Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Tolerance of Fungal isolates to Some Heavy Metals

Monira Zhran *, Asmaa El-Hosainy and Shadia Sabry

Al-Azhar University, Faculty of Science (Girls) Nasr city, Cairo, Egypt,

Abstract

Twenty-five fungal isolates were isolated from the collected soil samples from contaminated area, of Ain Helwan, Cairo, Egypt. All isolates could grow on media containing 1.0 g/L of Nickel, Lead or Cobalt. The most potent fungal isolates that tolerate high heavy metal concentrations were morphologically identified as *Penicillium sp.*, *Asperagillus niger*. and *Rhizopus sp*. These fungal isolates have recorded high tolerance index toward the tested heavy metals. Dispersive X-ray microanalysis (SEM-EDX) was used for the detection of the fate of metals present in fungal cells (before and after heavy metal treatment). The obtained data as a result of Dispersive X-ray microanalysis (SEM-EDX) application revealed the presence of high concentrations of the experimental heavy metal ions (Pb^{2+}, Ni^{2+} and Co^{2+}) in the isolated fungal cells. Also, their replacement of Ca^{++} ions in some cases and the detection of monovalent ions interruption such as K^{+} and Na^{+}, in the cell of the treated fungal isolate.

Keywords: Heavy metals -lead-nickel -cobalt -tolerance index -fungi and SEM-EDX.

1. Introduction

Because of human activities, harmful heavy metals and organic pollutants contaminate soil and water, which has become a major concern in environmental and health issues. The interaction of toxic electronics waste with fungi has been a key environmental setting that is hastening natural environmental pollution and is of importance due to the occurrence of dominating fungi in metal-polluted environments.

The translocation of harmful metals and radionuclides into the fruit bodies of edible higher fungi, as well as the use of fungal biomass for the detoxification of metals or radionuclide-containing industrial effluents, are examples of "Biotechnological Potential" (**Burgstaller and Schinner, 1993; Gadd, 1994**).

Until now, fungi have been primarily involved with the manufacture of antibiotics, enzymes, and organic acids. Fungi's capacity to solubilize large amounts of metals from solid objects has opened new possibilities for their use.

Carboxyl, amine, hydroxyl, phosphate, and sulfhydryl groups are among the functional groups found in fungal cell walls. These functional groups act as ligands, allowing metal ions to be chelated.

The sensitivity and protective mechanisms of different fungal species and strains to heavy metals vary. Metal removal is a complicated process that is influenced by metal ion chemistry, microorganism cell wall compositions, organism physiology, and physicochemical parameters such as pH, temperature, time, ionic strength, and metal concentration (**Mishra et al., 2010**).

Lead, one of the most extensively used heavy metals, is primarily utilized in the production of electric batteries, paint, lead smelting, internal combusting engines, fueled aviation engines, and explosives. Lead is very hazardous, and exposure to high levels can result in encephalopathy, hepatitis, and nephritic syndrome (**Lo et al. 1999).**

Nickel and cobalt are heavy metals that can enter the body through the respiratory tract and accumulate to dangerous amounts. They can be found in soil, water, and air. Nickel compounds have been shown to cause cancer in humans and animals, whereas cobalt compounds cause tumours in animals and are likely to cause cancer in humans (**Patel et al., 2012**).

Aluminum (Al (III)), chromium (Cr(III)), and lead (Pb(II) are the most common hazardous elements found in polluted water (**EPA, 2013; Simonescu and Ferdes, 2012**). Smelting, leather tanning, paint

*Corresponding author e-mail: Monira@azhar.edu.eg; (Monira Zhran).
Receive Date: 08 April 2022, **Revise Date:** 05 May 2022, **Accept Date:** 08 May 2022, **First Publish Date:** 08 May 2022
DOI: 10.21608/EJCHEM.2022.132266.5822

manufacture, and textile dyeing are just a few of the sectors that emit these metals (**Iskandar et al., 2011**).

Chemical and physical methods were used to remove metals from wastewaters. Precipitation, membrane technology, ion exchange, and adsorption on activated carbon are all examples of this (**Kavita and Keharia, 2012**). These traditional methods are generally effective for removing metals in moderate to high concentrations in a aqueous solutions, but they are less effective for removing metals in low concentrations (10–100 ppm) (**Simonescu and Ferdes, 2012**).

Metals are known to be removed successfully by fungi, either by biosorption or bioaccumulation. Metal ions are sequestered onto the cell wall by surface bonding during biosorption. Metal ions are assimilated intracellularly in bioaccumulation (**Kavita and Keharia, 2012; Gönen and Aksu, 2009**). Metal tolerance and metal removal capacity have been established in fungal species such as *Aspergillus*, *Rhizopus*, *Trichoderma*, and *Penicillium* (**Iskandar *et al.*, 2011; Kavita and Keharia, 2012; Sim *et al.*, 2016; Ezzouhri *et al.*, 2009).**

The objective of this work was to study the resistance, removal capacity of heavy metals and determination of minimum inhibitory concentration (MIC) by the most metal tolerant fungal isolates beside the study of SEM-EDX

2. Materials and methods

Isolation and maintenance of fungi:

Isolation of fungi were carried out using contaminated soil from Ain Hellwan, Egypt. The fungal isolates were sub-cultured on malt extract agar medium containing (g/L): Malt extract, 20; Glucose, 20; Peptone, 1 and Agar25 at pH 7 (**Smith and Onions, 1983)**.

Serial dilution of contaminated soil and standard spread plate techniques were employed for fungal isolation **(Sanders, 2012)**. Aliquots of 1 ml from different dilution were spread on the medium plates (in triplicates). The inoculated plates were incubated at 28°C ± 2 for 5 days. The spores of hyphal tips of fungal isolates were detached and allowed to develop on agar surface of sterilized media for purification. Then the purified cultures were maintained at 4°C till used. Fungal isolates were transferred once every two weeks to maintain availability and stability.

Fungal isolates response to heavy metal ions:

Lead, Nickel and cobalt were applied separately (1.0 g /L) on malt extract agar medium for investigation of the isolated fungal growth and sporulation**.**

Influence of different heavy metal concentration on growth of fungal isolates

Malt extract agar medium supplemented with different heavy metal ion (Lead, nickel and cobalt) concentrations; (0.5, 0.7, 1, 2, 3, 4, 5 and 6 g/L) were used. The initial pH value of the medium was adjusted at pH 7 before and after autoclaving. Triplicate sets of agar plates were carried out for each concentration for each fungal isolate. Mean colony area were determined.

Identification of fungal isolates:

Fungal isolates which appeared highest minimum inhibitory concentration of Lead, Cobalt and Nickel were morphologically identified for further study.

he three fungal isolates were identified morphologically according to **Supramanian, 1971; Domsch *et al*., 1980; Schipper and Stalpers, 1984.**

Determination of mycelial and tolerance index of the fungal isolates at different concentrations of Lead, Nickel and Cobalt.

Malt extract liquid medium supplemented with different, lead concentrations(g/L) (0.5, 3.0 and 5.0 for isolate 9 and isolate 14 and 0.5, 3.0 and 4.0 g/L for isolate 17), Nickel concentrations (g/L)(0.5, 1 and 3.0 g/L for isolate 9 and isolate 14 and 0.5, 1 and 2.0 for isolate 17) and Cobalt concentrations(g/L) (0.5, 3.0 and 6.0 for isolate 9, 0.5, 1.0 and 3.0 g/L for isolate 14 and 0.5, 1.0 and 2.0 for isolate 17) were used. The initial pH value of the medium was adjusted at pH7 before and after autoclaving. 50 ml media were allotted among conical flasks of 250 ml capacity. Triplicate sets of flasks were sterilized at 1.5 atmosphere, for 20 min then inoculated with one disk (of each fungal isolate) and incubated at 28 ± 2ºC for 5 days. The mycelial dry weights were determined.

The Tolerance index (TI):

is a ratio of fungal growth under the stress of heavy metal to control (without heavy metal) for the same period was calculated according to **(Valix *et al.*, 2001; Valix and Loon, 2003**).

