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Tolerance potential of different Species of *Aspergillus* as bioremediation tool - Comparative analysis

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Due to increase of industrialization in public and private sectors along with urbanization, are reflected in varying degree of pollution in the different compartments (water, soil and air) of environment. The present study has been carried out to understand the tolerance potential of different species of *Aspergillus* (*Aspergillus niger, Aspergillus flavus, Aspegillus fumigatus*) isolated from agriculture soil of Kasur irrigated with water contaminated with sewage and industrial effluent. The degree of tolerance of fungi was measured by minimum inhibitory concentration in the presence of different concentration of metals (Cr and Pb) and compared to control sample. Results are shown the variation in the tolerance level of different isolates of *Aspergillus.* Few isolates are tolerant, moderately tolerant and some are sensitive.

Keywords: Heavy metals; Tolerance potential; *Aspergillus*; MIC; Chromium; Lead

INTRODUCTION

Contamination of sediments and natural aquatic receptors with different pollutant is a major environmental problem all over the world (Baldrian and Gabriel, 2002; Gavrilesca, 2004; Malik, 2004). Industrialization and urbanization especially in developing countries have led to the accumulation of heavy metals and petroleum hydrocarbons in the environment (Adveniyi, 1996; Bamgbose and Sibanjo, 1998; Byomi *et al.,* 1999; Manay *et* al., 1999; Ngodigha *et* al., 1999; Yamasoet *et* al., 2000; Adedniyi and Folabi, 2002). Metals that are released into the environment tend to persist indefinitely, accumulating in to living tissues and posing a serious threat to the environment and public health. These harmful substances accumulate in crops via food chain; find their way into our bodies, where they can cause a variety of illness.

They are cytogenic, mutagenic, and carcinogenic in nature and are posing threats to the urban population, which rely on vegetables and foliage crops grown in pre-urban lands. Introduction of heavy metal in environment generally induces morphological and physiological changes in microbial communities (Vadkertiova and Slavikova, 2006), hence exerting a selective pressure on the microbiota (Verma *et al.,* 2001). These contaminated sites are source of metal resistance microorganisms (Gadd, 1993).

Among microorganisms fungi are very important microorganism; it can tolerate heavy metals to a limit and can also help to remove heavy metals from contaminated soil. Fungi and yeast biomasses are known to tolerate heavy metals (Khan, 2001, Baldrian, 2003; Gavrilesca, 2004). They are a versatile group, as they can adapt and grow under various extreme conditions of pH, temperature and nutrient availability, as well as high metal concentrations (Anand *et al.,* 2006).They offer the advantage of having cell wall material which shows excellent metal-binding properties (Gupta *et al.,* 2000). They are used for bioremediation because of their mycelia nature and ability to accumulate metals of all families.

Many fungal species such as *Rhizopus arrhizus*, *Penicilium spinulum* and *Aspergillus niger* have been extensively studied for heavy metals biosorption and the process mechanism seems to be dependent upon species (Zhou and Kiff, 1991; Hafez *et al*., 1997 ; Kapoor and Viraraghavan, 1997). The accumulation of heavy metals in agricultural soils is of increasing concern due to food safety issues and potential health risks as well as its

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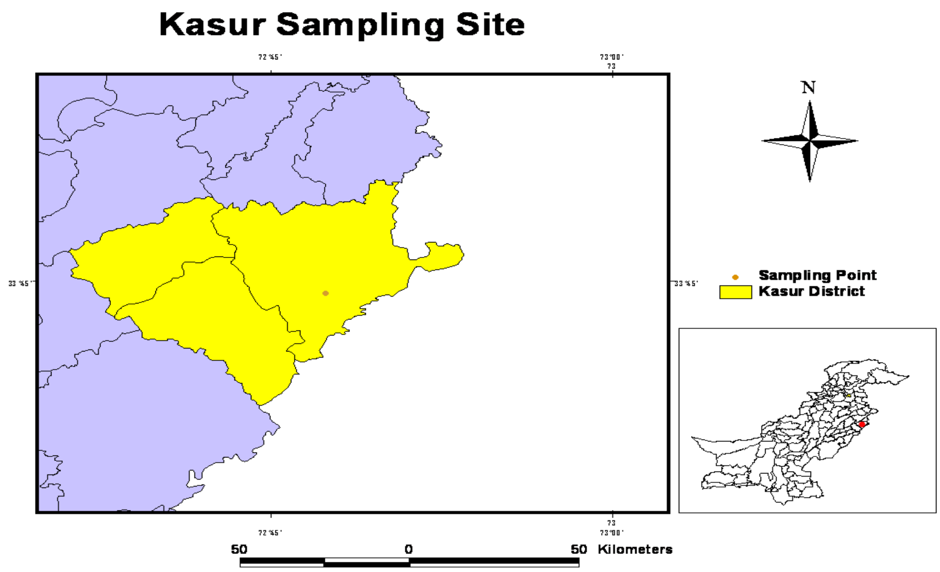

