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Evaluation of Some Fungal Endophytes of Plant Potentiality as Low-cost Adsorbents for Heavy Metals Uptake from Aqueous Solution

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Abstract: Wastewaters resulted from different industries in Egypt such as food, painting, service, pharmaceuticals, water valves, packing materials and cartoons & inks industries which are drained on sewage waste and underground water contain different harmful concentrations of the most toxic heavy metals to humans and wildlife, copper and cadmium. This study aim to screen and apply endophytic fungal strains of plant grow in industrial regions that are capable of degrading and accumulate recalcitrant heavy metals to decrease the environmental loading, solve the problems of pollution and improve the quality of drinking and irrigated water in industrial regions. Ten endophytic fungi were isolated and screened for uptake experiments. All endophytic fungal strains under study showed cadmium and copper resistance with varying level. Among them, endophytic *Penicillium lilacinum* showed the highest removal for copper (85.4%) with good removal for cadmium heavy metal ions (31.43 %). The Langmuir model was more able to describe the experimental equilibrium data for biosorption of Cd^{2+} and Cu^{2+} ions on fungal pellets under given experimental conditions than Freundlich isotherm model. High value of Langmuir constant b (0.121 and 1.54, L/mg) indicates the affinity of biosorbent for the binding of metal ions Cu^{2+} and Cd^{2+}, respectively resulting in a stable adsorption product.

Key words: Biosorption, Endophytic fungi, Factory effluents, Heavy metals, Uptake.

INTRODUCTION

Heavy metal pollution represents harmful environmental problem due to the toxic effects of metals and their accumulation throughout the food chain leads to serious ecological and health problems, especially water is the most vital element among the natural resources (Babel and Kurniawan, 2003). In Egypt, the environment, economic growth and developments are highly influencing by the quality of surface and groundwater. In terms of quality, the surface water of the country is vulnerable to pollution from untreated industrial effluents and municipal wastewater; this also depends on effluent types and discharge quantity from different type of industries (Gadd, 1986; Babel and Kurniawan, 2003 and Saiano *et al*., 2005). The rate of bio-sorption decreases with decreasing accessible surface area on the cell walls that might attract and sequester metal ions, so in the early stage the rate of bio-sorption is fast since most of the binding sites on cell surface are freely available (Volesky and Holan, 1995 and Galli *et al*., 2003).

According to the World Health Organization (WHO, 1984), the metals of most immediate concern are cadmium, copper, lead and zinc. The presence of such metals in aquatic environments will lead to severe damage to aquatic life, killing microorganisms during biological water purification process. Moreover, these metals have exacting consequences on humans such as brain damage, reproductive failures, nervous system failures and tumor formation (Volesky, 1995 and Volesky and Holan, 1995). Exposure to cadmium is associated with renal disfunction and high exposure can lead to obstructive lung disease, lung cancer and damage of human respiratory systems (Matis and Zouboulis, 1994 and Bayramoglu and Arica, 2008) as well as copper in high doses can cause anemia, liver and kidney damage, stomach and intestinal irritation (Galli *et al*., 2003 and Mukhopadhyay *et al*., 2006).

Conventional processes for removal of metals from industrial wastewaters include chemical precipitation,

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oxidation-reduction, filtration, electrochemical techniques are expensive. Biological methods such as bio-sorption strategies may provide an attractive alternative to existing technologies. Roy *et al*.,1993 reported the adsorption of heavy metals by green algae and ground rice hulls as well as Selatnia *et al*. 2004 (a, b) supported the biosorption of Ni^{2+} and lead (II) from aqueous solution by a NaOH-treated bacterial *Streptomyces rimosus* biomass. However, Volesky (1995), Volesky and Holan (1995), Vianna *et al*., 2000 and Tobin *et al*., 1984 reported the biosorption of heavy metals by different fungi such as *Saccharomyces cerevisiae, Aspergillus oryzae* and *Rhizopus arrhizus*.