Microanalysis by X-ray examination

Malt extract agar medium supplemented with different concentrations of Lead (Pb $(CH_3COO)_2$), Ni^{2+} ($NiCl_2$) and Co^{2+} ($CoCl_2$) were applied separately, the isolated colony were incubated at 28± 2ºC for 5 days. The concentrations used were 0.0, 5.0, 5.0 and 4.0 g/L for lead acetate for (control, isolate 9., isolate 14 and isolate 17, respectively); 0.0, 3.0 and 2.0

g/L for $NiCl_2$ (control, isolate 14. and isolate 17, respectively) and 0.0, 6.0 and 3.0 g/L for $CoCl_2$ (control, isolate 9 *and* isolate 14, respectively). Microanalysis by X-ray used for detecting elemental composition in the isolate cells. Samples were examined under X-ray microanalyzer (Model Oxford 6587 INCA x-sight) attached to JEOL JSM-5500 LV scanning electron microscopy at 20 KV after gold coating using SPI Model sputter coater. All methods were done at Regional Center for Mycology and Biotechnology, Al Azhar University, Cairo Egypt.

Statistical analysis

Statistical analysis, (mean ± SD) was carried out using software GraphPad InStat 3.06 Guide.

3. Results and Discussion

Fungal isolates response to heavy metal ions

Many microorganisms require metals such as Fe, Mn, Cu, Ni, Co, and Zn for growth, however these metals are hazardous when present in the cytosol at high levels (**Finney and O'Halloran, 2003**). Nickel (often referred to as Ni (II)) is employed in a number few of biologically significant processes, including the formation of complexes with polypeptide chains (**Cerruti et al., 1998**). For microbial growth and function, many heavy metals are essential. As microelements, trace elements like manganese, nickel, iron, copper, zinc, and molybdenum are required in very low concentrations (**Doelman, 1986; Hughes and Poole, 1989). Oladipo et al. (2018) i**nvestigated the tolerance to Cu, Pb, and Fe of *Rhizopus* and *Trichoderma* isolated from gold and gemstone mine site soils. The utilization of microorganisms for heavy metal removal and recovery from contaminated waste waters is of significance. The ability of biological materials to absorb heavy metals even at very low concentrations is their main use (**Volesky,1990**). Biosorptive characteristics of many biomaterials have been investigated, and various forms of biomasses have exhibited high levels of metal uptake **(Merroun et al., 2005; Anand et al., 2006; Tsekova et al., 2006; Perez et al., 2009; Gadd, 2009**). Fungal biomass appears to be an excellent sorption material among the various forms of biosorbent examined. Because it often demonstrates considerable tolerance to metals and other harsh circumstances, both living and dead fungal biomass can be used in biosorptive processes (**Kapoor and Viraraghavan, 1995**).

Table (1) shows the response of 25 fungal isolates against 3 heavy metal ions (Nicle, Lead and Cobalt) as a colony area (cm) and sporulation (high or low). In this experiment not all fungal isolates grew. Fungal isolates No. 9, 14 and 17 revealed the highest tolerance isolates to the used heavy metals which expressed by the colony area diameter (Table 1).

These results of qualitative screening corroborate with that of **Dwivedi *et al.* (2012), Abd El Hameed *et al.* (2015)**, **Rose and Devi (2018**) **and Imran *et al*. (2020).** Growth inhibition was seen in the isolates, which could be due to metal toxicity in the developing cells. The reduction in fungal growth at higher heavy metal concentrations could be attributed to a longer lag phase compared to the control (heavy metal free) (**Iram et al., 2012**) or a combination of biological variables (**Dwivedi et al., 2012**). **Eliseo and Bitacura (2021)** studied the ability of filamentous fungi to tolerate high concentrations of heavy metals.

Influence of different concentrations of heavy metals used on growth of fungal isolates:

The growth of most potent fungal isolates on different concentrations of used heavy metals was shown in Table (2). Generally, the growth of fungal isolates was decreased gradually by the increase of heavy metal concentration. Fungal isolates (9 and 14) were continued their growth till the concentration of 5.0 g/L that represented a minimum inhibitory Concentration. (the growth diameters were 6.11 ± 0.21 and 3.07 ± 0.29 cm, respectively). Whereas growth of fungal isolate (17) was persistent to a minimum inhibitory concentration 4.0 g/L as 2.01 ± 0.30 cm**.** In case of Nickel, the fungal isolates 9 and 14 continued growth to a minimum inhibitory concentration of 3.0 g/L by 3.28 ± 0.30 and 3.78 ± 0.19 cm, respectively but at 2.0 g/L for fungal isolate 17 with growth of 2.01 ± 0.30 cm. The growth of fungal isolates 9, 14, and 17 in case of Cobalt, was differed, according to the isolate that continued at minimum inhibitory concentrations 6, 3 and 2 g/L by 2.13 ± 0.30, 3.89 ± 0.14 and 4.19 ± 0.30 cm, respectively. This data was agreed with study of **Eliseo and Bitacura (2021)** on *Rhizopus sp., Mucor sp.* and *Trichoderma sp.*, where the concentration of the heavy metal was increased, the colony extension decreased. The MIC of Rhizopus sp. to Cu was 10<MIC≤15mM, according to **Eliseo and Bitacura (2021) and Ezzouhri et al. (2009)**, which was lower than that of *Aspergillus sp*., which was 15–20mM. This may mean that the fungus developed tolerance or adaptation during incubation as suggested by **Ezzouhri et al (2009)**. **Ahmad et al. (2005)** found that *Aspergillus* and *Rhizopus sp*. isolated from wastewater-treated soil showed multi-metal resistance with varying level as evident from the MIC value of different metal.

Anahid et al. (2011) discovered that *Aspergillus niger* and *Aspergillus foetidus* have a lower tolerance for Ni (II). According to **Gadd (2008)**, metal toxicity is dependent on metal type and concentrations, the organism, and environmental psychochemical factors.

Table (1): Preliminary response of fungal isolates to some heavy metal ions.

Fungal isolates	Heavy metal ions (1g/L)					
	Lead		Nicle		Cobalt	
	Colony area mean (cm)±SD	Sporulation	Colony area mean (cm)±SD	Sporulation	Colony area mean (cm)±SD	Sporulation
1	7.27 ± 0.32	High	2.37 ± 0.25	High	5.22 ± 0.12	Low
2	2.53 ± 0.47	Low	5.47 ± 0.55	High	7.77 ± 0.33	High
3	6.97 ± 0.15	High	5.33 ± 0.32	High	6.78 ± 0.56	High
4	7.63 ± 0.52	High	7.70 ± 0.21	High	3.26 ± 0.29	Low
5	5.00 ± 0.30	Low	7.27 ± 0.55	High	5.78 ± 0.21	High
6	7.10 ± 0.36	High	5.00 ± 0.20	High	4.26 ± 0.21	High
7	5.13 ± 0.15	Low	5.53 ± 0.32	High	6.41 ± 0.31	High
8	2.47 ± 0.25	Low	7.30 ± 0.20	High	6.11 ± 0.44	High
9	8.30 ± 0.30	Low	7.97 ± 0.31	Low	8.19 ± 0.32	High
10	2.83 ± 0.15	Low	6.13 ± 0.12	High	6.84 ± 0.29	High
11	2.40 ± 0.56	Low	5.20 ± 0.20	High	7.0 ± 0.11	High
12	Ng	Ng	Ng	Ng	Ng	Ng
13	Ng	Ng	Ng	Ng	Ng	Ng
14	7.99 ± 0.23	High	7.90 ± 0.15	High	8.04 ± 0.15	High
15	5.17 ± 0.31	Low	6.80 ± 0.26	High	7.69 ± 0.35	High
16	7.10 ± 0.36	High	5.47 ± 0.15	High	7.08 ± 0.28	High
17	8.77 ± 0.25	High	8.01 ± 0.36	High	8.55 ± 0.23	High
18	7.07 ± 0.25	High	5.20 ± 0.20	Low	Ng	Ng
19	Ng	Ng	Ng	Ng	Ng	Ng
20	Ng	Ng	Ng	Ng	Ng	Ng
21	4.77 ± 0.25	High	Ng	Ng	Ng	Ng
22	4.33 ± 0.35	High	5.33 ± 0.31	High	6.47 ± 0.17	low
23	7.77 ± 0.68	High	5.20 ± 0.20	High	6.90 ± 0.22	Low
24	Ng	Ng	Ng	Ng	Ng	Ng
25	7.60 ± 0.40	High	5.50 ± 0.44	High	Ng	Ng

Ng=No growth

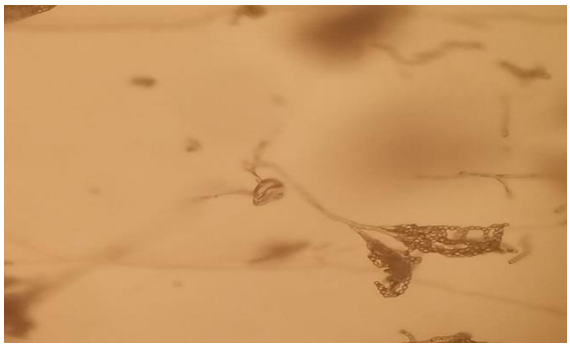
Table (2). Effect of different concentrations of Lead, Nickel and Cobalt on growth of fungal isolates.