Figure 1: Map showing sampling sit

determinate effects on soil ecosystem. Farmers in big cities where water from natural surface rain is not available use sewerage water and water of natural drains for crop production, due their being less expensive.

In most parts of Pakistan, untreated city effluent is utilized for growing vegetables around large urban settlements such as Kasur. Farmers use it as a source of irrigation water and plant nutrients. However, its continuous use may have serious environmental implications, since it also contains heavy metals. Use of untreated city effluent for irrigation without risk assessment and management could be a serious hazard, impacting soil and crop quality and ultimately human health.

In Pakistan, heavy metals contaminated land is becoming an environmental and economic issue. Combination of inadequately plan effluent disposal techniques and a rapidly growing population has lead to gradual accumulation of heavy metals in the soils and water of Pakistan. According to EPA, the industrial effluents being dumped into the land, by electroplating, tanner industries and textile mills and contains toxic metals such as Cr, Ni, As, Hg, Cu, and Pb, which have contaminated the soil and the biota residing in and around soils in Pakistan (EPA, 1990). Over the years there have been increasing concerns over the rising level of heavy metal contamination, as they tend to persist in the environment, accumulating in the biota, thereby entering the food chain causing deleterious harm to higher animals and humans (Zhang *et al*., 1998).

The aim of present study was to check the tolerance potential of different species of *Aspergillus* was investigated against heavy metals. For the present study soil samples were collected from peri-urban agricultural areas of Kasur which is irrigated by the contaminated industrial water and containing heavy metals and toxic chemicals. Fungi were isolated from the contaminated soil and studied for their tolerance analysis.

Materials and Methods

Sampling and Sampling site: The main purpose of the present study was to evaluate the tolerance potential of different isolated fungal strain towards heavy metals (Pb, Cr). For this purpose, soil samples were collected from peri-urban agricultural areas of Kasur (Figure 1). The agriculture land of Kasur is contaminated by sewage and industrial effluents from the nearby industries and contains heavy metals and toxic chemicals. From the contaminated soil fungi were isolated and preserved for further detail investigation of heavy metal tolerance.

Sterilization of Apparatus: Petri plates, media bottles, distilled water, McCartney bottles and syringes were sterilized in autoclave. For sterilization purpose all apparatus were autoclaved for 40 minutes at 121℃. After autoclaving all sterilized material dried in oven at 95℃.

Media Preparation and Fungal Isolation: Potato Dextrose Agar (PDA) media was used for the isolation of fungi. For the preparation of PDA, potatoes (200g) were peeled, sliced and boiled, and then sieved through a clean muslin cloth to get a broth in which agar (7.5g) and dextrose sugar (7.5g) was added. The media was then autoclaved for 30 minutes at 121°C (Martin, 1995). Fungi were isolated on Potato Dextrose Agar (PDA) by soil dilution method (Razak *et al*., 1999).

Table 1: List of fungal isolates

Isolated fungus	Sample code
Aspergillus fumigatus	K3, K4, K12, K18, K26, K27, K28, K29, K30
Aspergillus niger	K 14, K 16, K 22, K 23, K 24
Aspergillus flavus	K 5, K 7, K 17, K 33, K 34, K 36, K 35, K 37, K 41

Preparation of plates

Fungi were isolated on Potato Dextrose Agar (PDA) by soil dilution method (Razak *et al*., 1999).Poured the media in Petri-dishes and allowed to solidify for 24 hour. To suppress the bacterial growth, 30 mg/lit of streptomycin was added in the medium (Martin, 1995). Once the agar was solidified, and then put plates in an inverted position for 24 hours at room temperature (Martin, 1995).

