ISOLATION AND IDENTIFICATION OF *BACILLIUS SUBTILIS* FROM FISH FARMS WATER AND EVALUATION OF ITS BIOREMEDIATION EFFECT AGAINST HEAVY METALS (CADMIUM AND CUPPER)

Nadia M. Awny[1], Abd El-Gawad A. S[2], Azza M. Abdelrahman[2] and Samar S. Negm[2]

1- Botany dept., Faculty of science, Zagazig University.
2- Central Laboratory for Aquaculture Research (CLAR), Agricultural Research Center (ARC).

Abstract

High concentrations of some heavy metals (cadmium (Cd) and cupper (Cu) were found in industrial and sewage water that supplied El Manzala fish farms in Egypt. The metal tolerant bacterial strain *Bacillius subtilis* was isolated and identified morphologically and biochemically followed by 16S rRNA gene sequence analysis. The minimum inhibitory concentrations of Cd and Cu against *B. subtilis* that were (6 and 20) mg/l, respectively. In the presence of sub lethal concentrations of Cd and Cu, the *B. subtilis* strain showed an obvious metal removing potential. The environmental conditions as pH value and incubation temperature were studied to estimate the more efficient up-taking of Cd^{+2} and Cu^{+2}, ions. The maximum uptake (5 and 10) mg/l of Cd and Cu achieved at pH**7**and 8 for at (25- 30)ºC, while pH 5 and 9 and incubation temp10 and 40ºC showed the lowest Cd^{+2} and Cu^{+2} sorption by bacterial biomass, respectively the obtained results could be an advantage for large scale treatment of contaminated sites.

Introduction

Nowadays, pollution of aquatic environment is a serious and growing problem throughout the world, and it is a result of increasing industrial operations and commercial chemicals discharged into the aquatic environment that release alarmingly higher amounts of heavy metals into the natural environment **(Al-Kenawy and Niema, 2015).** Heavy metals such as Cd^{+2} and Cu^{+2} are potentially harmful to most aquatic organisms even in very low concentrations and have been reported as hazardous environmental pollutants (**Kaoud and El-Dahshan, 2010).** In addition, the exposure of some metals such as cadmium and cupper may cause development of reducing immunity and diseases of the kidneys, circulatory system, nervous system, and damaging of the fetal brain (**Imo *et al.*, 2014**). Heavy metals are inorganic chemicals that are non- biodegradable and will not break down into harmless forms since they leave biological cycles very slowly. Therefore, due to this fact it has become extremely important to find an eco-friendly option to cleanup metal contaminated environment and consequently to preserve the health of the deteriorating environment (**Golovanova, 2008**). There were several eco-friendly approaches that have been attempted focusing on heavy metals removal from environment but are expensive, disruptive and less practical under natural environmental conditions. In contrast, bioremediation, a relatively young, inexpensive and socially acceptable technology involves the use of renewable resources like microbes and plants (bioremediation) to dissolve heavy metal problems **(Abouzeid *et al.*, 2009 and Oves *et al.*, 2013)**.

In bioremediation process using of microorganisms not only absorb the heavy metals but also convert the highly toxic metals to the less toxic compounds through oxido-reduction processes **(Naik *et al*., 2013**). Bacteria have been reported as efficient bio-sorbents for heavy metals due to their small size and ability to grow in a wide range of environments **(Ghosh *et al*., 2015).**

Material and methods

Samples collection and heavy metal determination:

Five different water sources were collected from aquaculture farms, namely (agricultural drainage water, fresh water, industrial waste water, sewage water and mixed water that supplied by agricultural and industrial waste water. one liter of each water sample was collected in sterile glass bottle and chilled on ice for transport to the laboratory or stored in refrigerator (4ºC), for total bacterial count, isolation of resistance bacteria to heavy metals and detection of some heavy metals (Cd^{+2} and Cu^{+2}). The samples were prepared and analyzed for determination of heavy metal by atomic absorption spectrophotometer (Thermo Electron Corporation S Series AA Spectrometer, England) according to **APHA (2000).**

Isolation of resistant bacteria to heavy metals

Serial dilutions (10^{-1} to 10^{-7}) were prepared for each water sample. Different concentration of Cd^{+2} and Cu^{+2} ions were prepared and seeded separately into the sterile tryptone soya agar medium using pour plating technique, then incubated at 25 ºC for 48 hrs.