Heavy metals	Metal Concentration g/L	Fungal isolates - Colony diameter(cm) - mean ±SD		
		isolate 9	isolate 14	isolate 17
Lead	0.5	8.70 ± 0.15	8.64 ± 0.35	8.93 ± 0.22
	0.7	8.37 ± 0.21	8.24 ± 0.13	8.89 ± 0.21
	1	8.30 ± 0.30	7.99 ± 0.23	8.77 ± 0.25
	2	7.71 ± 0.29	7.17 ± 0.18	6.21 ± 0.12
	3	6.57 ± 0.27	6.09 ± 0.33	5.67 ± 0.42
	4	6.45 ± 0.30	5.57 ± 0.22	**2.01 ± 0.30**
	5	**6.11 ± 0.21**	**3.07 ± 0.29**	Ng*
	6	Ng	Ng	Ng
Nickel	0.5	8.33 ± 0.11	8.71 ± 0.30	8.42 ± 0.17
	0.7	8.10 ± 0.18	8.35 ± 0.21	8.11 ± 0.17
	1	7.97 ±0.31	7.90 ± 0.15	8.01 ± 0.36
	2	5.86 ± 0.28	6.06 ± 0.22	**4.17 ± 0.22**
	3	**3.28 ± 0.30**	**3.78 ± 0.19**	Ng
	4	Ng	Ng	Ng
	5	Ng	Ng	Ng
	6	Ng	Ng	Ng
Cobalt	0.5	8.76 ± 0.19	8.67 ± 0.19	8.88 ± 0.22
	0.7	8.45 ± 0.23	8.19 ± 0.24	8.62 ± 0.19
	1	8.19 ± 0.32	8.04 ± 0.15	8.55 ± 0.23
	2	8.05 ± 0.19	6.06 ± 0.31	**4.19 ± 0.30**
	3	7.83 ± 0.22	**3.89 ± 0.14**	Ng
	4	6.29 ± 0.20	Ng	Ng
	5	4.33 ± 0.18	Ng	Ng
	6	**2.13 ± 0.30**	Ng	Ng
	7	Ng	Ng	Ng

Ng=No growth

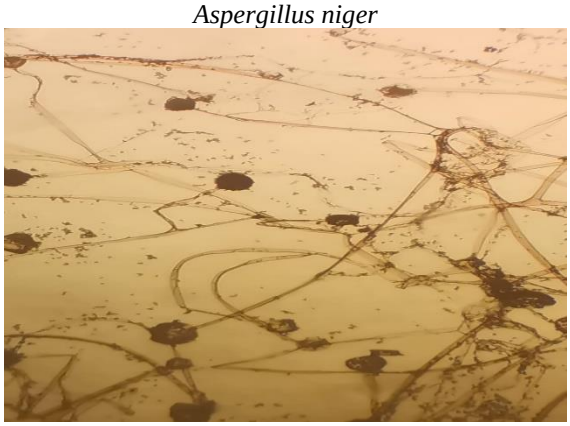
Identification of fungal isolates:

The three fungal isolates were identified morphologically as *Penicillium sp.*, *Aspergillus niger* according to (**Supramanian, 1971; Domsch *et al.*, 1980**) and *Rhizopus sp.* according to **Schipper and Stalpers (1984)** (Fig. 1).



Penicillium

Aspergillus niger



Rhizopus sp.

Fig. (1): Images of preliminary identification of fungal isolates.

Aspergillus and *Penicillium* species are the most fungal isolates tolerate high amounts of heavy metals as recorded by many authors as **Rose and Devi (2018**) they were identified four fungal isolates as *Aspergillus awamori*, *Aspergillus flavus*, *Aspergillus niger* and *Penicillium sp.* Heavy metals resistant filamentous fungi were tested by **Congeevaram et al. (2007)**, **Zafar et al. (2007)**, and **Ezzouhri et al. (2009)**, who discovered that the most tolerant fungi belonged to the genera *Aspergillus* and *Penicillium*. According to **Congeevaram et al. (2007)**, *Aspergillus* species are effective heavy metal reducers. **Dwivedi *et al.* (2012**) isolated and identified various Ni (II) tolerant fungal species as *Aspergillus niger* (Ni19, Ni27, Ni33) and *Aspergillus flavus* (Ni35, Ni36). Furthermore, **Iram et al. (2012)** isolated fifty-four fungal strains from a Faisalabad (Chakera Chack) industrial effluent irrigated agricultural area, thirteen of which were identified as *Aspergillus niger* and twenty-two as *Aspergillus flavus*.

Manguilimotan and Bitacura (2018) investigated eight Cd-tolerant filamentous fungi found in coastal waters and sediments near industrial plant effluent sites in Barangay Ibo, Lapu-Lapu City, Cebu. Only three isolates were identified to be the most effective in terms of Cd biosorption activity. Two of them belonged to the *Aspergillus* genus and one to the *Penicillium* genus.

Tolerance index of fungal isolates at different concentrations of heavy metal ions:

Figure (2) showed the fungal dry weights of the fungal isolates at different concentrations of the metals used and Fig. (3) revealed the metal tolerance indices. *Aspergillus niger* was the most sensitive one while *Rhizopus sp.* was the most tolerant one. On the other hand, the mean mycelial dry weight of fungal isolates was decreased by increasing metals concentrations. Highest decrement of mean mycelial dry weight was recorded in lead metal by *Rhizopus sp.;* 980 ± 147 mg/50 ml medium at concentration 4 g/L; *Aspergillus niger* on Nickel by*;* 316.7 ± 151 mg/50 ml medium at concentration 3 g/L while that of *Rhizopus sp. on* Cobalt revealed 475 ± 153 mg/50 ml medium at concentration 2 g/L.

Several investigations have demonstrated the tolerance of some fungal species to heavy metals, as well as the physiological reaction to them (Yang et al., 2009; Zaidi and Pal, 2017; Fawzy et al., 2017; Rivas-Castillo et al., 2017). *Simplicillium chinense*, *Penicillium simplicissimum*, and *Trichoderma asperellum* have shown tolerance to Al (III), Pb (II), and Cr (III) in previous studies at lower concentrations (**Ting et al., 2011; Sim et al., 2016; Chen et al., 2017**).

Aspergillus sp., according to Srivastava and Thakur (2006) and Rose and Devi (2018), can accumulate harmful heavy metals in excess of nutritional requirements. The effect of varying heavy metal concentrations on fungal growth was measured using a tolerance index (TI). Fungi with TI values more than one are resistant or tolerant, whereas those with TI values less than one are vulnerable or non-tolerant to a certain heavy metal and its concentration.

Pal et al. (2010) found that a heavy metal tolerant strain of *Rhizopus arrhizus* collected lead in cell walls and that cellular metabolism may be involved in the fungus' heavy metal bioaccumulation.

According to **Tsekova and Galabova (2003)**, acid phosphatase synthesis in and out of the cells after exposure to Cu2+ ions is required for heavy metal tolerance and bioaccumulation in *Rhizopus delemar*.