Identification of fungi:

The fungal cultures were identified on the basis of macroscopic (colonial morphology, color, texture, shape, diameter and appearance of colony) and microscopic (septation in mycelium, presence of specific reproductive structures, shape and structure of conidia, and presence of sterile mycelium) characteristics (Table 1) (Zafar *et al.,* 2006). Pure cultures of fungi isolates were identified with the help of literature (Domsch *et al.,* 1980; Barnett and Hunter 1999).

Heavy metal tolerance test experiment

For the evaluation of tolerance potential among isolated fungal strains, Potato Dextrose Agar (PDA) medium was prepared and amended with various concentrations(0, 200, 400, 600, 800 and 1000 mg/l) of heavy metal{$Cr(NO_3)_3.9H_2O$ and $Pb(NO_3)_2$} and 5.6 pH was maintained by adding 5 molar solution of NaOH. Media was autoclaved for 20 minutes at 121°C and poured in Petri plates. The plates were incubated at 29ºC for 7 days. The growth of fungi was monitored from the point of inoculation or centre of the colony. Tolerance is measured by observing minimum inhibitory concentration (MIC) and tolerance index.

Heavy metals analysis of soil

Each soil sample (1g) was taken in the conical flask (50ml), added 10ml of HNO_3:$HClO_4$ (1:2) solution (50ml) and heated for half an hour. Solutions were filtered through Whatman No. 1 filter paper and volume was made to 50ml by adding distilled water. Soil samples were digested in triplicates and analyzed for Zn, Cd, Cr, Cu, Ni and Pb. The blank was prepared for quality assurance of samples. The blank sample contained 10ml of HNO_3:$HClO_4$ (1:2) solution and heated for half an hour and volume was made 50ml by adding distilled water. For the determination of heavy metals the atomic absorption spectrophotometer was powered on and warmed up for 30 minutes. After the heating of cathode lamp, the air acetylene flame was ignited and instrument was calibrated or standardized with different working standards (Vanloon and Lichwa, 1973)

Statistical analysis

The experiments were set up with three replicates. Analysis of variance was performed by using statistical software (SPSS 17) to compare resistance to metal among individual isolates.

RESULTS AND DISCUSSION

Heavy metals released to the environment have been increasing continuously as a result of the industrial activities and technological development and poses significant threat. Application of domestic and industrial effluents to nearby agricultural fields is a common practice of irrigation and wastewater disposal in Pakistan (Hashem, 1995; Hafez *et al.,* 1997). When wastewater is applied to agricultural fields, heavy metals enter the soil and get fixed into the soil components. Thus continuous application of wastewater tends to accumulate large quantities of heavy metals in soil, which persists there for an indefinite period to have long lasting effects in the soil.

With increasing environmental awareness and legal constraints being imposed on discharge of effluents, different type of techniques presently in existence for removal of heavy metals from contaminated waters including reverse osmosis, electrodialysis, ion-exchange, chemical precipitation, phytoremediation, etc. However, all these methods have disadvantages like incomplete metal removal, high reagent and energy requirements, generation of toxic sludge or other waste products that require careful disposal (Ahalya *et al.,* 2003). Recently research interest has diverted towards use of

Table 2: Concentration of Heavy Metals in Kasur Soil (mg/kg)

	Pb	Cd	Cr	Cu	Ni	Zn
Min	42.1	2.0	54.1	31.2	32.6	81.4
Max	70.2	3.4	210.2	60.8	56.6	156.7
Mean	50.6	2.6	99.6	44.6	46.4	125.6
Median	47.2	2.6	93.2	43.9	50.0	131.4
S.D	±10.0	±0.5	±49.4	±8.3	±8.2	±28.4
Kurtosis	0.852364	-0.95102	4.182443	2.326493	-0.60081	-1.26948
Skewness	1.285644	0.437593	1.824419	0.578925	-0.75136	-0.57778

Table 3: Tolerance index of fungal isolates against lead.