Identification of resistant bacteria to heavy metals

All bacterial isolates which survived at sub lethal concentration of Cd^{+2} and Cu^{+2} ions were purified using nutrient agar pure colonies and identified according to morphological and biochemical characterizes following Bergey's Manual of Determinative Bacteriology **(Holt *et al.*, 1994).**

16S rRNA based identification

The partial sequencing of 16S rRNA gene of the selected bacterial strain was carried out using DNA Sequencing Service, QIAamp DNA Mini Kits, Catalogue no.51304 using primer AAGTCGAGCGGACAGATGG and CCAGTTTCCAATGACCCTCCCC. The amplified product occurred as band with molecular size 595 bp.

Minimum inhibitory concentration (MIC)

Tryptic soya broth medium (100 ml) which contained different concentrations of Cd^{+2} (0.0, to 60.4) and Cu^{+2} (0.0, to 316.5) mg/l**,** were inoculated with 0.1 ml of previously identified isolates of *B. subtilis* (24 hr. old) in 250 ml flask in three replicates, were incubated for 24 hrs at 25 ºC. The growth of each bacterial isolate in absence or presence of the different concentration of metal salt was measured by the spectrophotometer as optical density at 600 nm.

Effect of pH values

0.1 ml aliquots of 24 h culture of *B. subtilis* were inoculated in 25 ml test tube containing 10 ml of tryptic soya broth medium at different buffering basal broth medium ranged from pH (5, 6, 7, 8 and 9). The broth medium was adjusted using HCl and NaOH buffering solutions and supplemented with concentration of (5 and 10) mg/l of Cd^{+2} and Cu^{+2}, respectively then incubated at 25ºC for 24 hrs.

Effect of incubation temperature

0.1 ml aliquots of 24 h culture *B. subtilis* suspension were inoculated in 25 ml test tube each contained 10 ml of tryptic soya broth medium (at pH 7) tubes was supplemented with concentration (5 and 10) mg/l, of Cd^{+2} and Cu^{+2} respectively, and then incubated at different temperature (10, 15, 25, 30, 35 and 40) ºC for 42 hrs.

Statistical analysis

Recorded data were subjected to the statistical analysis of variance by using one-way analysis of variance (ANOVA). It was performed according to **Murray (1975).**

Results and discussion

The result in table (1) showed high concentration of Cd^{+2}, and Cu^{+2} in industrial, sewage water different water supply of fish farms in Egypt. It is higher than permissible limit of **WHO 2004** (0.003) ppm for Cd **WHO (2008)** (0.05) ppm for Cu^{+2} (table 1). This result is in agreement with **Abdel-Khalek (2015)** who revealed that heavy metals of (Cd^{+2} and Cu^{+2}) in industrial and sewage waste water higher than recommended by **WHO (2004)**. The increase in heavy metals occurs because of increasing in industrial operations, agricultural fertilization and commercial chemicals discharged into the aquatic environment release alarmingly higher amounts of heavy metals into the natural environment **(Oliveira *et al*., 2011; Tian *et al*., 2012).**

Table (1): Contents of heavy metals ions in different sources of (in water) Fish farms water.

Water source	Agriculture Water (Abbassa farms)	Agriculture Water (Abbassa farms)	Industrial waste water (EL Manzala lake)	El-Genka Sewage water (EL Manzala lake)	Agriculture and industrial waste water (EL Manzala lake)
Cd^{+2}	0.0009 ± 0.003^{b}	0.0002 ± 0.0011^{b}	0.0177 ± 0.0035^{a}	0.0049 ± 0.0003^{b}	0.0056 ± 0.0006^{b}
Cu^{+2}	0.0450 ± 0.0020^{c}	0.0037 ± 0.0004^{e}	0.0944 ± 0.0028^{a}	0.0842 ± 0.0011^{b}	0.0253 ± 0.0021^{d}

*Mean with different letters within column differ significantly, $p\leq0.05$.

Table (2): Morphologically and biochemical tests used for identification of selected bacteria.

Characteristics	Result
Colony on agar shape and size: Margin: Elevation: Color: Texture:	irregular, large Undulate (wavy), Umbonate white, dull dry or (rough)
Gram stain	+ ve
Catalase test	+ ve
Voges- prokauer test	+ ve
Formation of indole	-ve
O/F test	fermentative
Acid form:	
D-Glucose	+ve
L-Arabinose	+ ve
D-Xylose	+ve
D-Mannitol	+ve
D-Fructose	+ve
D-Maltose	+ve
D-Lactose	-ve
Sucrose	+ve
Utilization of citrate	+ve
Nitrate reduction test	+ve
Hydrolysis of gelatin	+ve
Hydrolysis of starch	+ve
Hydrolysis of casein	+ve
Production of H2S	+ve
Oxidase	Variable
Indole test	-ve
Citrate	+ve
KOH	-ve
Urease	-ve
Methyle red	-ve
Motality	+ve

Estimation and identification of bacterial resistance heavy metal toxicity Thirty different bacteria isolated from the collected industrial and sewage samples were purified and preliminary identified using conventional methods. Seventeen isolates were Gram + ve and 13 isolates were Gram – ve. Screening the bacterial isolates for (Cd^{+2} and Cu^{+2}) biosorption capability revealed a highly bioactive isolate identified as *Bacillus subtilis* depending on biochemical characteristics (table 2).

