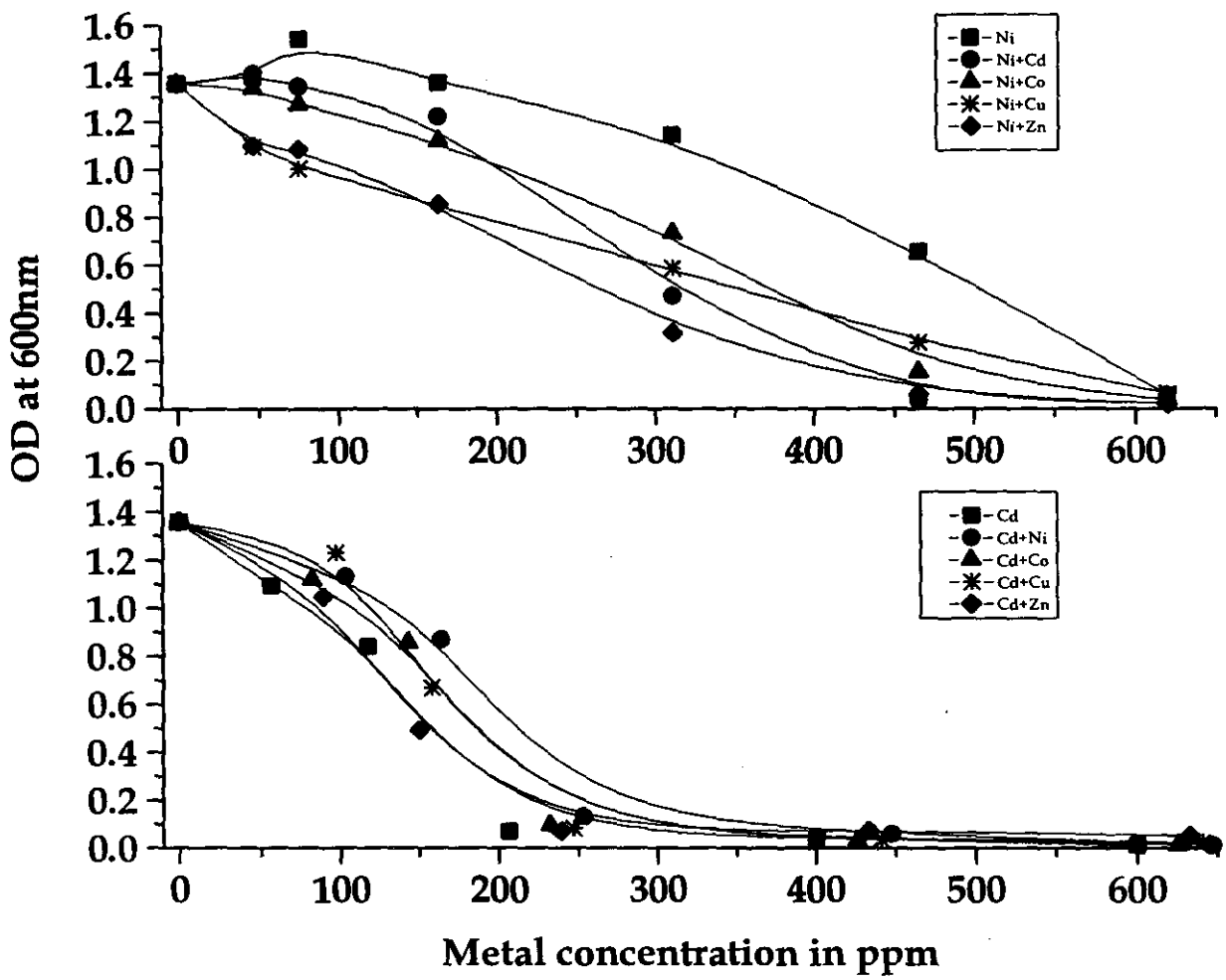


Fig. (6): The effect of 0.5mM different metals on Ni toxicity (a) and Cd toxicity (b) for Staphylococcus sp. RA90.

Nickel accumulation by Staphylococcus sp.

From the accumulation study of Ni at different Ni concentrations, it was found that with increasing Ni influent concentration, the accumulated amount was also increased. These results were presented graphically in Figure (7). The accumulated Ni concentration was measured and found to be around 0.5 mg Ni/g biomass.