Isolated fungus	Pb (TI)	Isolated fungus	Pb (TI)
Aspergillus fumigatus(K3)	0.29	*Aspergillus fumigatus*(K26)	0.28
Aspergillus fumigatus (K4)	0.30	*Aspergillus fumigatus*(K27)	0.17
Aspergillus flavus (K 5)	0.47	*Aspergillus fumigatus* (K28)	0.23
Aspergillus flavus (K 7)	1.09	*Aspergillus fumigatus* (K29)	0.28
*Aspergillus niger(*K 14)	0.71	*Aspergillus fumigatus* (K30)	0.25
Aspergillus niger (K 16)	0.48	*Aspergillus flavus* (K 33)	0.89
Aspergillus flavus (K 17)	1.18	*Aspergillus flavus* (K 34)	1.26
Aspergillus niger (K 22)	0.62	*Aspergillus flavus* (K 36)	0.12
Aspergillus niger (K 23)	0.54	*Aspergillus flavus* (K 35)	0.14
*Aspergillus niger(*K 24)	0.63	*Aspergillus flavus* (K 37)	0.39
		Aspergillus flavus (K 41)	0.55

biomaterials like microbial biomass has emerged as an alternative technique.

Fungi are natural inhabitants of soil and have greater potential for remediation by virtue of their aggressive growth; greater biomass, production and extensive hyphal reach in soil. They are a versatile group, as they can adapt and grow under various extreme conditions of pH, temperature and nutrient availability, as well as high metal concentrations (Anand et al., 2006). They offer the advantage of having cell wall material which shows excellent metal-binding properties (Gupta *et al*., 2000). Describing the ability to grow at high metal concentrations, (Malik, 2004) distinguished fungi tolerant to heavy metals. Generally, the contaminated sites are the sources of metal resistant micro-organism (Gadd, 1993).

The results of present study depicted that all tested isolates shows different tolerance behavior for different metals. Some isolates were sensitive, moderately tolerant and tolerant (see table 2). Most of the isolates were sensitive and very few were tolerant. Among all tested isolates of *Aspergillus, A.flavus* was more tolerant specie while *A.niger* was moderately tolerant. As Baldrian and Gabriel, 2002 reported that various genera and also isolates of the same genus did not necessarily have the same heavy metal tolerance.

It is apparent that these fungal strains exhibit considerable tolerance towards Cr and Pb and can become dominant microorganisms in some polluted habitats. *Aspergillus .flavus* and *Aspergillus niger* showed tolerance against Pb but it showed sensitiveness for Chromium metal. On the other hand, *A.fumigtus* shows exactly contrasting behavior.

This response (i.e. resistance to Cr and Pb) of *Aspergillus flavus* can be exploited for the remediation of heavy metal contaminated soil (McGrath, 2002).

The variation in the metal tolerance may be due to the presence of one or more strategies of tolerance or resistance mechanisms exhibited by fungi (Ezzouhri *et al.,* 2009). The level of resistance depended on the isolate

(A)

(B)