Metal tolerance in fungal communities is thought to be mediated by a variety of processes, including extracellular sequestration (binding to the cell wall) or intracellular binding to particular proteins **(Anahid et al., 2011; Liu et al., 2017).**

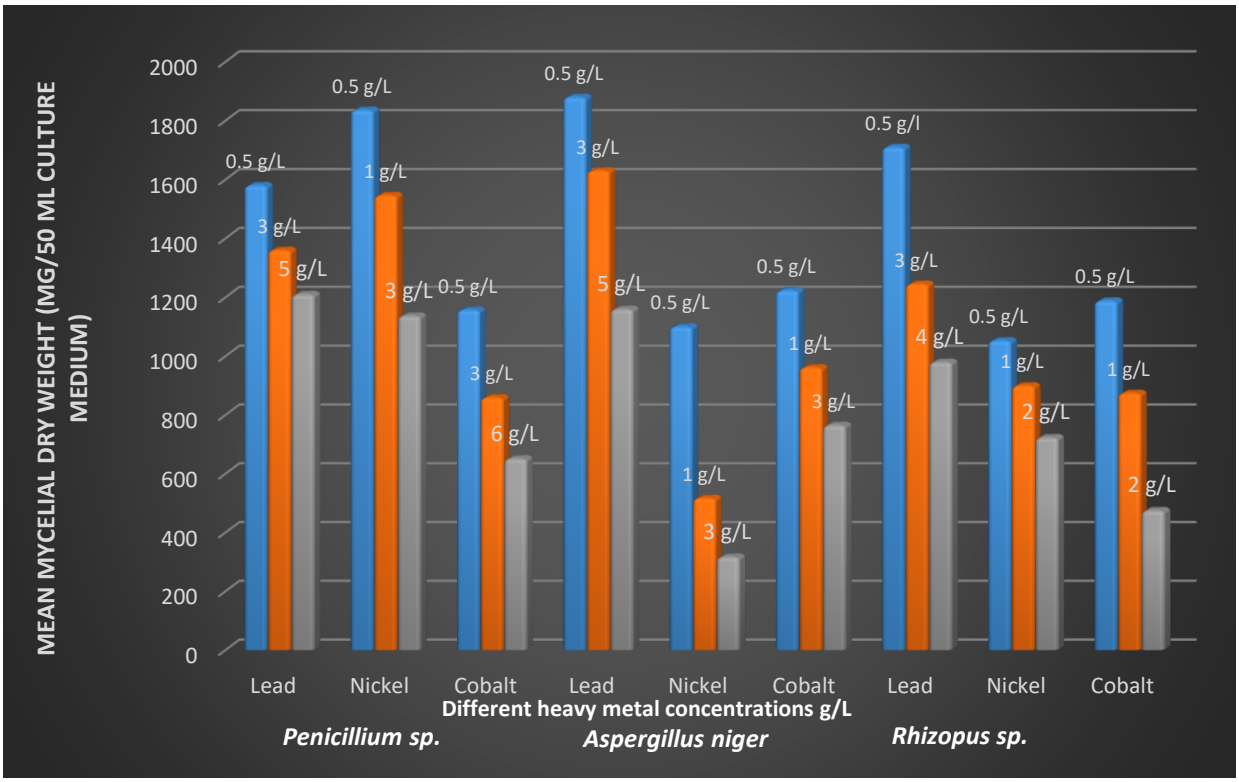


Fig. (2): Mean mycelial dry weight of the most tolerant fungal isolates at different concentrations of heavy metals.

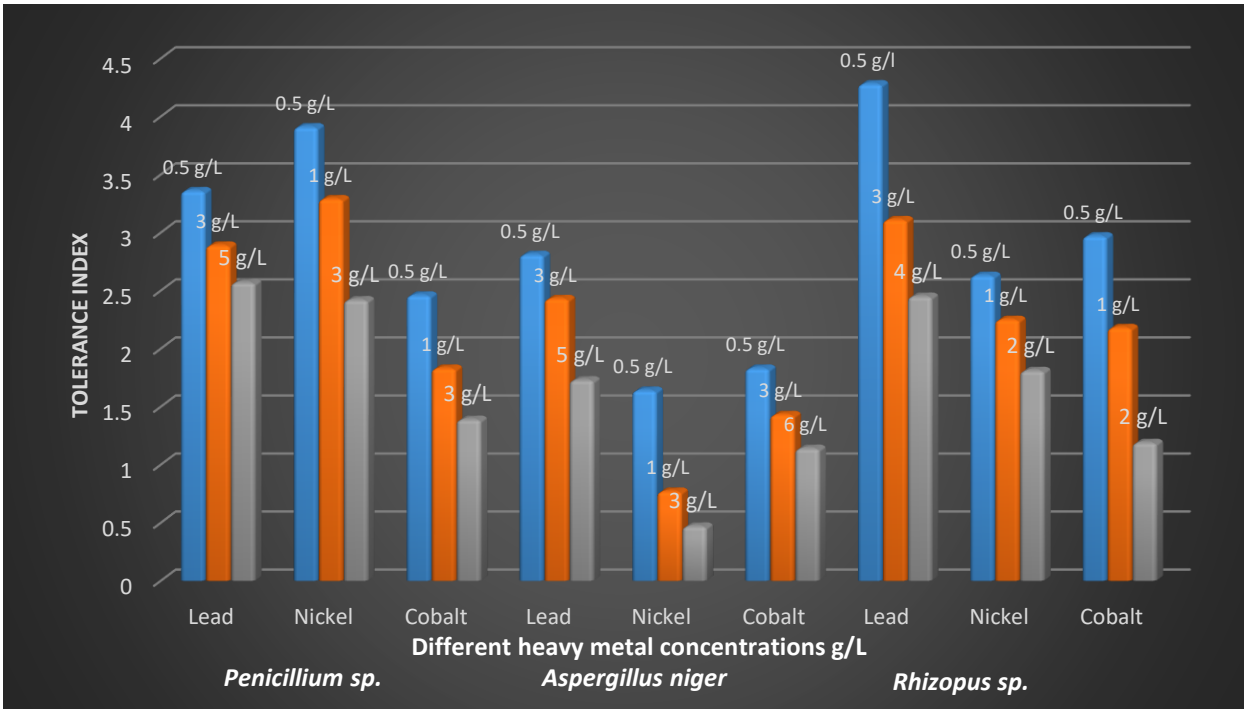


Fig. (3): Tolerance index of the most tolerant fungal isolates at different concentrations heavy metals.

Microanalysis by X-ray examination:

It is worth to detect the presence of these metal ions (Lead, Nickel and Cobalt**)** in fungal cells, which made using elemental analysis by scanning electron microscopy/energy dispersive X-ray microanalysis (SEM-EDX). Table (3) appeared that, Pb ions are not detected in all fungal cells cultivated on medium free of Pb^{++} ions. Lead ions were noticed in all cells cultivated on lead. High amounts of lead ions were accumulated in cells of *Aspergillus niger* while smaller amounts were in cells of *Penicillium sp.* and *Rhizopus sp*.

In Table (3) and figs. (4 and 5) remarkable amounts in Cl^{+} ions present in cells of treated *Aspergillus niger* while Cl^{+} ions not detected in Pb^{++} free fungal cells. Mn^{++} and Cu^{++} ions detected only in *Rhizopus sp.* cells cultivated on Pb^{++} ions free medium while they were disappeared in treated cells. AL^{+++} ions were recorded in *Aspergillus niger* and *Rhizopus sp*. cells treated with Pb^{++} whereas they not detected in *Penicillium sp.* cells.

Ni $^{++}$ions not detected in fungal cells cultivated on Ni $^{++}$ ions free medium, whereas the cells of *Aspergillus niger* that cultivated on Ni $^{++}$ ions had considerable amounts of the ion (Table 4 and Figs 4 and 6). Ca++ and K+ ions were decreased in the treated cells while there was considerable amount in untreated cells. Na^{+} ions were increased in treated cells; this increase in Na^{+} ions and decrease in K^{+} ions may appear as a result of interruption in the protein of the channel ions of Na^{+} and K^{+}. S^{++} ions were increased in treated cells to some extent while Ca^{++} ions were decreased sharply in the treated cell which may be due to the substitution of Ni^{++} ions to that of Ca^{++} in the fungal cells.

