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Mycoremediation of a mixture of heavy metals by a local marine *Fusaruim solani*

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Abstract

A heavy metal resistant marine fungus was isolated from a polluted sea spot in the Mediterranean, Alexandria, Egypt during June 2009. It was identified to the species level as *Fusaruim solani*. The fungal isolate effectively uptake mixtures of Pb (II) and Zn (II) from aqueous solution. Transmission Electron Microscopy (TEM) studies showed that, the fungus exhibited both adsorption of the metals on the fungal cell wall and accumulation within the cells. So removal of lead and zinc by the marine fungus *Fusaruim solani* was investigated in a batch system. pH 5 proved to be the most favorable for metal ions removal (88% and 91%) for Pb (II) and Zn (II) ,respectively . The maximum uptake of Pb (II) and Zn (II) occurred when using biomass of 3.75 mgL^{-1}. Statistical analyses revealed that there was a direct relationship between lead removal and zinc removal ($P<0.01$) .A 2^2 full factorial statistical design with three center point replicates was employed in the metal uptake experiments. The tested metals at two different initial concentration ranges (low and high), were used. After 75 minutes, metal removal at low concentration (100 mgl^{-1} for each) was 97 and 85 % for Pb (II) and Zn (II) respectively. While, at high concentration (150 mgl^{-1}) it was 92% for each . A statistical interpretation of the results was substantiated by the factorial analysis of variance (ANOVA). It revealed that, some of the main effects and two way interactions between Pb (II) and Zn (II) were significant ($P< 0.001$) and affect the two metal removal by *F. solani*. Overall, the results indicated that both metals taken up by the marine *F. solani* with lead may be accumulated extracellular in the mycelia .These metals may enter the marine ecosystem food web, of which marine fungi are integral members.

Keywords: marine fungal isolate; 2^2 full factorial design; lead; zinc; statistical analysis

1-Introduction

Pollution by heavy metals was accelerated dramatically during the last few decades. The primary sources of heavy metals pollution in coastal lagoons are input from rivers, sediments and atmosphere, which can affect aquaculture profitability in certain areas (Krishnani *et al.,* 2004). The anthropogenic sources of heavy metals include mining, smelting, manufacturing, treatment of agricultural soils with agro-chemicals and soil sludge, etc(Abdel-Saheb and Schwab, 1994). Aquatic ecosystem is the ultimate recipient of almost all the contaminants including heavy metals which are molecules of specific gravity>5.0 and non-biodegradable in nature. (Gupta *et al*., 2007). Pollution of heavy metals in aquatic ecosystem is growing at an alarming rate and has become an important worldwide problem. As they are persistent, their concentrations often exceed the permissible levels normally found in soil, water ways and sediments and are permanent additions to the marine environment (Igwe and Abia, 2006). On the other hand, Abdallah (2008) reported that lead exceeded the permissible limit in the coastal water of the Mediterranean Sea *(*1.0-39.8 mgL^{-1}). The ability of microorganisms to accumulate metal ions from aqueous solutions has been widely reported (Tangaromsuk *et al.,* 2002; Lopez-Errasquin and Vazquez, 2003; AL-Garni, 2005; Svecova *et al*., 2006; EL-Sherif *et al*., 2008 and Zamil *et al*., 2009). The ability of fungi species to adsorb lead ion from aqueous solution through batch experiments was demonstrated by (Awofolu *et al*., 2006). The adsorption of zinc (II) ions on *Rhizopus arrhizus*, a filamentous fungus, was investigated in a batch reactor by Preetha and Viruthagiri, (2005). Fungi have been proven more efficient and economical for removal of toxic metals from aqueous solutions by biosorption because the majority of fungi show filamentous or hyphal growth (Preetha and Viruthagiri, 2005 and Oghenekaro *et al*., 2008). Cell walls of fungi present a multi-laminate architecture where up to 90% of cell walls can be considered as two phase system consisting of chitin framework embedded on an amorphous polysaccharide matrix (Yan and Viraraghavan, 2000)**.** They tend to bioaccumulate more molecules because of their mycelia (Turketul *et al*., 2004).