Endophytes live within their host plants in closed relation without causing any apparent disease symptoms (El-Gendy, 2010 and El-Bondkly and El-Gendy, 2010). Due to the removal of heavy metal ions from wastewater is a challenge to overcome environmental pollution, this work aim to screening and evaluating of fungal endohytes derived from plant grow in industrial regions as bioactive adsorbents, based on two facts: 1) a variety of relationship exists between endophytes and their hosts ranging from mutualistic, symbiotic or antagonistic, 2) these host plants tolerate and reduce the heavy metal contents in their industrial environment. Certainly, these fungal endophytes of plant adsorbents have acquired efficient means of resisting, tolerating, detoxification or processing metal ions in industrial effluents. For our knowledge, there are no reports in the current literatures dealing with the bio-sorption of heavy metals from aqueous solutions by endophytic fungi of plant grow in industrial regions up tail now.

MATERIALS AND METHODS

Chemicals:

Sodium hydroxide, nitric acid and metal ion standard were of analytical grade and they were performed according to a previous report (Saiano *et al*., 2005).

Plant samples:

Fresh plant samples were collected from garden of fruits in industrial region irrigated by factory effluents. Each sample was tagged and placed in separate polythene bags, brought back to the lab and kept at 4 °C until processing within 24 hours of collection.

Isolation and Identification of Fungal Endophytes:

Plant samples were washed thoroughly in running tap water for five minutes before processing, surface disinfested by sequential washes in 70% (v/v) ethanol (1 min) and 3.5% (v/v) NaOCl (2 min), rinsed with sterile water and plated 10 to 15 segments on potato dextrose agar medium (PDA, 30g/L) supplemented with 100 mgL^{-1} streptomycin to inhibit the bacterial growth. Plates were incubated for a month at 12 hours light/dark cycles at 28ºC. Another segment of the same origin without surface sterilization was cultured as a negative control to check the presence of contaminated microbes on the sterilized segment surface (Kumar and Hyde, 2004; Lee *et al.*, 2006 and El-Gendy, 2010). Single colonies were transferred periodically to the same medium and after 10 days of incubation, a pure culture of the fungal strains were obtained and examined for biosorbtion activity. Fungal spore formation was encouraged by inoculating cultures on sterilized banana leaf pieces amended with the PDA medium. For identification, Steyaert, 1949; Guba, 1961; Gilman, 1971; Samson, 1979; Samson and Gams, 1984; Kumar and Hyde, 2004 and Lee *et al.*, 2006 methods were followed.

Factory Effluents:

The present study was undertaken using the wastewaters resulted from different industries in Egypt such as food, painting, service, pharmaceuticals and water valves, packing materials and cartoons and inks industries that are draining on sewage waste water or underground water (Table 1). Their contents of Cu^{2+} and Cd^{2+} were evaluated by using flame unit of atomic absorption spectrophotometer (AA, Model- M series Thermo scientific, NIOSH) after digesting by microwave using standard protocols as described earlier by Hayat *et al*., 2002 before removing studies by endophytic bio-sorbets. The results of wastewater analyses are presented in Table 1.

Determination of Cadmium and Copper Tolerance of Endophytic Fungi:

Metal tolerance level was determined in terms of Minimum inhibitory concentration (MIC) by spotting inoculation of fungal inoculum (10^6 CFU/ml) on heavy metal supplemented agar plates and incubated at 30 ºC for 10 days. Presence or absence of growth was observed on the spotted area. MIC was defined as the lowest concentration of heavy metal ml^{-1} of medium inhibited the visible growth of the tested fungi.

Preparation of Mycelium for Uptake Experiments:

Spores (10^6 CFU ml^{-1}) of 10 days old culture incubated on potato dextrose agar slant at 30°C for each fungal strain were inoculated into 250 ml Erlenmeyer flasks containing 100 ml of yeast malt extract glucose medium (YMG) composed of (g L^{-1}): yeast extract, 5; malt extract, 10; glucose, 20. The cultures were grown under static conditions at 30°C for 10 days in dark. Thereafter the growth of such fungi were harvested by filtration and dry cell weight measurements were carried out by passing 100 ml cell culture through a previously weighted filter papers (Whatman No. 1). The harvested biomass of each strain was washed with deionized water to remove non-biomass particles. Mycelia were dried in an oven set at 60°C until constant weights, then stored at -20 °C until processing.