The cells of *Rhizopus sp.* appeared the same trend in case of Na^{+}, K^{+} and Ca^{++} ions, while that of cultivated in Ni^{++}, Cl^{+} and Mn^{++} were disappeared in the used concentration whereas Ni^{++}, S^{++} and Al^{+++} were detected and may replace the disappeared metals (Table 4 and Figs 4 and 6). The appearance of small amounts of Cu^{++} ions in the cells of treated Ni^{++} reflected the stress conditions system in which the fungus cells occurred, so it is a process of detoxification. Surprisingly the Al^{+++} and S^{++} ions in the treated cells while the disappearance of Mn^{++} ions which may be substituted by Ni^{++} ions in the cells.

Co^{++} ions not detected in fungal cells cultivated on Co^{++} ions free medium. Al^{+++}, Co^{++}, Mn^{++} and Cu^{++} ions not detected in *Penicillium sp.* cells cultivated on Co^{++} free medium (Table 5 Figs 4 and 7). Cl^{+} ions disappeared from the cells cultivated on Co^{++} ions while Al^{+++} ions appeared in high concentration. Na^{+} ions decreased whereas K^{+} and S^{++} ions increased dramatically. The decrease of Ca $^{++}$ ions may be due to the replacement of Co^{++} ions to that of Ca $^{++}$ ions in fungal cells.

There is a greater decrease in Ca^{++} ions in *Aspergillus niger* cells that cultivated on Co^{++} ions, which may be substituted by Co^{++} ions (Table 5 and Figs 4 and 7). K^{+} and S^{++} ions were decreased in the cells cultivated on Co^{++} ions. The appearance of large amount of Al^{+++} in cells cultivated on Co^{++} ions detect the interruption of plasma membrane channel proteins.

The presented data was harmonized with **Chen *et al.* (2017)** who recorded that, the EDX analysis of hyphae surfaces of *S. chinense*, *P. simplicissimum* and *T. asperellum* revealed the presence of low amounts of Al (III) (0.52, 4.02 and 3.41 wt%, respectively). No heavy metal elements were detected on the hyphae of the isolates in control. Instead, elements that were commonly found on fungal cells, such as C (53.86–56.83 wt%), O (32.47–38.54 wt%), Na (2.46–2.87 wt%), P (1.97–2.75 wt%) and Pt(2.85–5.61 wt%), were detected. In *Bacillus firmus* and *Bacillus subtilis*, the presence of Co^{2+} and Ni^{2+} ions in the cultivation medium metamorphosed cells, affect all cells constituents and increased the Co^{2+} and Ni^{2+} ions presented in the cells (**El-Meleigy et al., 2011**)

Metals bind to the cell wall (biosorption) prior to bioaccumulation or metal sequestration via channels and transporters, according to EDX tests (**Damodaran et al., 2013**). These ions are assumed sequestered to vacuolar compartments as aluminium polyphosphate complexes, with the help of metal chelators (such as glutathione) to restrict cellular interactions with hazardous metals (**Farrag, 2009**).

After connecting with several proteins, calcium ions play a crucial role in cellular metabolism and function (**Pidcock and Moore, 2001**). Ca2+ is structurally identical to the divalent metal cations (Cd2+, Cu2+, Ni2+, and Zn2+) (**Huheey et al., 1993**). Because of their comparable ligand, it is possible that they substitute Ca2+ from the binding sites, as evidenced by the greater amount of Ca2+ observed in the culture filtrate of *Acidocella sp.* GS19hrs treatment (**Chakravarty et al., 2007**).

Table (3): Elemental analysis by scanning electron microscopy/energy dispersive X-ray microanalysis (SEM-EDX) of *Penicillium sp.*, *Aspergillus niger* and *Rhizopus sp.* at minimum inhibitory lead concentration.

Fungal isolates / Element	*Penicillium sp.*		*Aspergillus niger*		*Rhizopus sp.*	
	Control	5 g/L	Control	5 g/L	Control	4 g/L
Na	9.727 ± 0.526	6.357 ± 1.362	17.493 ± 2.124	18.210 ± 3.031	9.610 ± 0.546	6.343 ± 0.883
P	25.353 ± 1.891	27.850 ± 0.650	22.023 ± 7.318	17.737 ± 2.268	26.177 ± 2.804	27.873 ± 0.311
S	1.557 ± 0.534	3.653 ± 0.510	11.057 ± 4.725	---	---	2.943 ± 0.474
Cl	1.293 ± 0.170	---	---	12.463 ± 4.111	1.453 ± 0.350	2.053 ± 0.304
K	44.743 ± 4.008	47.280 ± 4.802	11.460 ± 4.031	14.363 ± 3.116	47.827 ± 5.846	44.507 ± 2.431
Ca	17.323 ± 1.777	10.980 ± 2.977	37.963 ± 13.382	10.343 ± 6.186	12.247 ± 4.676	11.080 ± 2.651
Pb	---	3.880 ± 1.767	---	21.600 ± 0.261	---	5.113 ± 0.512
Al	---	---	---	5.287 ± 3.889	---	0.083 ± 0.122
Mn	---	---	---	---	0.533 ± 0.751	---
Cu	---	---	---	---	2.157 ± 0.967	---

Table (4): Elemental analysis by scanning electron microscopy/energy dispersive X-ray microanalysis (SEM-EDX) of *Aspergillus niger* and *Rhizopus sp.* at minimum inhibitory nickel concentration.

Fungal isolates / Element	*Aspergillus niger*		*Rhizopus sp.*	
	Control	3 g/L	Control	2 g/L
Na	17.493 ± 2.124	29.923 ± 7.561	9.610 ± 0.546	17.370 ± 4.115
P	22.023 ± 7.318	15.287 ± 5.365	26.177 ± 2.804	23.860 ± 4.960
S	11.057 ± 4.725	12.313 ± 4.235	---	5.363 ± 2.148
Cl	---	---	1.453 ± 0.350	---
K	11.460 ± 4.031	8.187 ± 2.758	47.827 ± 5.846	37.903 ± 5.078
Ca	37.963 ± 13.382	27.360 ± 13.069	12.247 ± 4.676	7.527 ± 4.539
Ni	---	6.937 ± 5.656	---	0.997 ± 1.613
Mn	---	---	0.533 ± 0.751	---
Al	---	---	---	4.220 ± 7.006
Cu	---	---	2.157 ± 0.967	2.760 ± 1.749

Table (5): Elemental analysis by scanning electron microscopy/energy dispersive X-ray microanalysis (SEM-EDX) of *Penicillium sp.* and *Aspergillus niger* at minimum inhibitory Cobalt concentration.

Fungal isolates / Element	*Penicillium sp.*		*Aspergillus niger*	
	Control	6 g/L	Control	3 g/L
Na	9.727 ± 0.526	7.443 ± 0.414	17.493 ± 2.124	16.073 ± 5.033
P	25.353 ± 1.891	25.630 ± 2.311	22.023 ± 7.318	17.250 ± 5.860
S	1.557 ± 0.534	6.837 ± 1.127	11.057 ± 4.725	7.817 ± 3.439
Cl	1.293 ± 0.170	---	---	---
K	44.743 ± 4.008	47.097 ± 4.911	11.460 ± 4.031	7.183 ± 1.320
Ca	17.323 ± 1.777	6.590 ± 2.208	37.963 ± 13.382	6.287 ± 0.664
Al	---	6.083 ± 7.800	---	38.540 ± 18.225
Co	---	0.317 ± 0.864	---	6.850 ± 2.560
Mn	---	---	---	---
Cu	---	---	---	---