Therefore, the present study was designed to evaluate the capability of *F. solani*, a marine fungal strain to remove [Pb (II) and Zn (II)] mixtures from aqueous solution. Statistically valid 2^2 full factorial design of experiments was employed to study the main and interaction effects between the two metals during their uptake by the marine fungal isolate. Transmission Electron Microscopy (TEM) studies was used as a tool to help in localization of metals taken up by *F. solani*.

2. Materials and methods

2.1 Fungal strain

Fusaruim solani used through bioremediation experiments was isolated from metal polluted spot in the Eastern part of Mediterranean Sea, Alexandria, Egypt. It was identified to the species level at the Regional Center of Mycology and Biotechnology (RCMB), Al- Azhar University, Cairo, Egypt, according to (Domsch *et al.*, 1993).

2.2 Sampling collection and isolation of marine fungi

Sea water samples were collected from heavy metals polluted spot in Mediterranean Sea, Alexandria, Egypt during June, 2009. The culture medium according to (Cooke 1963) with slight modification was used .It has the following composition (gl^{-1}): rose bengal (3.0 gl^{-1}); glucose 10; peptone 5; and agar 15; sea water 1 L. The medium was poured onto the samples within Petri dishes and the plates were allowed to solidify, then incubated at 28 °C for 7 days. After incubation, species were isolated into pure culture and used for screening. Furgil were maintained on Malt Extract Agar medium (MEA) and kept at 10°C.

2.3 Metal solutions

The stock solutions of Pb (II) and Zn (II) ($1000mgL^{-1}$) were prepared by dissolving a weighed quantity of $Pb (NO_3)_2$ and $Zn (NO_3)_2.7H_2O$ (Merck) in deionized water and sterilized by filtration. Working standard solutions used throughout the different experimental runs were prepared by dilution of the stock. Deionized water was used during the whole work.

2.4 Biomass preparation and metal uptake studies

Cells of *F. solani* were grown in 50 ml of malt extract liquid medium dispensed in 250 ml Erlenmeyer flasks and incubated shaken at 150 rpm at 30 °C for 7 days. Cells were removed by centrifugation and washed well with deionized water. A standard weight (3.75 gL^{-1}) of fungal biomass was suspended in 100 ml of metal solution (in 250 ml Erlenmeyer flasks) and shacked at 150 rpm as described in results section**.**

2.5 Screening and identification of heavy metal resistant fungal isolate

The most promising fungus in terms of heavy metal Pb (II) and Zn (II)) removal efficiency was identified at (RCMB), Al-Azhar University – Cairo – Egypt by using the most documented keys in fungal identification (Domsch *et al.*, 1993). Fungal isolate was subjected for certain morphological studies by an Image Analysis System using Soft-Imaging GmbH software (analysis Pro ver. 3.0) at (RCMB), . As mentioned before the fungal isolate was identified to the species level as *Fusaruim solani.* It was grown on Malt Extract Agar medium (MEA) for approximately 4days.

2.6 Metal determination

Fungal suspension was filtered using 0.45 µm membrane filter. Residual metals were determined using atomic absorption spectrophotometer. The amount of metal taken up by the biomass was calculated as the difference between the initial and final concentration of the metal in the aqueous solution. (*Anand et al.*, 2006).

2.7 Effect of pH

Uptake of metal ions by mycelia biomass was studied at pH values of 3, 4, 5 and 6. A fixed biomass of 3.75 gL^{-1} was added to 100 mL of heavy metal solution containing Pb (II) and Zn (II) at an initial concentration of 150 mgL^{-1} each for up to 120 min. To avoid shifts in pH due to biomass addition, the pH was adjusted with 0.1N HCl or 1N NaOH after the solution had been in contact with the fungal biomass .Duplicate samples were analyzed as previously mentioned.

2.8 Effect of biomass concentration

To evaluate the effect of biomass concentration on the uptake behavior of Pb and Zn, biomass concentrations of 1.25; 2.5; 3.75; 5.0; 6.25 gL^{-1} were separately added to 250 mL Erlenmeyer flasks. Aliquots (50 mL) of heavy metal solution ($150mgL^{-1}$) were added to each flask, and the flasks were left 120min on a rotary shaker at 180 rpm (30 °C) before being analyzed as above.