Removal Studies:

Thirty mg of biomass were inoculated into 55 ml metal ion solution containing 5 and 3.5 mgL^{-1} of copper and cadmium, separately respectively and adjusted to pH 6.0 ± 0.1. The flasks were kept under magnetic stirring, the time dependence of metal ion concentration could be measured within 3 hrs at 30°C. Afterwards, the suspension was filtered and the filtrates were acidified with HNO_3. The contents of the filtrate of each fungal strain after each treatment were analyzed after proper digestion and dilution by atomic absorption spectrophotometer. Metal solution without biomass addition was served as control. Uptake and removal of each metal ion was expressed as mgL^{-1} and %, respectively. Experiments were carried out in duplicate and average values were recommended.

Amount of metal ions (mg) bioadsorped per gm dry biomass was calculated using the following equation: Q=(C○-C / M) V. Where Q =mg of metal ion bioadsorped per gm of biomass, Co = initial metal ion concentration mg/L, C = final metal ion concentration mg/L after biosorption, M = mass of biomass in the reaction mixture (g), V = volume of the reaction mixture (L) (Bai and Abraham, 2001 and Bayramoglu and Arica, 2008).

Adsorption Isotherms:

For adsorption isotherms, 30 mg of biomass was inoculated into 55 ml metal ion solution containing 0.5, 1, 5, 10, 15 and 20 mg/L of cadmium or copper. The preparation of different metal ion concentrations of 0.5, 1, 5, 10, 15, 20 mg/L were obtained by diluting a corresponding standard. All preparations were adjusted to pH 2.0 ± 0.1. Two isotherm equations have been tested in the present study, namely Langmuir and Freundlich equations.

Langmuir equation represented as $q_e= Q_\circ bC_e/1+bC_e$, where q_e is the amount of biosorbed metal ions at time t (mg/g), Ce is the equilibrium concentration (mg/L). Q_o (mg/g) and b (L/mg) are the maximum biosorption capacity and energy of adsorption, respectively. $K_a =1/K_d = b$, $\ln K_a =-\_G\max/RT$ (R is the gas constant, 8.314 J/mol K). The essential characteristics of a Langmuir isotherm can be expressed in terms of a dimensionless constant separation factor called the equilibrium parameter, R_L, which is used to predict if an adsorption system is "favorable" or "unfavorable. It is by the following relationship: $R_L =1/1+bC_\circ$, where R_L and Co are the dimensionless constant separation factor or equilibrium parameter and initial metal ions concentration, respectively. The value of R_L indicates the shape of isotherm to be either unfavorable ($R_L > 1$) or linear ($R_L = 1$) or favorable ($0 < R_L < 1$) or irreversible (RL = 0).

The Freundlich expression is an empirical equation based on adsorption on a heterogeneous surface. The equation is commonly presented as: $q_e = nC_eK_F$, where q_e is the amount of adsorbed metal ions at time t (mg/g), C_e is the equilibrium concentration (mg/l).K_F (mg/g) and n (g/L) are the equilibrium constants indicative of biosorption capacity and biosorption intensity (Bayramoglu and Arica, 2008).

Equilibrium Experiments:

The metal biosorption capacity was determined at different initial metal ion concentration of 0.5, 1, 5, 10, 15 and 20 mg/L and a fixed concentration of biomass (30 mg, dry weight) was used to calculate biosorption constants by using different isotherms. 55 ml of each solution was tested at pH 2.0 ± 0.1. The solutions were kept under magnetic stirring for 24 hrs to allow the biomass to adsorb the metal ions until the solution reached equilibrium. The initial and the final concentration of the metal ion were measured. The obtained data were used to calculate the adsorption capacity of the adsorbent. Finally, a diagram of adsorption capacity against equilibrium concentration was plotted (Mukhopadhyay *et al.*, 2006).