Effect of increasing concentration heavy metals on the growth of *Bacillus subtilis*

From figure (2) it was observed that the growth rate of *B. subtilis* decreased by increasing Cd^{+2} and Cu^{+2} concentrations in linear relationship in tested water samples with different tolerance degrees. Minimum inhibitory concentrations of Cd^{+2} and Cu^{+2} against *B. subtilis* were (6, and 20) ppm respectively. Results are in accordance with **Vijayadeep and Sastry (2014)** who found that bacterial growth decreased by increasing heavy metal concentrations. Also, **Oves *et al.* (2013)** reported that while MIC of *Bacillus. Sp* was 3 ppm for Cu^{+2} and 2ppm in case of Cd^{+2}. **Cabrera *et al.* (2006)** stateded that heavy metals toxicity caused denaturation and inactivation of enzyme and consumption of dissolved oxygen

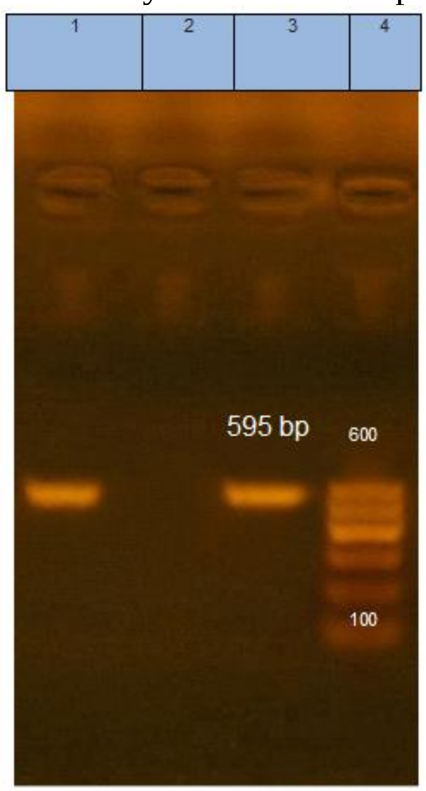

Fig. (1): Agarose gel electrophoresis showing the 16 sRNA gene amplicate of positive control (lane 1), negative control (lane 2) and tested bacteria (lane3) 500 bp DNA ladder marker in lane (4).

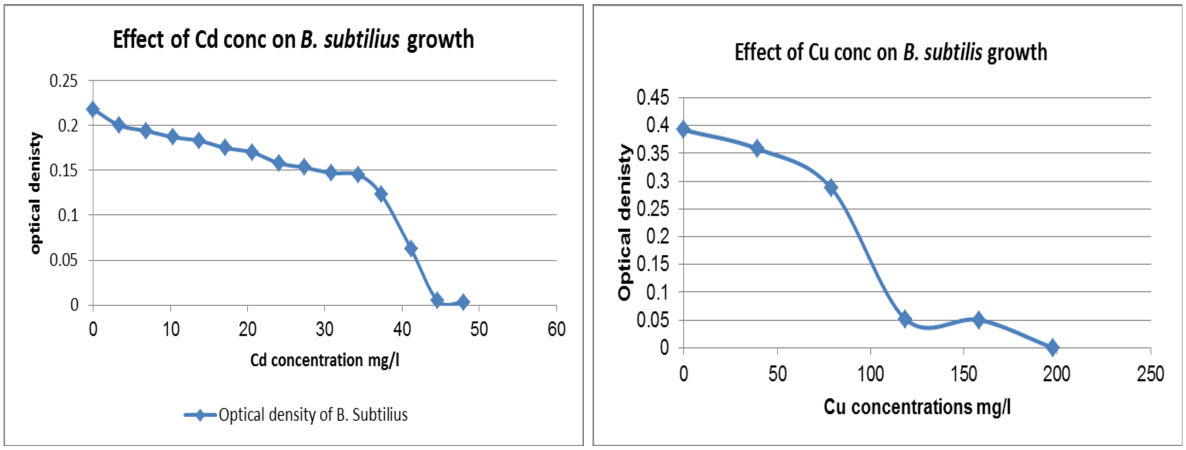

Fig (2): Effect of increasing concentration of Cd^{+2} and Cu^{+2} ions on the growth of *Bacillus subtilis*.