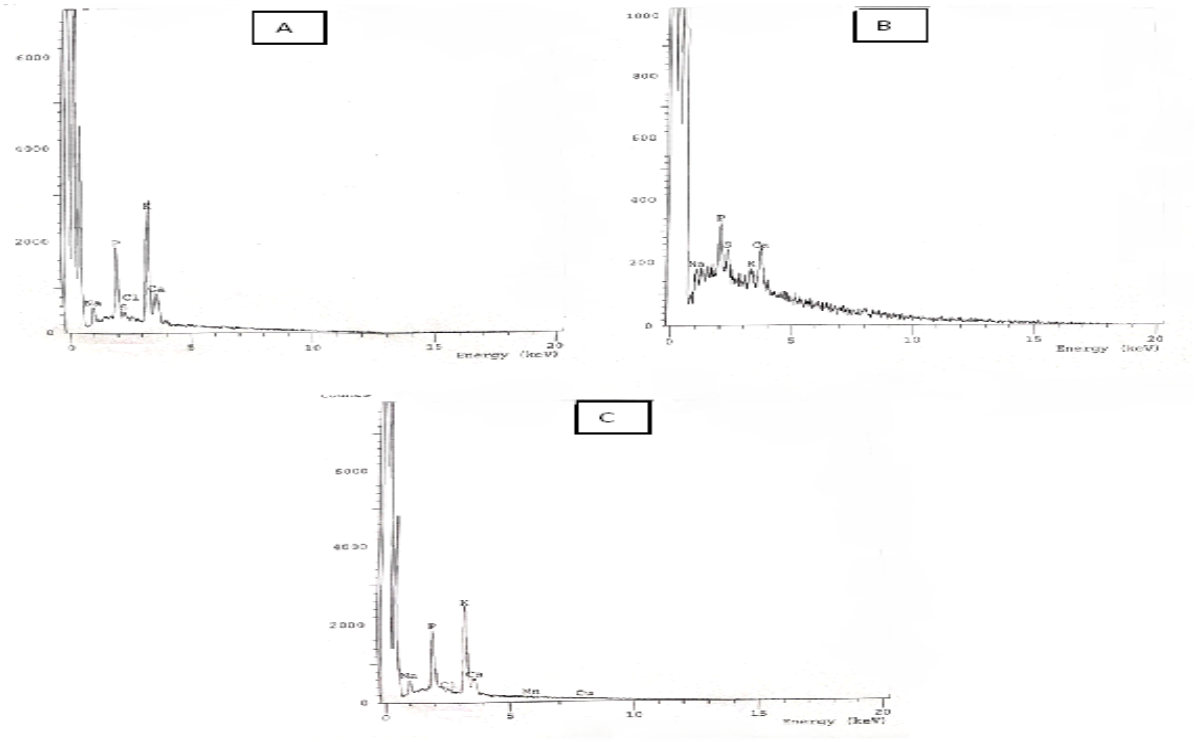


Fig. (4): Elemental analysis by scanning electron microscopy/energy dispersive X-ray microanalysis (SEM-EDX) of *Penicillium sp.*, (A) *Aspergillus niger* (B) and *Rhizopus sp.* (C) growing at medium free of heavy metal ions.

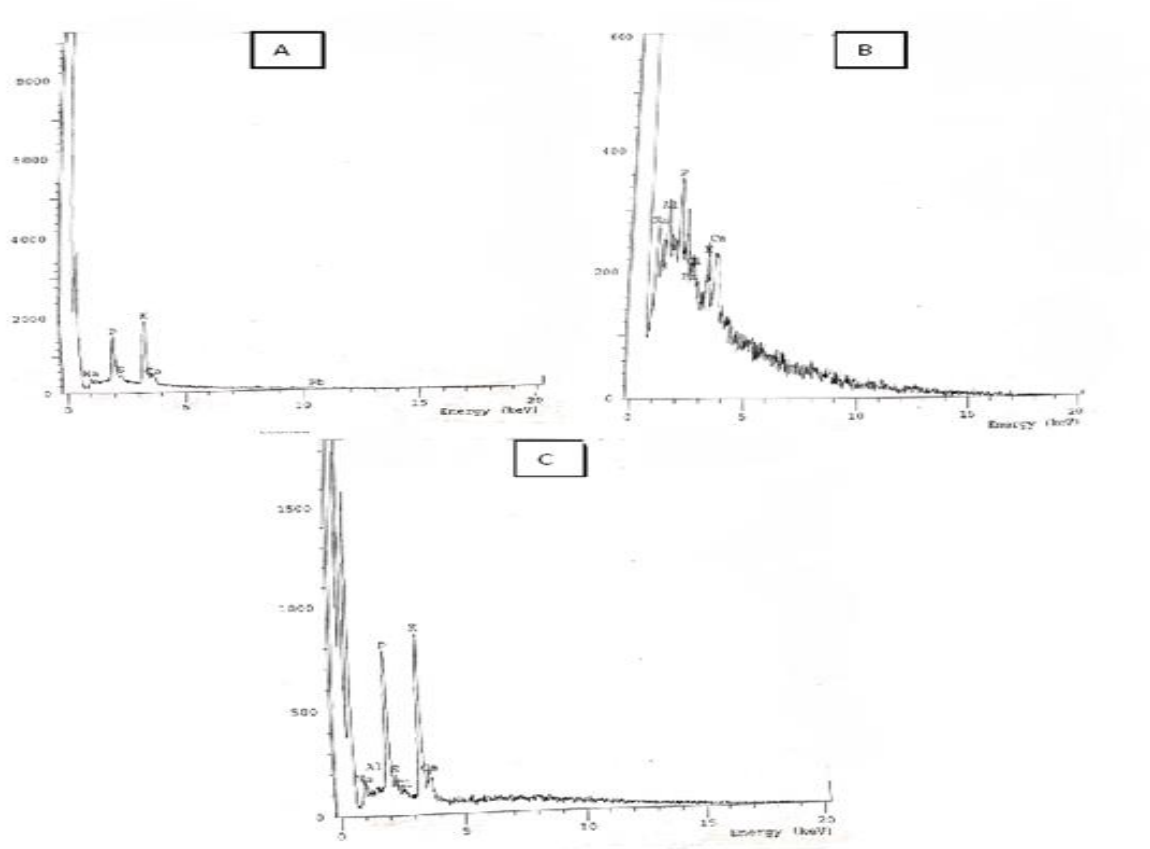


Fig. (5): Elemental analysis by scanning electron microscopy/energy dispersive X-ray microanalysis (SEM-EDX) of *Penicillium sp.* (A), *Aspergillus niger* (B) and *Rhizopus sp.* (C) at different concentrations of Pb^{++} ions.

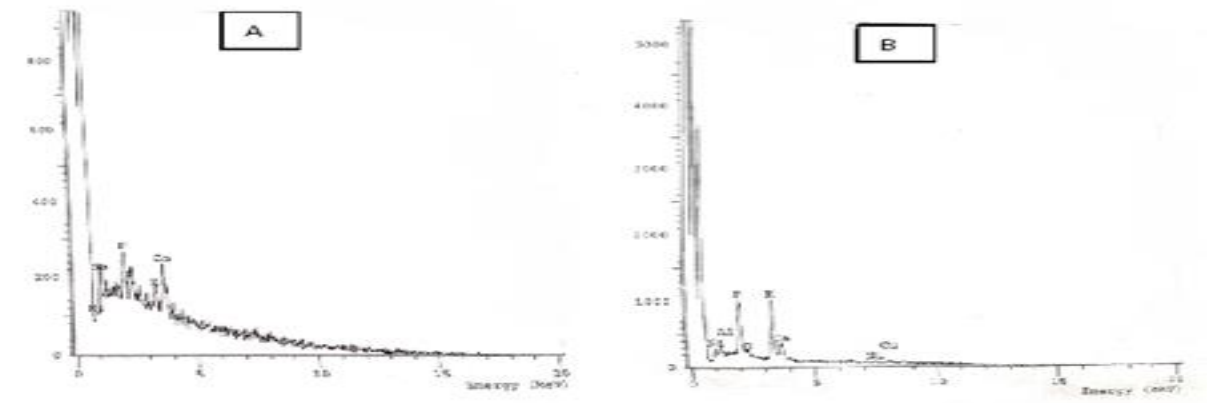


Fig. (6): Elemental analysis by scanning electron microscopy/energy dispersive X-ray microanalysis (SEM-EDX) of *Aspergillus* niger (A) and *Rhizopus sp.* (B) at 1g/l of nickel of Ni^{++} ions.