RESULTS AND DISCUSSIONS

Analysis of Industrial Wastewaters Resulted from Different Industries in Egypt:

As shown in Table 1, analysis results of different industrial wastewaters resulted from different industries in Egypt such as food (Green Land Co., Halwany Co. and Bisco Misr Co.), painting (Sipes Co.), service (Cairo International Air Port Co., central and middle workshop), pharmaceuticals (Gedco Co.) and water valves industries (Jacop Delafon Co.) which are drained on sewage waste water as well as packing materials (Misr pack Co.), painting (Infit, International Co for casting & fitting Co.) and cartoons and inks industries (El-Baddar Co.) that drained on underground water were contain different harmful concentrations of copper and cadmium (the most toxic heavy metals to humans and wildlife). Copper was not detected in packing materials industries but cadmium was not detected in water valves industries and Bisco-Misr company effluents. Whereas the highest copper concentration were reported in cartoons & inks industry (10.31 mg/L), followed by water valves industry, service industry and painting industries (6.71, 3.90 and 2.002 mg/L, respectively), the highest cadmium were recorded in the industrial waste water of painting, service, pharmaceuticals industries followed by cartoons & inks industry (0.284, 0.12, 0.106 and 0.1 mg/L, respectively). Harmful concentrations of different metals ions previously reported in wastewaters such as mercury, cadmium , zinc (Luef *et al*., 1991; Matis and Zouboulis, 1994 and Bayramoglu and Arica , 2008); Copper (Galli *et al*., 2003 and Mukhopadhyay *et al*., 2007); lead and nickel (Volesky, 1995 and Selatnia *et al*., 2004 a and b). Thus treatment of these industrial wastewaters to remove these toxic heavy metals is an argent and necessary process to protect the environment and public health by decreasing the environmental load.

Table 1: Analysis of industrial waste water collected from different factories in Egypt.

Industry	Region	Drained on	Cu^{2+} (mg/L)	Cd^{2+} (mg/L)
1. Cartoons & inks Industries				
El-Baddar Co.	Gesr EL-Swes	underground water	10.310	0.100
2. Painting industries				
Sipes Co.				
Infit, International Co for	10th of Ramadan	sewage waste water	0.282	0.284
casting & fitting Co.	Abo Rawash –Giza	underground water	2.002	Nil
3. Pharmaceuticals industries				
Gedco Co.	free zone, Nasr city	sewage waste water	0.214	0.106
4. Water valves industries				
Jacop Delafon Co.	10th of Ramadan	sewage waste water	6.710	Nil
5. Packing materials industries				
Misr pack Co.	El-Khanka, Kalyobia	underground water	Nil	0.030
6. Service industries				
Cairo International Air Port Co.				
(Central workshop)	Cairo	sewage waste water	2.400	0.120
(Middle workshop)		sewage waste water	3.900	0.020
7. Food industries				
Green Land Co.	10th of Ramadan	sewage waste water	0.200	0.034
Halwany Co.	10th of Ramadan	sewage waste water	0.526	0.056
Bisco Misr Co.	El-Amerya	sewage waste water	0.240	Nil

Isolation and Identification of Endophytic Fungal Strains:

According to the previous international taxonomic keys (Steyaert, 1949; Guba, 1961; Gilman, 1971; Samson, 1979; Samson and Gams, 1984; Kumar and Hyde, 2004 and Lee *et al*., (2006). Ten endophytic fungal species belonging to different 8 genera namely, *Rhizopus oryzea, Aspergillus luchuensis, Aspergillus tubingensis, Monacrosporium elegans, Penicillium duclauxi, Penicillium lilacinum, Curvularia lunata, Drechslera hawaiiensis, Verticillium Fungicola, Pestalotiopsis clavispora* were isolated and identified (Table 2)

Determination of Cadmium and Copper Tolerance of Endophytic Fungi:

All endophytic fungal strains under study showed cadmium and copper resistance with varying level as evident from the MIC value of these metals (Table 3). The MIC of cooper towards *Penicillium lilacinum, Drechslera hawaiiensis and Pestalotiopsis clavispora* was 1000, 950 and 890 µg ml^{-1}, respectively. *Monacrosporium elegans, Rhizopus oryzea* and *Penicillium lilacinum* exhibited the highest tolerance towards cadmium (MIC of Cd^{2+} were 800, 716 and 650 µg ml^{-1}, respectively). Data in Table (3) supported *Penicillium lilacinum* as potent multi-metal resisted fungus. It seems that continuous exposure of endophytic fungi of plants

Table 2: Bio-sorbents endophytic fungal strains in this study.

Isolation number	strain name
Merv2	*Rhizopus oryzea*
Merv3	*Aspergillus luchuensis*
Merv4	*Aspergillus tubingensis*
Merv5	*Monacrosporium elegans*
Merv6	*Penicillium duclauxi*
Merv7	*Curvularia lunata*
Merv8	*Penicillium lilacinum*
Merv9	*Drechslera hawaiiensis*
Merv10	*Verticillium Fungicola*
Merv11	*Pestalotiopsis clavispora*

Table 3: Minimum inhibition concentration (MIC) of heavy metals against endophytic fungal strains.

Fungal isolates	MIC (µg ml $^{-1}$)	
	Cu^{2+}	Cd^{2+}
Rhizopus oryzea	200	716
Aspergillus luchuensis	417	590
Aspergillus tubingensis	350	280
Monacrosporium elegans	630	800
Penicillium duclauxi	299	630
Curvularia lunata	214	400
Penicillium lilacinum	1000	650
Drechslera hawaiiensis	950	290
Verticillium Fungicola	318	512
Pestalotiopsis clavispora	890	480

grows in industrial area against heavy metals of wastewater might have exerted selection pressure on to endophytic fungal population resulting in the development of multi-metal resistance of fungi. Our results are in agreement with the previous study of Ahmad *et al*., 2005, they reported that two isolates belonging to the predominant genera *Aspergillus* and *Rhizopus* isolated from agricultural field treated with sewage/ industrial effluents showed potent resistance against different metal ions such as Cd, Cr, Cu, Co and Ni.

Evaluation of Fungal Endophytes Isolates as Bio-sorbents:

Studies tabulated in Table (4) represent the amount of heavy metal uptake and removal occured (in mg/L and %, respectively) by the biomass of each fungal isolate. Whereas the biomass of the endophytic *Rhizopus oryzea, Monacrosporium elegans, Penicillium duclauxi, Aspergillus luchuensis* and *Penicillium lilacinum* supported the highest uptake (1.7, 1.6, 1.51, 1.3 and 1.1 mg/L, respectively) with removal of 48.58, 45.72, 43.15, 37.15 and 31.43 %, respectively of cadmium, the biomass of the endophytic *Penicillium lilacinum, Drechslera hawaiiensis* and *Pestalotiopsis clavispora* showed uptake of 4.27, 4.18 and 4.01 mg/L, respectively and removal (85.4, 83.6 and 80.2 %, respectively) of copper. *Penicillium lilacinum* which showed the highest uptake of copper with good uptake of cadmium as the most cytotoxic heavy metals was selected for further adsorption isotherms studies.

Table 4: Removal and uptake of Cu^{2+} and Cd^{2+} metal ions from aqueous solution by different fungal endophyte biomass.

Source of biomass	Uptake and removal of Cu2+ and Cd2+ from aqueous solutions*			
	Cu2+		Cd2+	
	mgL -1	%	mgL -1	%
Rhizopus oryzea	2.3	46	1.7	48.58
Aspergillus luchuensis	3.1	63	1.3	37.15
Aspergillus tubingensis	2.5	50	0.9	25.72
Monacrosporium elegans	3.5	70	1.6	45.72
Penicillium duclauxi	1.4	30	1.51	43.15
Curvularia lunata	1.04	79.2	1	28.58
Penicillium lilacinum	4.27	85.4	1.1	31.43
Drechslera hawaiiensis	4.18	83.6	0.9	25.72
Verticillium Fungicola	1.7	34	1.2	34.29
Pestalotiopsis clavispora	4.01	80.2	1.1	31.43

*Initial concentration of copper and cadmium were 5 and 3.5 ,respectively.