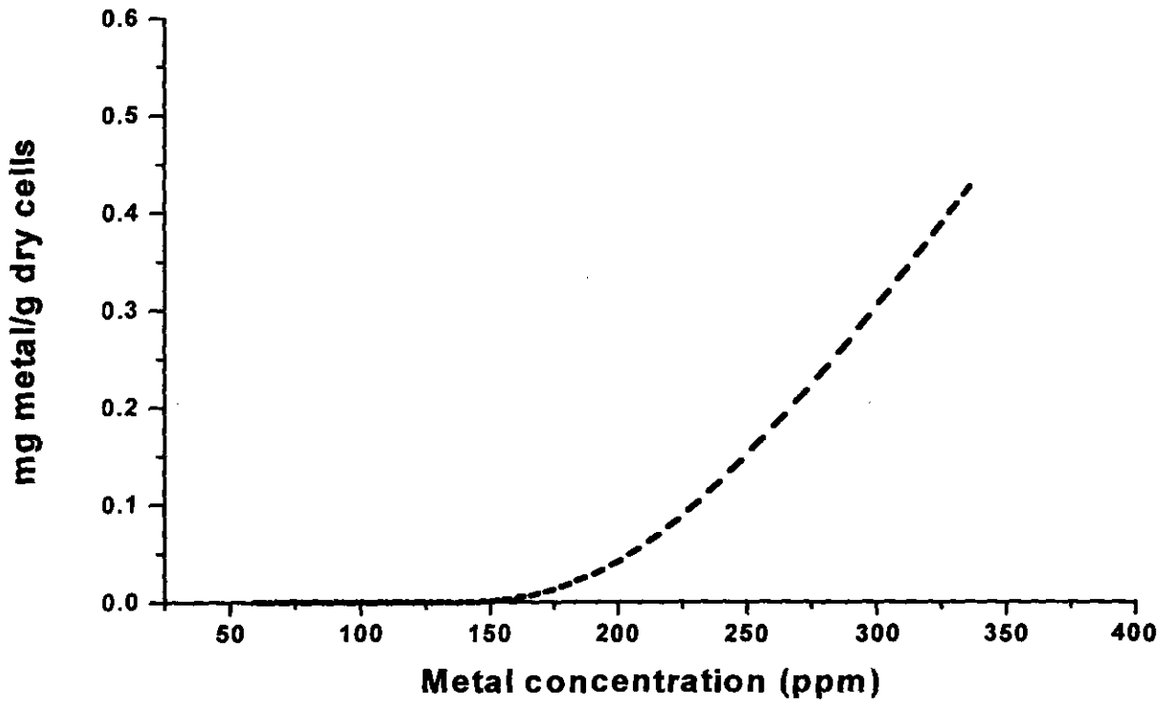


Fig. (7): Ni accumulation by Staphylococcus sp. RA90.

CONCLUSIONS

The cumulative resistant patterns of the 22 isolated stains to 5 different metals Ni, Co, Cd, Cu and Zn was made by the standard inhibition zone method. Strain 90 showed high resistance to all metals, but strains 85 and 86 are more sensitive. Cd was shown to have the highest toxicity, hence almost 50% of the tested isolates were sensitive to this metal. Strain 90 was identified as *Staphylococcus sp.* using sequence similarities and phylogenetic analysis of the 16S rDNA gene. The strain shows the highest resistance levels to the tested metals. The MIC of Ni was 750 ppm, which is equivalent to 10 mM $NiCl_2$ and MIC of Cd was 400 ppm equivalent to 4 mM $CdCl_2$. On the other hand, the strain could only tolerate up to 250 ppm of the other three metals, Zn, Co and Cu. High salt concentration

(15%) increased the ability of the strain to tolerate Ni, probably due to the formation of less toxic metal species. From the accumulation study it was found that with increasing Ni influent concentration, the accumulated amount was also increases.

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الملخص العربي

نمط البكتريا المقاومة للمعادن الثقيلة فى البكتيريات المحبة للملوحة المتوسطة

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**المركز القومي للبحوث

*** قسم النبات كلية العلوم- جامعة الإسكندرية

تعتبر البكتريا التي تتحمل درجات ملوحة متوسطة واحدة من أهم مجموعة الكائنات الدقيقة التي تؤقلم نفسها علي النمو في البيئات ذات الملوحة العالية . هذه البكتريا تستطيع أن تنمو بكفاءة في تركيزات من كلوريد الصوديوم تتراوح بين ٠,٥ و ٢,٥ عياري .

في هذه الدراسة تم مسح ٢٢ سلالة بكتيرية محبة للملوحة المتوسطة و التي لها مقاومة عالية للعناصر الثقيلة و قد حدد أقل تركيز من العناصر ثقيلة له تأثير مثبط لنشاط هذه السلالات و أوضحت الدراسة أن أكثر عنصر تتحمله البكتيريا هو عنصر النيكل وأن وجود الكادميوم والنحاس فى تركيزات قليلة مع النيكل يؤدى إلى زيادة السمية عن طريق قياس تركيز النيكل خارج وداخل خلايا البكتريا باستخدام جهاز الامتصاص الذرى تبين انه تراكم داخل الخلية.

وتم عزل الجين المسئول عن إنتاج ال 16SrDNA بواسطة تفاعل البلمرة المتسلسل وتحديد تتابع الوحدات المكونة له أدى ذلك إلى تعريف السلالة على أنها سلالة *Staphylococcus*