2.9 Transmission Electron Microscopy (TEM)

The fungal isolate was grown on – (MEA) medium as a control and on (MEA) amended with 150 mgL^{-1} of each Pb (II) and Zn (II) (treated). It was prepared for fixation and dehydration procedures using the programmable Electron Microscopy Science (EMS) tissue processor model (Lynx TMel) at the Regional Center for Mycology and Biotechnology (RCMB) at Al-Azhar University, -Cairo, -Egypt according to

Bozzola and Russell (1999). Stained sections were examined with a JEOL 1010 Transmission Electron Microscope at 80 kv. Transmission Electron Micrographs (TEM) of the control hyphae and conidia of *Fusaruim solani*, were compared with those loaded with Pb (II) and Zn (II).

2.10 Factorial experimental design

A 2^2 full factorial experimental design was employed in this study. Thus a total of 7 experimental runs with three center-point replicates were performed(Saravanan *et al.*,2008).The analysis focused on how the Pb (II) and Zn (II) removal efficiency is influenced by an independent variable named metal concentration (X). Coded levels and actual values of the variables investigated in this study are given in Table 1.

Table 1: 2^2 full factorial design with three center point replicates employed in metals removal for both the low and high concentration ranges by marine *F. solani*.

Experimental run number	Factor and level	
	Lead	Zinc
1	-1	-1
2	-1	+1
3	+1	-1
4	+1	+1
5	0	0
6	0	0
7	0	0

-1: low level (100mgl^{-1}), +1: high level (150 mgl^{-1}).
0: center point or middle value of the factors (125mgl^{-1}).
Time intervals: 15, 30,45,60,75 min, Fungal dosage 3.75 gL$^{-1.}$

3. Results

3.1 Screening for heavy metal removal efficiency

In this study, several marine fungal isolates were submitted to a preliminary experiment to screen their efficiency for the two metal removal. One of the isolates named *F solani* was selected for further investigations due to its highest activities in terms of heavy metals Pb (II) and Zn (II) removal efficiency.

3.2 Effect of pH on Pb (II) and Zn (II) uptake by marine *Fusaruim solani*

The effect of pH 3, 4, 5, 6 (up to 120 min) on Pb (II) and Zn (II) removal efficiency by marine *F. solani* (Figure 1) indicate that the more acidic pH 3 was not favorable for the two metal ions removal , not exceeded 46% and 53 % for the removal of Pb (II) and Zn (II),respectively. However, at less acidic pH 4, the removal efficiency was nearly doubled (90%) for Pb (II) .On the other hand, pH 5 prove to be the most favorable for metal ions removal (88% and 91%) for Pb (II) and Zn (II) , respectively . As the pH raised to 6 a significant decrease of Pb (II) and Zn (II) removal efficiency was recorded.

3.3 Effect of biomass concentration on Pb (II) and Zn (II) uptake by marine *Fusaruim solani*

Metal uptake varied with biomass concentration. The maximum uptake of Pb (II) and Zn (II) occurred at a biomass of 3.75 mg/L (Figure.2).Insignificant increase in the metals uptake were recorded at 5.00 and 6.25 gL^{-1} biomass.

3.4 Simultaneous uptake of zinc and lead in the multi - metal system

The metal uptake patterns due to the local marine *F. solani* for Pb (II) and Zn (II) removal during the experimental runs, are presented in Fig 3. The data depict that, the fungal culture exhibited parallel patterns of the metals uptake of Pb (II) and Zn (II) for a given concentration of the metal. It should be noted here that each experimental run in the study always contained both of the two metals. The metals lead and zinc were completely removed within 75 min when present at low concentrations (100 mgL^{-1} for each), and at high concentration (150 mgL^{-1} for each), a maximum removal was 92% for both metals after the same contact time Data obtained were analyzed and the results were used to develop the regression equation relating the removal efficiency and process parameters and expressed as: $Y=1.2063X+22.049$, ($P<0.001$ and $R^2=0.7485$), (Figure 4).

