Screening of Soil for Lead-Tolerant Fungi

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Soil samples were screened for fungi tolerant to lead (lead nitrate) using poisoned food technique. Control media as well as media supplemented with 40 ppm, 200 ppm and 400 ppm concentrations of lead (in the form of lead nitrate) were used to isolate fungi by serial dilution plate method. In total, 19 species of fungi were isolated. Out of these, six species, *i.e. Aspergillus niger* var. *niger* Tiegh, an unidentified sterile species, *Sepedonium chrysospermum* (Bull) Fr., *Trichoderma lignorum* var. *lignorum* (Tode) Harz, *Penicillium implicatum* var. *implicatum* Biourge and *Aspergillus candidus* var. *candidus* Link could tolerate the highest concentration (*i.e.,* 400 ppm) of lead. In addition to the above six fungal strains, *Aspergillus ustus* var. *ustus* (Bainier) Thom and Church could tolerate and grew well on the media with moderate concentrations (200 ppm) of lead nitrate. *Cladosporium herbarum* var. *herbarum* (Pers.) Link, *Curvularia* sp. Boedijn, *Scopulariopsis brevicaulis* var. *brevicaulis* (Sacc.) Bainier and *Scopulariopsis communis* var. *communis* Svilv. are moderately resistant as these could grow and flourish at 40 ppm concentration only. *Fusarium* sp. (isolate-III) Link cannot tolerate even low doses of lead nitrate. Out of these 19 species, 5 fungal species, *i.e., Aspergillus candidus, Aspergillus niger, Penicillium implicatum, Sepedonium chrysospermum* and *Trichoderma lignorum* were selected for studying the effect of different concentrations of lead nitrate on the *in vitro* growth of individual species. Thus, these species can be further tried for their utilization as biosorbents for remediation of lead in the effluents.

Key Words: Lead tolerance, *Aspergillus*, Serial dilution plate method

Introduction

Significant amount of heavy metals including lead are introduced into the aquatic systems as a result of sludge disposal, ore-refining, metal-smelting, welding, refining, agricultural sources and manufacturing of pesticides (Singh, 2001). Even if these metals are present in undetectable quantities, their recalcitrance and consequent persistence in water bodies might ultimately, through biomagnification, increase their concentrations to such an extent that these begin exhibiting toxic characteristics. Heavy metals, once released into the soil matrix, also find their way into the food web through ground water aquifers (Walton, 1995). Hence, it is necessary to remove these metals before these enter the complex ecosystem. Physio-chemical treatments developed to deal with very diluted metal-containing effluents (precipitation, flocculation, coagulation and ion-exchange etc.) are very expensive (Lacina, 2003). The ability of fungi to serve as biotraps for heavy metals and biosorb them has attracted the attention of a number of workers for purification of such water (Bellion *et al*., 2006; Duruibe *et al*., 2007). The purification of the metal-containing water using fungal biomass is not only cheaper but also presents several advantages such as: (i) fast removal, (ii) production of residual small volume, (iii) easy installation of the process, (iv) possibility of valorization of fungal waste biomasses from industrial fermentations. Since soil constitutes reservoir of immense variety of the fungi, it would be pertinent to exploit the possibilities of getting suitable fungal strains from soil which are capable of removing metals from the wastewater effectively. The present communication deals with an attempt to discern the soil fungal species capable of surviving lead nitrate *in vitro*.

Materials and Methods

Serial dilution plate method (Waksman, 1927) was followed to isolate fungi from the soil samples. 20 g of the sample collected from C.C.S. University Campus, Meerut were placed in 200 ml of sterile water and stirred for fifteen minutes using a magnetic stirrer to get a stock solution (1:10 dilution). 10 ml of this solution are immediately transferred to a conical flask containing 90 ml of sterile distilled water to get a suspension of 1:100 dilution. This suspension was used for the preparation of further serial dilutions (1:1000 and 1:10,000). The suspension of 1:10 dilution was discarded. From the suspension of each of the remaining three dilutions (1:100, 1:1000, 1:10,000), 1 ml aliquots were transferred to each of a set of three Petri dishes followed by the addition of approximately 20 ml of sterilised and cooled (45°C) Czapek Dox Agar medium (Raper and Thom, 1949) with 30 ppm of rose bengal and 30 mg of streptomycin. In addition to the normal medium, media amended with different concentrations (40 ppm, 200 ppm and 400 ppm) of Pb (as lead nitrate) were also prepared. Thus, 36 Petri dishes were used, *i.e.*, three dilutions × four types of media (control amended with 40 ppm, 200 ppm and 400 ppm) × three replicates. These

Petri dishes containing the media and the inocula were incubated at 25 ± 1°C for 6 to 8 days. The total number of colonies of individual fungal species growing in each Petri dish were recorded. The fungal strains obtained were identified using standard keys (Gilman, 1957; Nagamani *et al.*, 2006).