These fungal strains have bio-sorption capability by being able to sequester substantial amounts of heavy metals from aqueous solution (Gadd, 1986; Luef *et al*., 1991 and Mukhopadhyay *et al*., 2007). The accumulation of heavy metals in the fungal biomass suggested that endophytic fungi are able to entrap the heavy metals as occur in the aqueous phase (Tobin *et al.*, 1984). However, adsorption capacity for each metal ion is related to the nature of biological action between the biomass surfaces and biohazards, which are depending on such different functional groups form complexes with metal ions resulting in chemical complexes formation as an uptake mechanism (Vianna *et al*., 2000; Volesky, 1995 and Mukhopadhyay *et al*., 2007). However, metal uptake can also be due to physical sorption or bioaccumulation (Saiano *et al*., 2005). Results of our evaluation could recommend the promising role of fungi in the search for an environmentally friendly approach dealing with removing and adsorbed pollutants from aqueous phase (Table 4). Results are in agreement with literatures, that supported fungal biomass in the field of decontaminates metal bearing wastewaters as an economic threshold for practical applications of biosorption as well as concentrate toxic metals as compared with alternative technique (Tobin *et al*., 1984; Gadd, 1986; Luef *et al*., 1991; Volesky 1995; Vianna *et al*., 2000; Saiano *et al*., 2005 and Mukhopadhyay *et al*., 2007).

Adsorption Isotherms Studies:

It is important to establish the most appropriate correlations for the equilibrium curves to optimize the design of a biosorption and removal system. Two isotherm equations have been tested in the present study, namely Langmuir and Freundlich equations. The Langmuir and Freundlich constants have been calculated from the corresponding plots for biosorption of Cd^{2+} and Cu^{2+} ions on the biosorbents. Figure 1 and 2 illustrates the curves for Freundlich and Langmuir isotherm models for Cu^{2+} and Cd^{2+}-*Penicillium lilacinum* biosorption system and results are presented in Table 5. The Langmuir model was able to describe the experimental equilibrium data for biosorption of Cu^{2+} and Cd^{2+} ions on fungal biomass under given experimental conditions (Table 5). Langmuir constant b (0.121 and 1.54, L/mg) indicates the affinity of biosorbent for the binding of metal ions Cu^{2+} and Cd^{2+}, respectively. So a high value of *b* indicates a steep desirable beginning of the isotherm which reflects the high affinity of the biosorbent for the sorbets (metal ions) resulting in a stable adsorption product. Moreover, the values of K_F in Freundlich isotherm model were determined to be 0.036 and 0.026 for Cu^{2+} and Cd^{2+} ions for fungal biomass, respectively. The other Freundlich constant (*n* value) is an empirical parameter which varies with the degree of heterogeneity indicating the degree of nonlinearity between metal ions biosorption capacity and equilibrium concentration of metal ions in aqueous phase due to the distribution of bonded ions on the sorbent surface. Data in Table (5) also indicated the highest value of *n* in the case of Cu^{2+}, indicating that copper metal ion is favorably adsorbed by the biosorbent than Cd^{2+} (Saiano F *etal*., 2005); Bayramoglu and Arica, 2008 and Mukhopadhyay *etal*., 2007).

Table 5: The Langmuir and Freundlich isotherm models constants for biosorption of Cd^{2+} and Cu^{2+} metal ions from aqueous solution.