3.5 Factorial experimental design

The results recorded in Table (2): reveal the interactions between metals (zinc and lead) during their removal in different experimental runs .The data were elucidated statistically through factorial analysis of variance. The smaller the *P* values, the bigger the significance of the corresponding coefficient (Rene *et al.*, 2007). This implied that the main effects and 2-way interactions of metal concentration are significant as is evident from their underlined *P* values (Table 2). For lead adsorption, the main effects, are highly significant (*t*=39.43, 105.00 and 29.5, *P*=0.007, 0.002 and 0.011), after 15, 30 and 45 mints, respectively. Also the interactions between lead and zinc concentrations were significant (*t*=97.73, *P*=0.002) after 75 mints. Similarly, during zinc uptake the main effects were (*t*=32.79 and 12.52 , *P*=0.009 and 0.035), after 60 and 75 mints, respectively . The interactions between lead and zinc concentrations were also significant (*t*=23.28, *P*=-0.017) after 60 min.

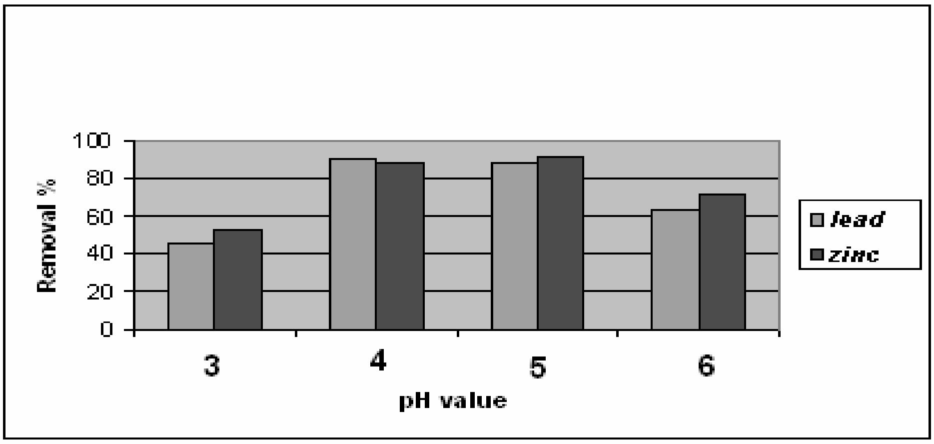

Figure 1: Effect of pH on Pb (II) and Zn (II) up take by marine *F.solani* .

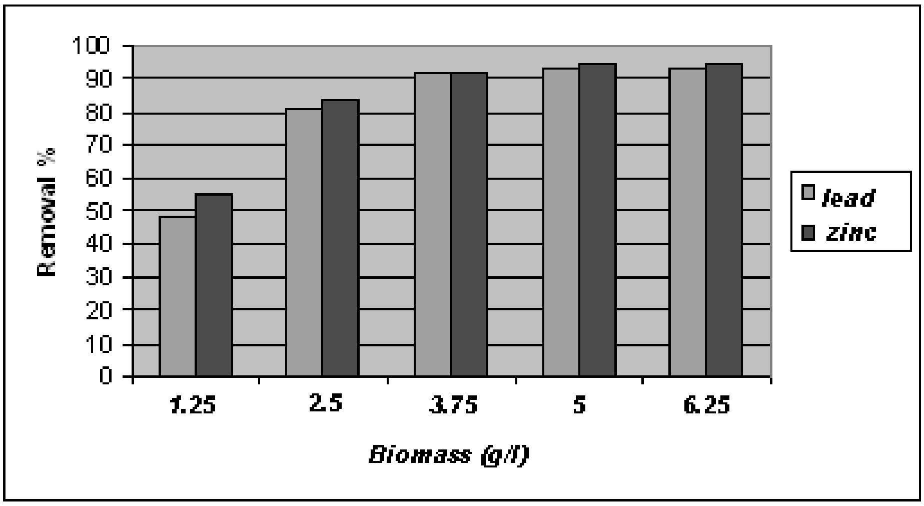

Figure 2: Effect of biomass on Pb (II) and Zn (II) up take by marine *F. solani*.