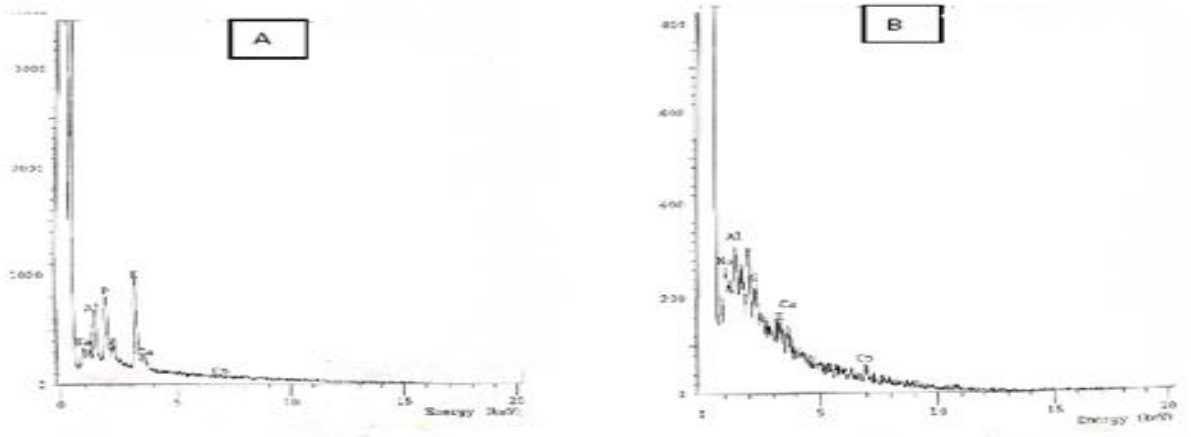


Fig. (7): Elemental analysis by scanning electron microscopy/energy dispersive X-ray microanalysis (SEM-EDX) of *Penicillium sp.* (A) and *Aspergillus niger* (B) at 6 g/L of Cobalt ion.

Conclusion:

Fungi are good absorbent for heavy metals, they remove considerable amounts of these metals from their solutions. *Penicillium sp.*, *Aspergillus niger* and *Rhizopus sp.* tolerate high amounts of lead, cobalt and nickel ions, they were screened for metal sequester in their cells by SEM-EDX analysis which reveal the presence of high amounts of the metals used in the cells. Next, we must study the other routs of tolerance of heavy metals in these fungi.

References

Abd El Hameed, A.H., Eweda, W.E., Abou-Taleb, K.A.A., Mira, H.I., (2015): Biosorption of uranium and heavy metals using some local fungi isolated from phosphatic fertilizers. Ann. Agric. Sci., 60: 345–351.

Ahmad I, Zafarn S and Ahmad, F. (2005). Heavy metal biosorption potential of *Aspergillus* and *Rhizopus sp.* Isolated from wastewater treated soil. J. Appl. Sci. Environ. Manag. 9(1):123-126.

Anahid, S.; Yaghmaei, S. and Ghobadinejad, Z. (2011): Heavy metal tolerance of fungi. Sci. Iran. 18: 502–508.

Anand, P.; Isar, J.; Saran, S.; Saxena, R.K., 2006: «Bioaccumulation of copper by *Trichoderma viride*. Bioresour. Technol., 97: 1018-1025.

Burgstaller, W. and Schinner, F. (1993). Leaching of metals with fungi. J. Biotechnol., 27(2): 91-116.

Cerruti, C.; Curutchet, G. and Donati, E. (1998): Bio-dissolution of spent nickel-cadmium batteries using *Thiobacillus ferrooxidans*. J. Biotechnol., 62: 209-219

Chakravarty, R.; Manna, S.; Ghosh, A.K. and Baerjee, P.C. (2007): Morphological changes in an *Acidocella* strain in response to heavy metal stress. Res. J. Microbiol., 2(10): 742-748.

Chen, L.; Feng, Q.; Li, C.; Wei, Y.; Zhao, Y.; Feng, Y.; Zheng, H.; Li, F. and Li, H. (2017): Impacts of aquaculture wastewater irrigation on soil microbial functional diversity and community structure in arid regions. Sci. Rep., 7: 11193. https://doi.org/10.1038/s41598-017-11678-z

Congeevaram, S.; Dhanarani, S.; Park, J.; Dexilin, M. and Thamaraiselvi, K. (2007): Biosorption of chromium and nickel by heavy metal resistant fungal and bacterial isolates. J. Hazard. Mater., 146: 270–277.

Damodaran, D.; Balakrishnan, R.M. and Shetty, V.K. (2013): The uptake mechanism of Cd (II), Cr (VI), Cu (II), Pb (II), and Zn (II) by mycelia and fruiting bodies of *Galerina vittiformis*. BioMed Res. Int., 2013 (2013). DOI: http://dx.doi.org/10.1155/2013/149120.

Doelman, P. (1986): Resistance to soil microbial communities to heavy metal. FEMS. Symp., 33: 369-384

Domsch, K. H.; Gams, W. and Anderson, T. H. (1980): Compendium of soil fungi. Academic Press, London. New York, Torroute, Sydeny San Francisco, 859Pp.

Dwivedi, S.N.; Mishra, R.P. and Alava, S. (2012): Phytochemistry, pharmacological studies and traditional benefits of *Trachyspermum ammi* (Linn.) Sprague. Int. J. Pharm. Life Sci., 3: 1705–1709.

Eliseo, R.M. and Bitacura, J.G. (2021): Heavy metal tolerance of filamentous fungi from the sediments of Visayas State University wastewater pond. Annals of Tropical Research 43 (1):88-101(2021). DOI: https://doi.org/10.32945/atr4317.2021 https://doi.org/10.32945/atr4317.2021.

El-Meleigy, M.A.; Abed, N.N. and Sari, I. P. (2011): Fate of cobalt and nickel in *Bacillus firmus* and *Bacillus subtilis*. Nature and Science., 9 (8) http://www.sciencepub.net/nature

Environmental Protection Agency (EPA) (2013): Code of federal regulations part423: steam electric power generating point source category, Environmental Protection Agency, United States of America.

Ezzouhri, L. (2009): Heavy metal tolerance of filamentous fungi Isolated from polluted sites in Tangier, Morocco. Afr. J. Microbiol. Res., 3(2):35-48

Ezzouhri, L.; Castro, E.; Moya, M.; Espínola, F. and Lairini, K. (2009): Heavy metal tolerance of filamentous fungi isolated from polluted sites in Tangier, Morocco. Afr. J. Microbiol. Res. 3: 35–48.

Farrag, R.M. (2009): Ultrastructure, glutathione and low molecular weight proteins of *Penicillium brevicompactum* in response to cobalt, Pol. J. Microbiol. 5: 327–338.

Fawzy, E. M.; Abdel-Motaal, F. F.; Soad, A. and El-Zayat, S. A. (2017): Biosorption of heavy metals onto different eco-friendly substrates. J. Bioremediation and Biodegradation, 8(3): 1–7.

Finney, L.A. and O'Halloran, T.V. (2003): Transition metal speciation in the cell: Insights from the chemistry of metal ion receptors. Sci., 300 (5621): 931-936.

Gadd, G. M. (1994): Interactions of fungi with toxic metals. In The Genus *Aspergillus* (pp. 361-374). Springer US.

Gadd, G.M. (2008): Bacterial and fungal geomicrobiology: a problem with communities. Geobiology., 6: 278-284

Gadd, G.M. (2009): Biosorption: critical review of scientific rationale, environmental importance and significance for pollution treatment. J. Chem. Technol. Biotechnol., 84: 13-28.

Gönen, F. and Aksu, Z. (2009): Single and binary dye and heavy metal bioaccumulation properties of *Candida tropicalis*: use of response surface methodology (RSM)for the estimation of removal yields. J. Hazard. Mater 172: 1512–1519.