Effect of environmental condition on biosorption of heavy metals

- Effect of pH value

The pH is an important parameter, which affects the degree of ionization and the surface functional groups of the bacterial cell wall **(Aryal and Liakopoulou-Kyriakides, 2015).** Figure (3) illustrated the efficiency of *B. subtilis* to uptake (5 and 10) ppm concentration of Cd^{+2} and Cu^{+2} at 35ºC after 24 h. in different pH solution, which at pH (7 and 8) showed high growth rate of *B. subtilis* and (39.4) % of Cd^{+2} uptaking and (24.02) % of Cu^{+2} was achieved at pH 7. The result agreed with **Oves *et al.*, (2013)** who mentioned that the optimum pH for Cd^{+2} and Cu^{+2} uptake was 6 by *B. thuringiensis* strain *OSM29*, and **García *et al.* (2016)** who reported that the optimum adsorption of Cd, Pb, Cr and Mn for the isolated strains *Bacillus sp. (C13 and C16)* occurred in pH ranges (7 to 10). In this work, the heavy metals uptake was reduced at pH 5 similar to **García *et al.* (2016**) who suggested that Cd^{+2}, Pb^{+2}, Cr^{+2} and Mn^{+2} adsorption by *Bacillus sp. (C13 and C16)* due to increasing in H^{+} ion concentration which changes in pH value effects on the solubility of the metal ions in the solution. **Vázquez (2005)** reported that H^{+} ions replace some of the positive ions from the biomass surface. Also, at low pH values, the binding sites of the cell wall are blocked and associated with hydrogen ions that hinder the access of metal cations due to repulsive forces to the surface functional groups (**Aryal and Liakopoulou-Kyriakides, 2015**).

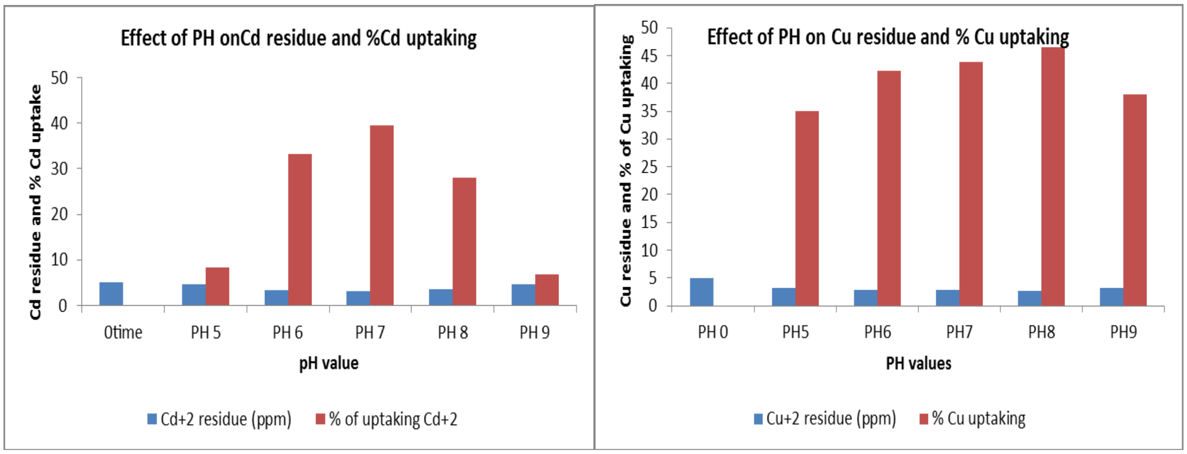

Fig (3): Uptake of cd^{+2} and Cu^{+2} ions by *B. subtilis* at different pH value

Effect of temperature

The temperature of the medium is one of the factors that effects on the sorption of metal ions (**Aryal and Liakopoulou-Kyriakides 2015**).

The result obtained from this work showed indicated that the optimum temperature for bioremediation of Cd^{+2} and Cu^{+2} by *B. subtilis* ranged from (25 to 30) (Fig 4). These results agree with most of the literature determined which the optimum temperature for heavy metal sorption between 20 and 35 °C (**Oves *et al*., 2013 and Veneu *et al*., 2013).** This occurs as a result of biosorption of heavy metals that is usually modified with increase in temperature due to the increase in surface activity and kinetic energy of the solute, but destruction of some binding sites available for metal ions can occur at higher temperatures (**Aryal and Liakopoulou-Kyriakides, 2013b).**

However, it was noticed from our study that the growth of these bacteria and the removal capicity of heavy metal decreased at 10 ºC and 40 ºC. This occurs as enzyme denaturation and organisms either die or become less active **(Trasar-Cepeda *et al.*, 2007). García *et al.* (2016)** study revealed that maximum remediation of Cd^{+2}, Pb^{+2}, Cr^{+2} and Mn by *Bacillus spp. (C13 and C16)* occurred at 27 ± 1ºC. Moreover**, Mansour (2005)** reported that maximum Cd^{+2} biosorption with *B. circulans* was attainted at 35 ºC.