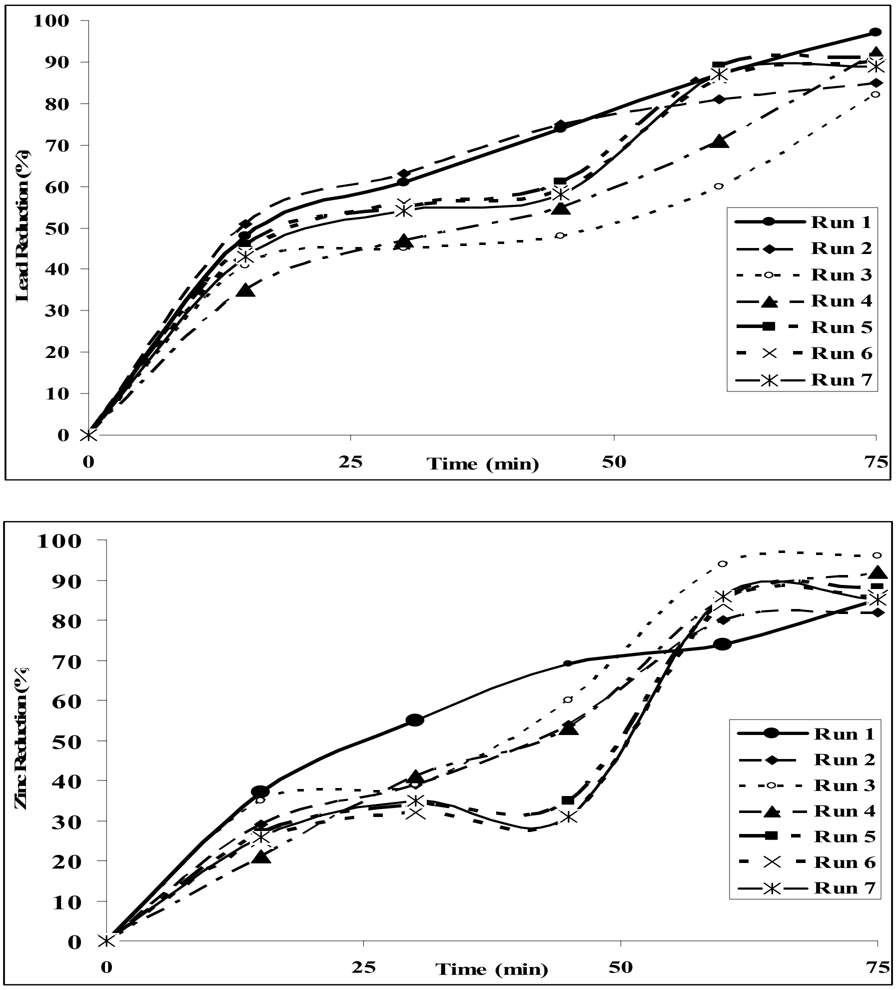

Figure 3: Percentage removal of Pb (II) and Zn (II) by *marine F. solani* during the different experimental runs.