Metal ions	Freundlich isotherm model			Langmuir isotherm model	
	Q_{exp} (mg/g)	n (g/L)	K_F (mg/g)	$b*10^1$ (L/mg)	Q_o (mg/g)
Cu^{2+}	2.765	1.09	0.036	0.121	0.199
Cd^{2+}	1.0006	0.86	0.026	1.540	0.211

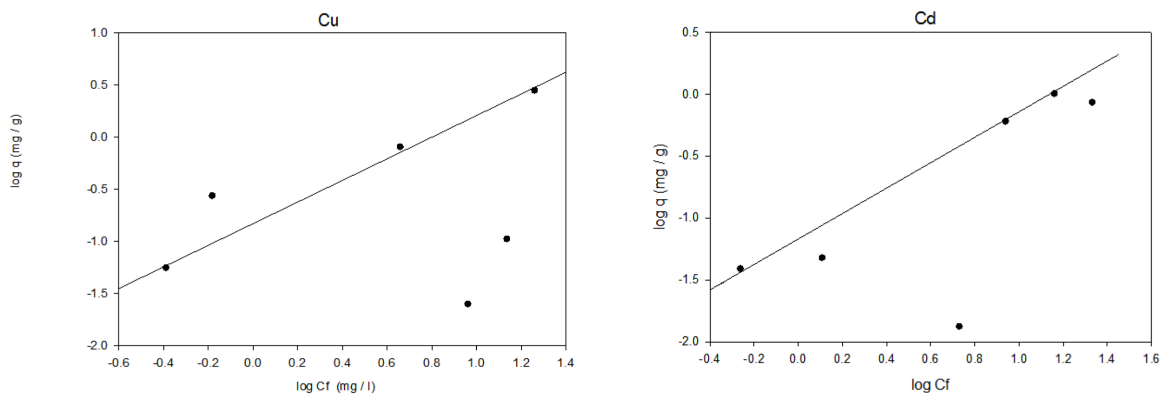

Fig. 1: Application of the Freundlich equation to the adsorption data of Cu^{2+} and Cd^{2+} onto *Penicillium lilacinum*.

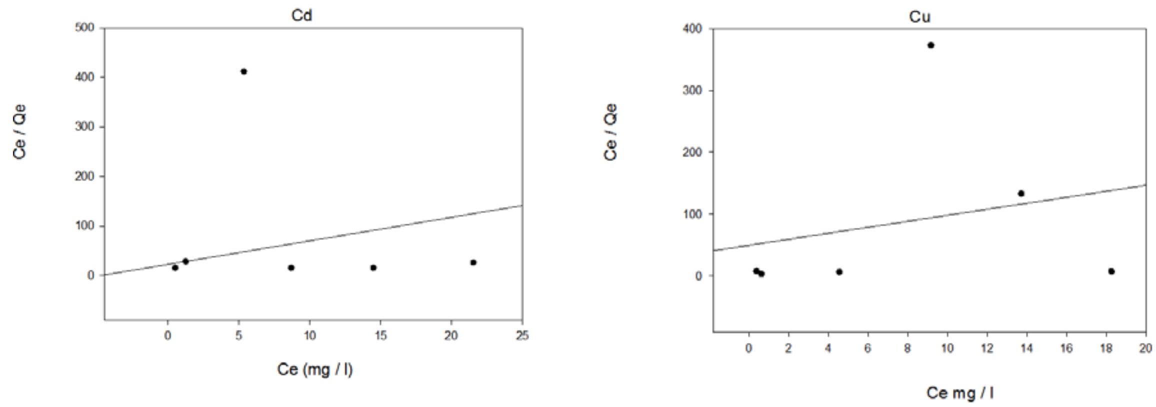

Fig. 2: Application of the Langmuire equation to the adsorption data of Cu^{2+} and Cd^{2+} onto *Penicillium lilacinum*.

Conclusions:

This work supported the potential role of endophytic fungal strains derived from plants grow in industrial area as cost-effectiveness bio-adsorbent of metals from aqueous solutions through a simple economical process. Our results could establish a basis for evaluating the role of fungi in the search for an environmentally friendly approach dealing with pollutants in aqueous phase. This fungal endophytes adsorbent can be a promising technology and good candidate for adsorption of cadmium and copper metal ions in wastewater stream. The application of these endophytic fungi in removal and adsorbtion of cadmium and copper from the industrial wastewater directly are under study by our group.

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