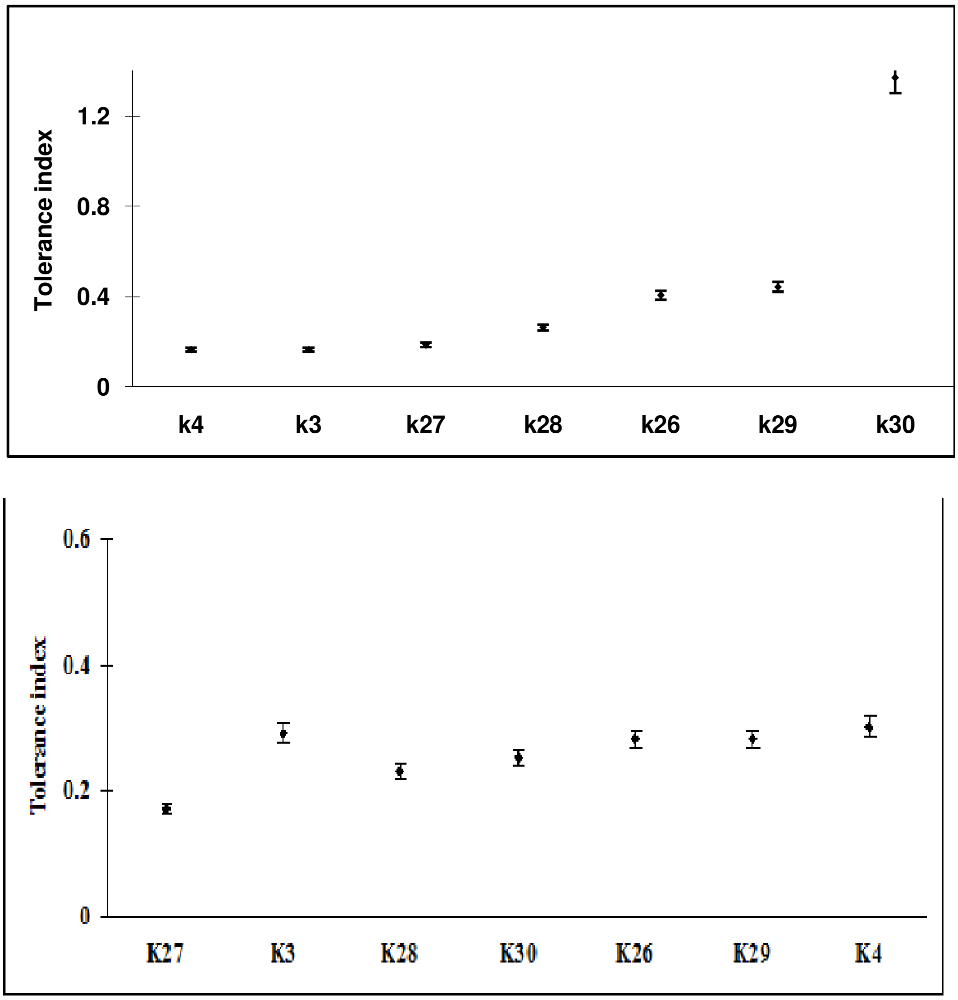

Figure 2: Tolerance index of isolates of *A.fumigatus* against (a) chromium and (b) lead

tested as well as the site of its isolation. The statistical analysis showed the diverseness in heavy metal tolerance of different isolates (see Figures 2, 3 and 4).

Fungi growth phase, which was observed from the tolerance index of the fungi with time upon exposure to heavy metals, was found to be a predictable pattern for each strain and metal concentrations. The determination of Minimum inhibitory concentration (MIC) of metals suggests that the resistance level against individual metals dependent on different fungal isolates. MIC was defined as the minimum inhibitory concentration of the heavy metal that inhibited visible growth of test fungi. MIC of Cr and Pb is determined. It ranged from 800mg/l to 1000mg/l for chromium and lead for all tested isolates (figure 3). The growth phase was characterized by a lag, retarded, similar and enhanced rates compared to control which appear to reflect the tolerance development or adaptation of fungi in the presence of heavy metals (Hashem *et al*., 1995).

The results revealed that growth rate of all tested isolates at lower concentration was higher but with but with passage of time and exposure of higher concentration density and growth of species were reduced leading to no growth. It was of interest to establish the effect of type of heavy metal, the metal concentration and the strain on this adaptive behavior. The same increment and reduction in growth was observed during study on filamentous fungi, which belonged to the genera *Aspergillus*, were more resistant to Cr at higher metal concentrations and suddenly the growth pattern changes (Ngodigha, 1999)

El-Morsy (2004) studied 32 fungal species isolated from polluted water in Egypt for their resistance to metals and found that *Cunninghamela echinulata* biomass could be employed as a biosorbent of metal ions in wastewater. However, although some authors found that micro-organisms isolated from contaminated sites were more tolerant than those from natural environments (Ngodigha *et al.,* 1999; Verma *et al*., 2001).

Table 4: Tolerance index of fungal isolates against chromium.

Isolated fungus	Cr (TI)	Isolated fungus	Cr (TI)
Aspergillus fumigatus(K3)	*0.16*	*Aspergillus fumigatus*(K26)	0.40
Aspergillus fumigatus (K4)	*0.16*	*Aspergillus fumigatus*(K27)	0.19
Aspergillus flavus (K 5)	*0*	*Aspergillus fumigatus* (K28)	0.26
Aspergillus flavus (K 7)	*0.14*	*Aspergillus fumigatus* (K29)	0.44
*Aspergillus niger(*K 14)	0.47	*Aspergillus fumigatus* (K30)	1.37
Aspergillus niger (K 16)	0.09	*Aspergillus flavus* (K 33)	0.39
Aspergillus flavus (K 17)	1.09	*Aspergillus flavus* (K 34)	0.26
Aspergillus niger (K 22)	*0.17*	*Aspergillus flavus* (K 36)	0.20
Aspergillus niger (K 23)	*0.39*	*Aspergillus flavus* (K 35)	0.12
*Aspergillus niger(*K 24)	*0.47*	*Aspergillus flavus* (K 37)	0.33
		Aspergillus flavus (K 41)	0.12