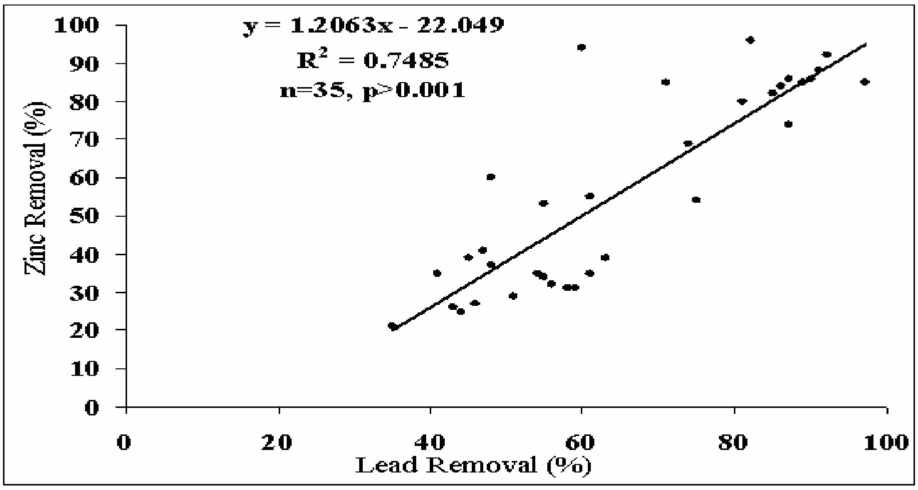

Figure 4: Regression Curve between percent removal of Pb (II) and Zn (II) by marine *F. solani*.

3.6 Localization of heavy metals Pb (II) and Zn (II))

Transmission Electron Microscopy (TEM) micrographs of the control hyphae and conidia of marine *F. solani , and* those loaded with Pb (II) and Zn (II) were compared in Fig (5) . Transmission Electron micrographs (TEM) clearly showed a layer of heavy metals on the cell wall surface, which was absent in control and accumulated metals within the cells. Thus the fungus exhibit both adsorption and accumulation of the metals.

Table2: Factorial analysis of variance for Pb (II) and Zn (II) uptake by marine *F. solani*.

Metal	**Time**	**R^2 (%)**	**2-Way Interactions**	
			F	***F***
Lead	**15**	96.72	39.43**	11.57*
	30	98.59	105.00**	00.00
	45	95.24	29.50**	0.97
	60	60.38	1.89	0.79
	75	97.38	8.50	97.73**
Zinc	**15**	80.85	5.96	0.75
	30	51.23	0.86	1.43
	45	11.64	0.18	0.04
	60	96.73	32.79**	23.28*
	75	89.32	12.52*	0.05

**: significant at 0.01 level of confidence.
*: significant at 0.05 level of confidence.

4. Discussion

Various micro-organisms have been screened for their heavy metal sorption capacity, but of particular interest are those isolated from the environment where these contaminants are found. Micro-organisms isolated from industrial processes and polluted environments with high metal concentrations exhibit considerable tolerance to these elements. This tolerance may be due to a biotic factor or the physiological and genetic adaptations of the microorganism (Barros *et al*., 2006).

In this investigation, a local marine fungal strain isolated from heavy metals contaminated sea spot, identified as *F. solani* was shown to bind efficiently and accumulate metals. The transmission electron microscope revealed that the fungal cells adsorbed metals on the cell wall and accumulate them within the fungal hyphae. In this respect it was reported that the fungus *F. solani* has the ability to actively remove heavy metals from aqueous solutions (Gadd, 1992). Taboski *et al.* (2006) had reported that lead accumulated extracellularly in the mycelia of marine fungi. Also, Igwe and Abia (2006) recorded that Pb (II) was preferentially adsorbed over Zn (II) and Zn (II) uptake was only towards depletion of Pb (II) in the media . The results in this study suggested that the fungus *F. solani* had enough potential to mitigate the excessive contamination of their surroundings and can be used to reduce the metal concentrations in aqueous solutions in a specific time frame. In this respect, El-Morsy (2004) reported that Maximum uptake for metals including Zn (II) and Pb (II) was achieved after 15 min. In this study, pH 5 proved to be the most favorable for metal ions removal (88% and 91%) for Pb (II) and Zn (II), respectively. It was reported that pH value has important role in metal ions biosorption, where the active biosorbing groups have the ability to accept or loss of protons that depend mainly in the pH value (Pinghe *et al.,* 1999; Yalcinkaya *et al*., 2002). The maximum uptake of Pb (II) and Zn (II) occurred at a biomass of 3.75 gL^{-1} .Insignificant increase in the metals uptake were recorded at 5.00and 6.25 gL^{-1} biomass. This may be attributed to the more number of adsorption sites available as a result of increase in amount of adsorbent (Subbaiah *et al.,* 2008).