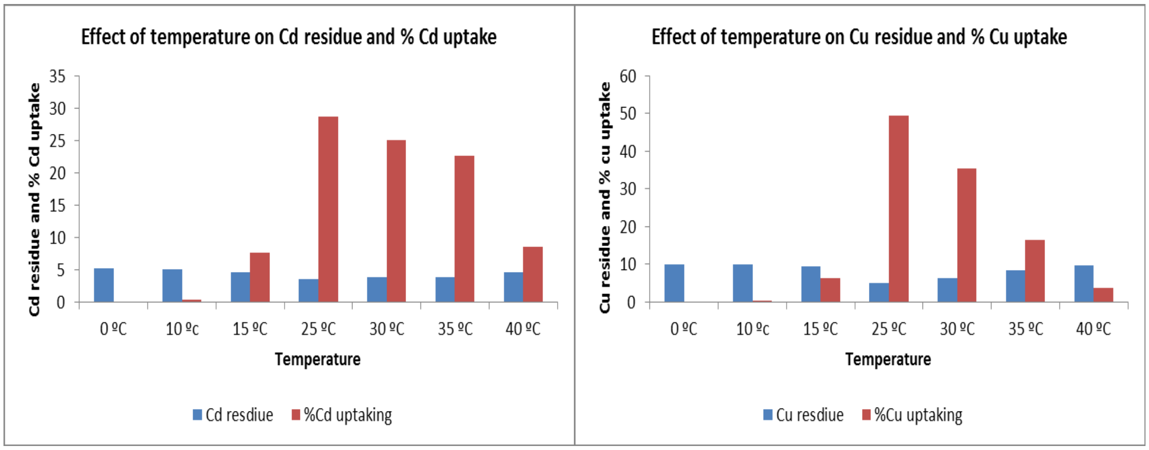

Fig (4): uptake of Cd^{+2} and Cu^{+2} ions *B. subtilis* at different temperature

Conclusion

The selected bacteria identified as *Bacillus subtilis* showed high resistance to heavy metal present in industrial waste water samples. The minimum inhibitory concentrations of heavy metal against strain were (6 and 20) ppm in case of $Cd^{+2}$and Cu^{+2}, respectively. This isolate had capacity to remove sub lethal concentration of Cd^{+2} and Cu^{+2} ions in solution successfully. The bioremediation of heavy metal by *B. subtilis* was affected by pH and temperature of incubation. The maximum absorption of Cd^{+2} and Cu^{+2} by *B. subtilis* was at 25 ºC and pH7 for Cd^{+2} and pH 8 for Cu^{+2}.

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عزل وتعريف سلالة باسيلس ساتليس العصوية المعزولة من مياه المزارع السمكيه وتقييم مدى معالجتها الحيويه لعنصرى الكادميوم والنحاس

الملخص العربى

لقد وجدت تركيزات عاليه من بعض المعادن الثقيله مثل (الكادميوم والنحاس) فى مصادر المياه من الصرف الصحى والصناعي-والتى تغذى المزارع السمكيه ببحيرة المنزله فى مصر. تم عزل وتعريف السلالة المختارة المقاومه لسميه المعادن الثقيله باستخدام 16 SRNA وتحليل التتابع الجينى على انها باسيلس ساتليس.

تم تعيين اقل جرعة مثبطه من المعادن الثقيله لبكتريا باسيلس ساتليس العصويه ووجدت انها 6 مليجرام/لتر لعنصر الكادميوم و20 مليجرام/لترلعنصر النحاس تم استخدام جرعات نصف مميته لاختبار مدى امتصاصها بواسطة البكتريا المقاومه لها مع دراسة الظروف البيئيه المختلفه مثل الاس الهيدروجينى واختلاف درجات الحرارة. حيث وجد ان اعلى امتصاص يحدث عند درجة حرارة 25 الى 30 درجه مئويه فى وسط متعادل و يزيد الامتصاص بزيادة الوقت بينما يحدث نمو ضعيف للسلالة البكتيرية المختبره عند درجه حرارة 10 إلى 40كما لوحظ انه عندما يكون الوسط البيئى حمضى يقل نمو البكتريا وبالتالى يقل امتصاص تللك المعادن.