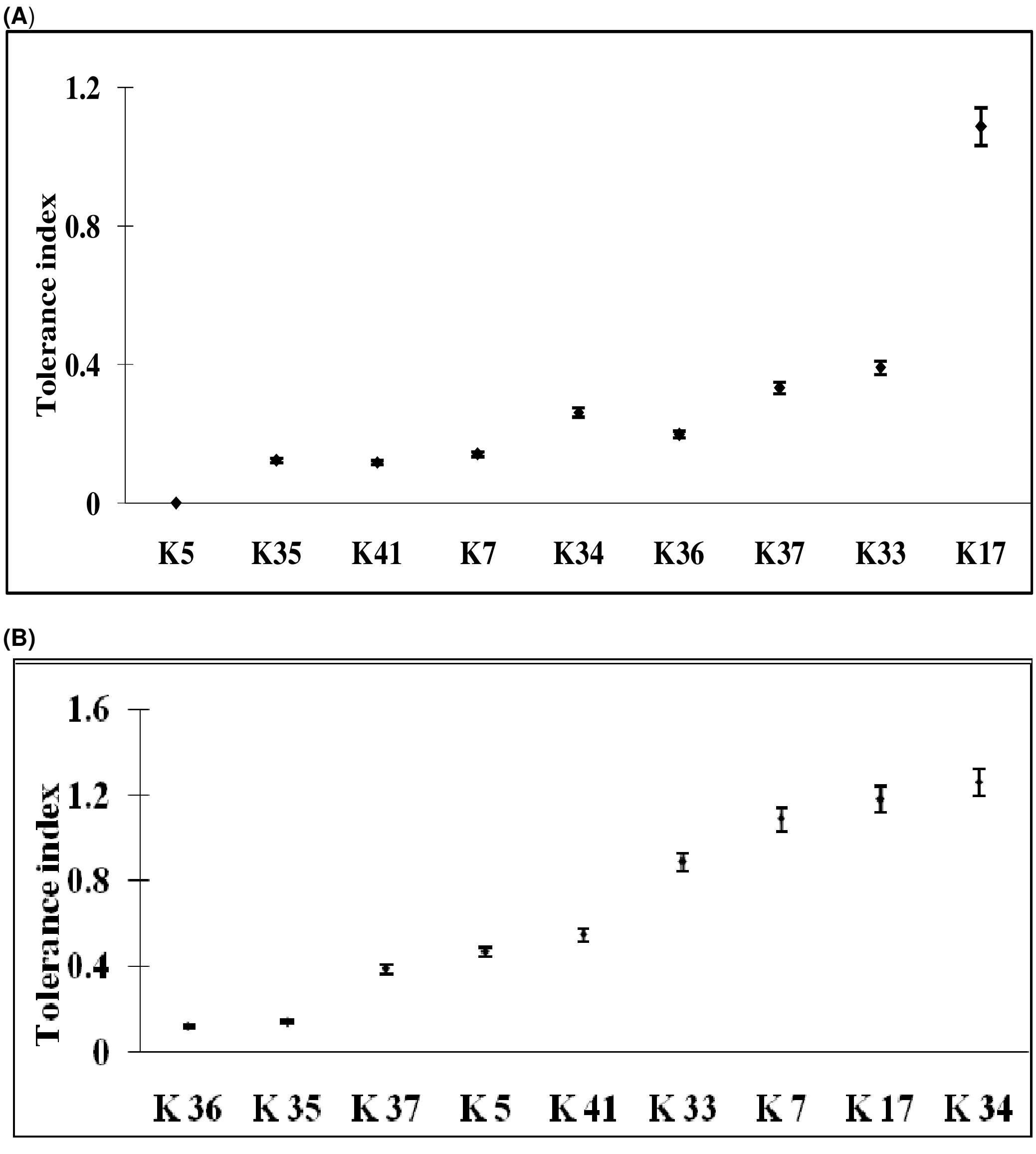

Figure 3: Tolerance index of isolates of *A.flavus* against (a) chromium and (b) lead