Compared to conventional one factor at a time experiments, statistical based factorial design of experiments give more meaningful information on the effects, main and interaction of the factor involved in a given study. Moreover, the added advantage of reduction in the number of experiments to be

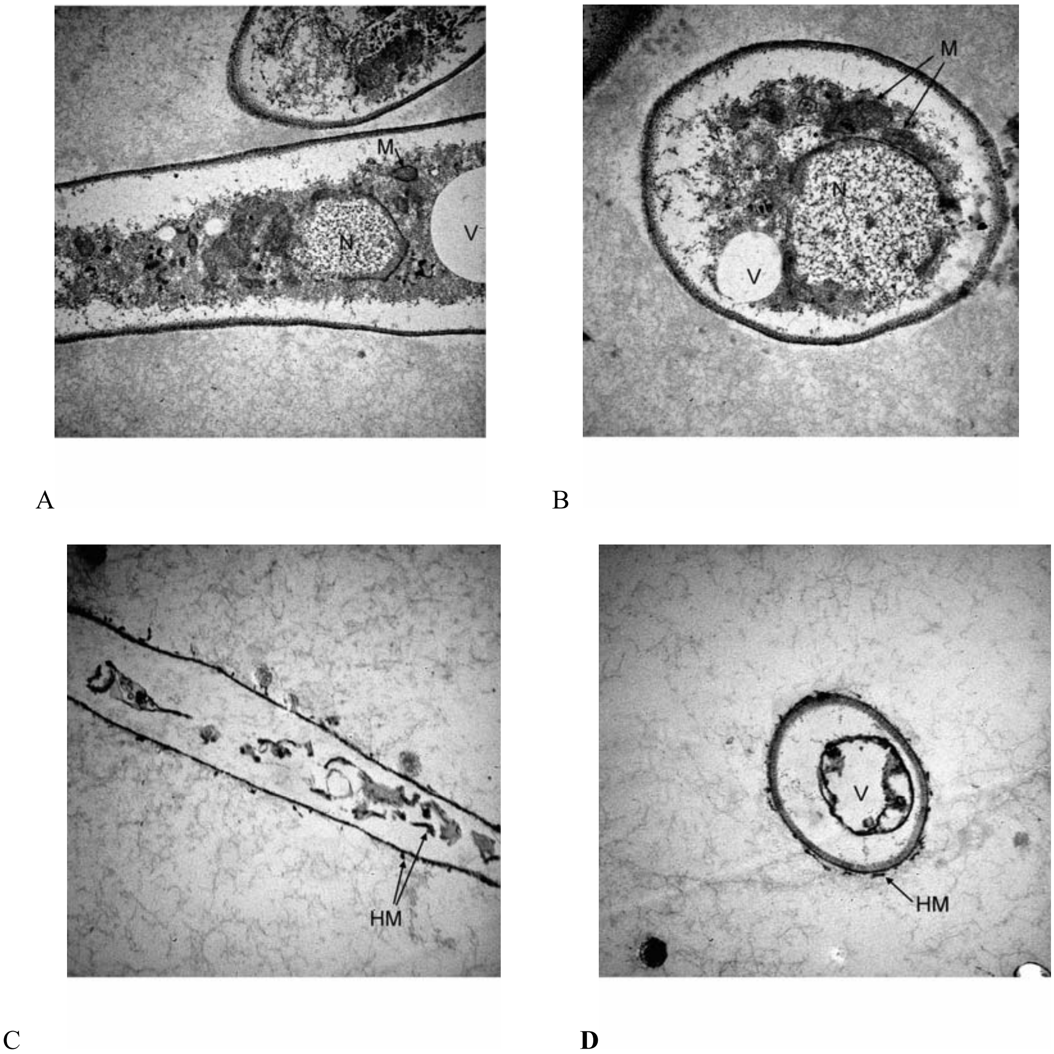

Figure 5: Transmission Electron Micrograph of marine *F. solani* (A-control hypha: B-control conidia)*;* (C-D) Electron micrographs of marine *F. solani* cells exposed to 125mgL^{-1}of each Pb (II) and Zn (II), (HM): heavy metals, (V): vacuoles, (N): nucleus, (M): mitochondria.(TEM Mag ranged from 15 000x to 30 000x)