Mycelial discs (8 mm diameter) cut from the margins of actively growing cultures of the given fungal species were centrally inoculated onto separate Petri plates containing media of normal, 40 ppm, 200 ppm and 400 ppm concentrations of lead nitrate with the help of corkborer aseptically on agar plate. Thus, for each fungal species and for a given metal, 12 Petri dishes were used, *i.e.*, 4 (normal medium + 3 media with varying heavy metal concentrations) × 3 replicates. The inoculated agar plates were incubated at 25 ± 1°C for 4 to 5 days (2-3 days in case of some fast-growing fungi). The diameters of fungal colonies were measured to observe the differences between the growth of test fungal species at different heavy metal levels.

Results and Discussion

A total of 19 species of fungi could be isolated from the soil using Czapek's Dox Agar normal medium and that amended with different concentration of lead nitrate (Table 1).

Nine species of fungi colonized agar plates containing normal medium. *Aspergillus niger,* an unidentified sterile species and *Fusarium* sp. (isolate-III) dominated the plates accounting for 42%, 27% and 15.3%, respectively of the isolates. These were followed by *A. fumigatus* var. *fumigatus* Fresen*, A. candidus, Fusarium* sp. (isolate-IV) Link and *Penicillium citrinum* Link each of which accounted for 3.2% of the isolates. *P. implicatum* and *Sepedonium chrysospermum* were megerly represented by only one isolate each.

Colonies belonging to twelve fungal species developed on the agar plates containing low concentrations (40 ppm) of lead nitrate. Six species, *i.e., A. sulphureus, Scopulariopsis communis, S. brevicaulis, Curvlaria* sp., *Cladosporium herbarum* and *Trichoderma lignorum* which failed to appear on the normal plates, did appear on these agar plates with low concentrations of lead nitrate. On the other hand, *Fusarium* sp. (isolate-III) failed to appear on plates with the low concentration as well as higher concentration for lead nitrate. The plates were dominated by unidentified sterile species, *A. niger* and *Sepedonium chrysospermum* which accounted for 30%, 23% and 16.7% of the isolates, respectively.

A. fumigatus, Fusarium sp. (isolate-IV), *Scopulariopsis communis, Curvularia* sp., *Scoplariopsis brevicaulis, Cladosporium herbarum, Trichoderma lignorum,*

A. candidus and *A. sulphureus* var. *sulphureus* (Fresen.) Thom and Church were represented by 1 to 3 isolates only.

Ten fungal species were isolated on agar plates with 200 ppm lead nitrate. *A. niger,* an unidentified sterile species and *Penicillium implicatum* dominated the plates comprising 39%, 30% and 10% plates of the isolates, respectively. These were followed by *A. ustus, Sepedonium chrysospermum* and *Trichoderma lignorum* which accounted for 4.2%, 6% and 6% respectively of the isolates. *A. candidus, A. flavus* var. *flavus* Link*, Alternaria alternate* (Fr.) Keissl. and *Torula* sp. Persoon appeared on the plates, though these were represented by one isolate each only.

Seven fungal species could survive on the agar plates containing 400 ppm lead nitrate. *A. niger* and the unidentified sterile species heavily dominated the plates comprising 33.3% and 27.5%, respectively of the isolates.

A. candidus, *P. implicatum*, *S. chrysospermum* and *Trichoderma lignorum* lagged far behind with only 7%, 13%, 9% and 9% of the isolates, respectively. *P. citrinum* was megerly represented by one isolate only. The number of species was greater in the plates containing 40 ppm and 200 ppm lead nitrate as compared to the control plates. However, there was a decrease in the number as the concentration of the metal increased. The number of isolates did not vary much with the change in metal concentration though it was much lesser in amended plates as compared to the control plates. It can be concluded that:

a) *A. niger*, an unidentified sterile species, *S. chrysospermum, T. lignorum, P. implicatum* and *A. candidus* are resistant to very high concentration (400 ppm) of lead nitrate.

b) *A. ustus* could tolerate and grow with moderate concentrations (200 ppm) of lead nitrate.

c) *Cladosporium herbarum, Curvularia* sp., *Scopulariopsis brevicaulis* and *S. communis* are moderately resistant as these could grow and flourish at 40 ppm concentration only.

d) *Fusarium* sp. (isolate-III) cannot tolerate even low doses of lead nitrate.