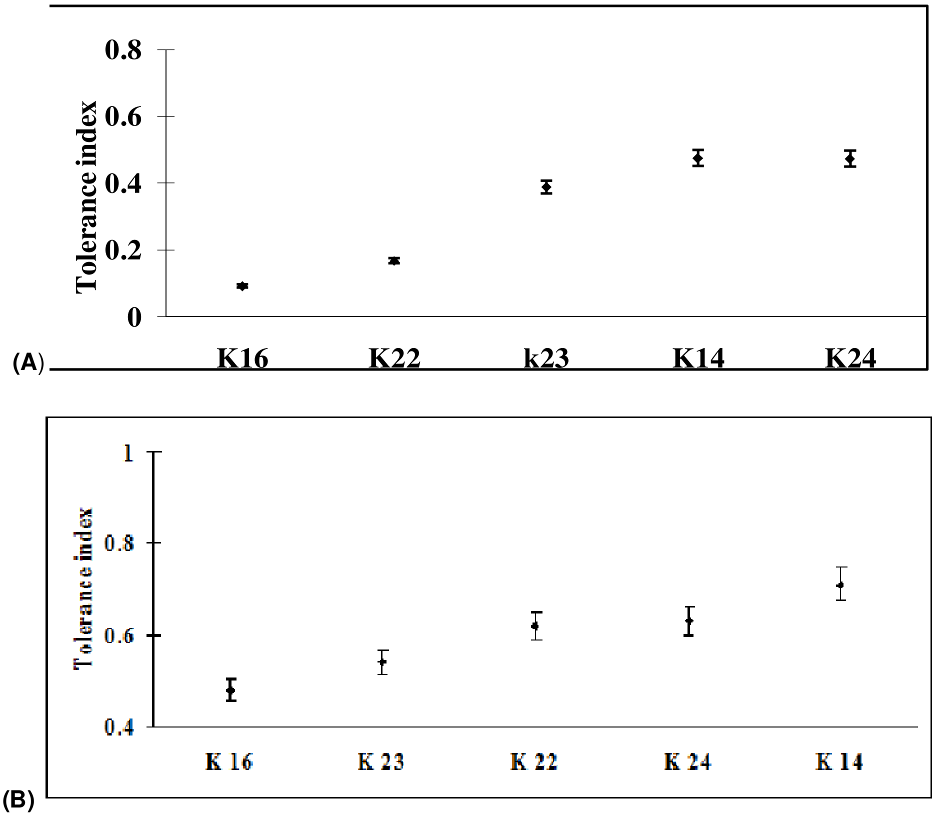

Figure 4: Tolerance index of isolates of *A.niger* against (a) chromium and (b) lead

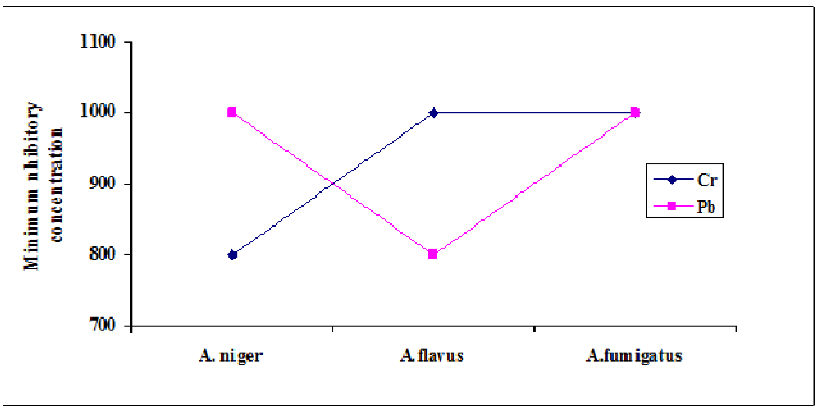

Figure 5: Minimum inhibitory concentration of different species of *Aspergillus* against metals

Our preliminary findings indicate that fungi from soil contaminated with heavy metals have tolerance potential due to their physiological adaptation and they have greater potential for remediation of metals. *A.flavus and A.niger* showed higher tolerance potential than other tested fungal strain which makes them more attractive potential candidates for further investigations regarding their ability to remove metals from contaminated sites.

Conclusion

The present study concludes that soil irrigated with waste water contains tolerant fungus. The findings are *A.flavus and A.niger* showed higher tolerance potential than other tested fungal strain which makes them more attractive potential candidates. It is affirmed that the response of the isolates to heavy metals depended on the metal tested; its concentration in the medium and on the isolate considered and can be used further for the purposes of bioremediation.

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