Hughes, M.N. and Poole, R. K. (1989): Metal mimicry and metal limitation in studies of metal-microbe interactions. (eds: Poole, R.K., Gadd, G.M.) Metal-Microbe Interactions. IRL Press, Oxford. 1-17p

Huheey, J.E.; Keiter, E.A. and Keiter, R.L. (1993): Inorganic Chemistry: Principles of structure and reactivity. 4th edition. Harper Collins College Publishers, New York. 577-622p

Imran, M.; Ahmad, I.; Barasubiye, T.; Abulreesh, H.H.; Samreen; Monjed, M.K. and Elbanna, kh. (2020): Heavy metal tolerance among free-living fungi isolated from soil receiving long term application of wastewater. *J. Pure Appl. Microbiol.*, 14(1), 157-170. DOI: https://doi.org/10.22207/JPAM.14.1.17.

Iram, S.; Parveen, K.; Usman, J.; Nasir, K. and Akhtar, N. (2012): Heavy metal tolerance of filamentous fungal strains isolated from soil irrigated with industrial wastewater. Biologija ,58: 107–116.

Iskandar, N.L.; Zainudin, N.A. and Tan, S.G. (2011): Tolerance and biosorption of copper (Cu) and lead (Pb) by filamentous fungi isolated from a freshwater ecosystem. J. Environ. Sci., (China) 23: 824–830.

Kapoor, A. and Viraraghavan, T. (1995): Fungal biosorption-An alternative treatment option for heavy metal bearing wastewaters: A review. Bioresour. Technol., 53: 195- 206.

Kavita, B. and Keharia, H. (2012): Biosorption potential of *Trichoderma gamsii* biomass for removal of Cr(VI) from electroplating industrial effluent, Int. J. Chem. Eng., DOI: http://dx.doi.org/10.1155/2012/305462.

Liu, S-H.; Zeng, G-M.; Niu, Q-Y.; Liu, Y.; Zhou, L.; Jiang, L-H.; Tan, X-F.; Xu, P.; Zhang, C. and Cheng, M. (2017): Bioremediation mechanisms of combined pollution of PAHs and heavy metals by bacteria and fungi: A

mini review. Biores. Technol., 224: 25-33. https://doi.org/10.1016/j.biortech.2016.11.095.

Lo, W.; Chua, H.; Lam, K.H. and Bi, S.P. (1999): A comparative investigation on the biosorption of lead by filamentous fungi biomass. Chemosphere, 39: 2723-2736.

Manguilimotan, L.C. and Bitacura, J.G. (2018): Biosorption of cadmium by filamentous fungi isolated from coastal water and sediments. J. Toxicol., 2018:1- 6.

Merroun, M.L.; Raff, J.; Rossberg, A.; Hennig, C.; Reich, T.and Selenska-Pobell. S. (2005): Complexation of Uranium by cells and S-layer Sheets of *Bacillus sphaericus* JG-A12. Appl. Environ. Microbiol., 71: 5532-5543.

Mishra,V.; Balomajumder, C. and Agarwal, V. K. (2010): Zn(II) ion biosorption onto surface of eucalyptus leaf biomass: isotherm, kinetic, and mechanistic modeling," Clean—Soil, Air, Water, 38(11): 1062–1073.

Oladipo, O.G.; Awotoye, O.O.; Olayinka, A.; Bezuidenhout, C.C. and Maboeta, M.S. (2018): Heavy metal tolerance traits of filamentous fungi isolated from gold and gemstone mining sites. Brazilian J. Microbiol., 49(1):29-37.

Pal, T.K.; Bhattacharyya, S. and Basumajumdar, A. (2010): Cellular distribution of bioaccumulated toxic heavy metals in *Aspergillus niger* and *Rhizopus arrhizus*. Int. J. Pharma and Bio. Sci., 1(2): 1-6.

Patel, E.; Lynch, C.; Ruff, V. and Reynolds, M. (2012): Co-exposure to nickel and cobalt chloride enhances cytotoxicity and oxidative stress in human lung epithelial cells. Toxicol. Appl. Pharmacol., 258(3):367-75. DOI: 10.1016/j.taap.2011.11.019

Perez, R.M.S.; Abalos, A.R.; Gomez, J.M. and Cantero, D.M. (2009): Biosorption of chromium, copper, manganese and zinc by *Pseudomonas aeruginosa* AT19 isolated from a site contaminated with petroleum. Bioresour. Technol., 100: 1533-15538.

Pidcock, E. and Moore, G.R. (2001): Structural characteristics of protein binding sites for calcium and lanthanide ions. J. Biol. Inorg. Chem., 6: 479-489.

Rivas-Castillo, A. M.; Guatemala-Cisneros, M. E. and Rojas-Avelizapa, N. G. (2017): Effect of aluminum in *Bacillus megaterium* nickel resistance and removal capability. Mexican J. Biotechnol., 2(2): 206–220.

Rose, K.P. and Devi, R. (2018): Heavy metal tolerance and adaptability assessment of indigenous filamentous fungi isolated from industrial wastewater and sludge samples. Beni-suef University J. Basic and Appl. Sci., 2018.**7**: 688-694. https://doi.org/10.1016/j.bjbas.2018.08.001.

Sanders, E. R. (2012): Aseptic laboratory techniques: plating methods. J Vis Exp. (63): 3064.doi: 10.3791/3064

Schipper, M.A.A. and Stalpers, J.A. (1984): A revision of the genus *Rhizopus*. Studies in Mycol., 25: 1–34.

Sim, C.S.F.; Tan, W.S. and Ting, A.S.Y. (2016): Endophytes from Phragmites for metal removal: evaluating their metal tolerance, adaptive tolerance behaviour and biosorption efficacy, Desalin. Water Treat. 57: 6959–6966.

Simonescu, C.M. and Ferdes, M. (2012): Fungal biomass for Cu (II) uptake from aqueous systems, Pol. J. Environ. Stud. 21: 1831–1839.

Smith, D. and Onions, H.S.(1983): A comparison of some preservation techniques for fungi. *Transactions of the British Mycological Society.*, **81** (3): 535-540.

Srivastava, S. and Thakur, I.S. (2006): Biosorption potency of *Aspergillus niger* for removal of chromium (VI). Curr. Microbiol., 53: 232–237.

Subramanian, C. V. (1971): Hyphomycetes, an account of Indian species, except cercosporae. ICAR, New Delhi, 930 Pp.

Ting, A.S.Y.; Lim, S.J. and Tan, W.S. (2011): Diversity and metal tolerance of filamentous fungi from analytical wastewater in laboratory, in: Proceedings of the International Congress of the Malaysian Society for Microbiology, Penang, Malaysia, December 8–11: 110–113.

Tsekova, K. and Galabova, D. (2003): Phosphatase production and activity in copper (II) accumulating *Rhizopus delemar*. Enzyme and Microbial Technol., 33(7):926-931

Tsekova, K.; Christova, D. and Ianis, M. (2006): Heavy metal biosorption sites in *Penicillium cyclopium*. J. Appl. Sci. Environ. Manag., 10: 117-121.

Valix, M. and Loon, L.O., 2003. Adaptive tolerance behaviour of fungi in heavy metals. Miner. Eng., 16: 193–198.

Valix, M.; Tang, J.Y. and Malik, R. (2001): Heavy metal tolerance of fungi. Miner. Eng., 14: 499–505

Volesky, B. (1990): Biosorption of Heavy Metals. CRC Press. Boca Raton, FL, 396 p.

Yang, J.; Wang, Q.; Luo, Q.; Wang, Q. and Wud, T. (2009): Biosorption behavior of heavy metals in bioleaching process of MSWI fly ash by *Aspergillus niger*. Biochemical and Engineering J., 46: 294–299.

Zafar, S.; Aqil, F. and Ahmad, I. (2007): Metal tolerance and biosorption potential of filamentous fungi isolated from metal contaminated agricultural soil. Bioresour. Technol., 98: 2557–2561

Zaidi, J. and Pal, A. (2017): Review on heavy metal pollution in major lakes of India: remediation through plants. Afr. J. Environ. Sci. Technol., 11(6): 255–265.