Table 1. Frequency and number of isolates of fungal species colonising agar plates containing unamended medium (Normal) as well as that amended with different concentrations of lead nitrate

S. No.	Fungal species	Normal medium			Medium amended with lead nitrate									Total isolates
					40 ppm			200 ppm			400 ppm			
		%F	FC	Isolates	%F	FC	Isolates	%F	FC	Isolates	%F	FC	Isolates	
1.	*Alternaria alternata*							11.1	I	1				1
2.	*Aspergillus candidus*	22.2	II	3	11.1	I	1	11.1	I	1	11.1	I	5	10
3.	*Aspergillus flavus*							11.1	I	1				1
4.	*Aspergillus fumigatus*	33.3	II	3	33.3	II	3							6
5.	*Aspergillus niger*	77.7	IV	38	66.6	IV	15	66.6	IV	27	66.6	IV	23	103
6.	*Aspergillus sulphureus*				11.1	I	1							1
7.	*Aspergillus ustus*							11.1	I	3				3
8.	*Cladosporium herbarum*				22.2	II	2							2
9.	*Curvalaria* sp.				11.1	I	3							3
10.	*Fusarium* sp. (isolate-III)	11.1	I	14										14
11.	*Fusarium* sp. (isolate-IV)	22.2	II	3	33.3	II	3							6
12.	*Penicillium citrinum*	11.1	I	3							22.2	II	1	4
13.	*Peniillium implicatum*	11.1	I	1				33.3	II	7	11.1	I	6	14
14.	*Scopulariopsis brevicaulis*				11.1	I	2							2
15.	*Scopulariopsis communis*				11.1	I	3							3
16.	*Sepedonium chrysospermum*	11.1	I	1	33.3	II	11	22.2	II	4	44.4	III	9	25
17.	*Torula* sp.							11.1	I	1				1
18.	*Trichoderma lignorum*				11.1	I	2	22.2	II	4	22.2	II	6	12
19.	Sterile species	77.7	IV	25	66.6	IV	20	77.7	IV	21	66.6	IV	19	85
		9		91	12		66	10		70	7		69	296

%F = Percentage frequency; FC = Frequency class

Five fungal species, *i.e., A. candidus, A. niger, P. implicatum, S. chrysospermum* and *T. lignorum* were selected for the study. The results are presented in the Table 2.

When grown individually in the medium containing different concentrations of lead nitrate, *S. chrysospermum* did not seem to be much affected either positively or negatively; while 40 ppm concentration had a positive effect on the growth of all the five fungal species *i.e.*, *A. candidus*, *A. niger*, *P. implicatum*, *S. chrysosperum* and *T. lignorum* tested at least in the early period of two days. Slightly longer exposure upto four days had either

Table 2. Radial growth (in mm) of the selected fungal species in control medium as well as media containing different concentrations of lead nitrate after 2 days and 4 days of incubation

S. No.	Fungal species	Days of incubation	Control media	Media amended with lead nitrate		
				40 ppm	200 ppm	400 ppm
1.	*Aspergillus candidus*	2	14.33	19.00 (+32.58)	17.00 (+18.63)	16.33 (+13.95)
		4	18.00	22.00 (+22.22)	17.00 (–5.55)	13.66 (–24.11)
2.	*Aspergillus niger*	2	16.33	17.00 (+4.10)	18.00 (+10.23)	17.33 (+6.12)
		4	28.00	24.33 (–13.10)	24.33 (–13.10)	32.00 (+14.28)
3.	*Penicillium implicatum*	2	10.00	12.66 (+26.60)	11.33 (+13.30)	11.66 (+16.60)
		4	16.33	18.33 (+12.24)	18.33 (+12.24)	17.33 (+6.12)
4.	*Sepedonium chrysospermum*	2	87.33	87.66 (+0.37)	87 (–0.37)	87.33 (=0)
		4	87.66	87.66 (=0)	87.66 (=0)	87.66 (=0)
5.	*Trichoderma lignorum*	2	62.33	64.00 (+2.67)	68.00 (+9.09)	72.66 (+16.57)
		4	81.66	82.00 (+0.41)	82.66 (+25.89)	86.33 (+31.48)

slightly inhibitory effect or the positive effect was diminished.

The growth of *T. lignorum* exhibited an increase with the increase in metal concentration, thus, corroborating the results obtained on isolation plates where also the frequency and isolates of *T. lignorum* were found to increase with an increase in the metal concentration. Low concentrations of metal stimulated the *in vitro* growth of *A. candidus* but as the concentration increased, either the growth was inhibited or the stimulatory effect was decreased. More or less similar trend was observed with *P. implicatum*. Of course, these results do not tally with those obtained on isolation plates.

Rai *et al*. (1995) observed 37.74% inhibition of *A. niger* and 21.1% inhibition of *T. harzianum* after seven days of incubation in the medium containing 200 ppm of lead. However, in the present investigation both the species exhibited more growth than that in the control in the first two days. Later, by the fourth day, the growth of *A. niger* was inhibited by 13.10% while that of *T. lignorum* was further stimulated (+25.89%).

The lead is the metal which is absorbed in very high amounts by the fungal biomass. This raises the possibility of reduced uptake as one of the factor responsible for the apparent metal tolerance of *A. niger*, *S. shrysospemum*, *T. lignorum* and other species. In the present investigation, rich nutrient medium amended with heavy metals has been used. Babich and Stotzky (1982) believed that this may provide overestimation of tolerant populations as a result of complexing and detoxification of the heavy metals by inorganic and organic constituents of the medium. Further studies therefore, must be carried out before branding these strains as lead-resistant.

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