performed. Employing such techniques proves more attractive for systematic investigations (Montgomery, 2004). The study was designed to investigate the interaction effects between Pb (II) and Zn (II) during their mycoremediation by the marine *F. solani.* The factorial analysis of variance revealed that, the main effects, and two way interactions greatly affect lead and zinc removal. Compared to single metal Zn (II) or Pb (II) biosorption, the combination of Zn (II) and Pb (II) as substrate additives exhibited parallel patterns of the metals uptake of Pb (II) and Zn (II), the same observations were recorded with transmission electron micrograph of the marine *Fusaruim solani* which revealed that the fungus exhibit both adsorption and accumulation of heavy metals, indicating the competition among the divalent ions, as reported by Kok *et al*. (2002). Similar to this present study, Dias *et al.* (2002) demonstrated that the presence of different metal species at different levels in the culture medium substantially affected the adsorption capacity of the fungus. Thus, *Fusaruim solani* appears to be a good candidate organism for industrial application in mycoremediation of Pb (II) and Zn(II) from contaminated water systems.

In conclusion, the foregoing discussion indicated the highest potential of marine *F. solani* for Pb (II) and Zn (II) removal. Living mycelia of the fungus exhibited the highest Pb (II) and Zn (II) removal capacity. The factorial analysis of variance (ANOVA) revealed that some of the main effects and two way interactions between metals are significant and affect lead and zinc removal by the marine *F. solani*. Overall, the results indicated that both metals taken up by marine fungi with lead may be accumulated extracellular in the mycelia. The (TEM) clearly showed a layer of heavy metals on the cell wall surface, and accumulated metals within the cells. Thus the fungus exhibit both adsorption and accumulation of the heavy metals. These metals may enter the marine ecosystem food web, of which marine fungi are integral members.

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تحقيق أفضل الظروف العمليةِ للتخلص من معدنِي الرصاص والزنك باستخدام السلالة البحرية فيوزاريوم سولاني

هالة القصاص

المعهد القومى لعلوم البحار والمصايد ـ فرع الإسكندرية

فيوزاريوم سولاني سلاله فطرية بحرية مقاومه للمعادن الثقيلة تم عزلها من بقعه ملوثه في البحر الأبيض المتوسط بالا سكندريه خلال شهر يونيو 2009 . و قد وجد أن هذا الفطر مقاوم لتراكم معدني الرصاص والزنك . ويتبع الفطر طريقتي الامتصاص و التكثيف في التخلص من المعادن الثقيلة المذابة في محلول مائي. و بعد دراسة تأثير الأس الهيدروجيني و الكتلة العضوية للفطر وجد إن الأس الهيدروجيني 5 و الكتلة العضوية 3.75 جرام لكل لتر تعطي أفضل النتائج حيث كانت نسب المعالجة لعنصري الرصاص و الزنك 91% and 88% على التوالي . و لمعرفة تأثير العلاقة بين العنصرين خلال معالجتهما بالفطر تم استخدام التصميم الإحصائي 2^2 الكامل لتحليلِ النَتائِج التجريبية و لقد تم معالجة العنصرين عند تركيزيين مختلفين (عالي و منخفض) و تم تسجيل النتائج على أبعاد زمنيه مقدارها 15 دقيقه .و لقد اظهرت النتائج أن نسبة التخلص من العنصرين عند التركيز المنخفض (100 مللجم/لتر) كانت 97% و 85% لعنصري الرصاص و الزنك على التوالي في حين أنها كانت 92% لكلا العنصرين عند استخدام التركيز العالي (150 مللجم/لتر) بعد 75 دقيقه . أما عن تأثير التفاعل بين العنصرين فقد أظهرت نتائج هذه الدراسة (باستخدام معادله الانحدار بين المعالجة الحيوية للرصاص و الزنك) إن هناك علاقة طرديه مهمة ($p<0.01$) بين المعالجة الحيوية للعنصرين باستخدام فيوزاريوم سولاني. و إجمالا فان نتائج هذه الدراسة تدل على ان العنصرين موضع الدراسة واللذان يلوثان البيئة البحرية يمكن التخلص منهما باستخدام الفطرة البحرية فيوزاريوم سولانى .