

Isolation and characterization of Arsenic tolerant fungal strains from contaminated sites around urban environment of Kolkata

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Abstract: Today arsenic is causing an alarming global problem due to its toxicity and day by day this problem will amplify if necessary actions are not taken. In this paper we have isolated two arsenic resistant fungal strains (*Aspergillus flavus* and *A. niger*) from some polluted sites of Kolkata. These two strains are capable of removing 50%-76 % of arsenic from different arsenic enriched medium, simultaneously also tolerant to different other heavy metals (Cd, Pb, Hg, Zn and Cr). In near future these tolerant fungal species could be used in remediation of Arsenic and other metal pollution to some extent.

Key words: Arsenic, *Aspergillus flavus* & *A.niger*, Bioremediation

I. Introduction

Development in terms of globalization and industrial expansion, heavy metal/metalloid pollution is attaining an alarming problem. Arsenic(As) pollution (a semi metallic element; metalloid) is one of the most concerned topic in recent years, as Arsenic is known to be a dangerous toxin that can lead to death when exposed to a large amounts. Arsenic, an ubiquitous metalloid found in groundwater due to its association with rocks, sediments and soils as well as industrial discharges and pesticide use which enters into the food chain through drinking water, crops and vegetables and causes different serious health problems.

Conventional physical and chemical methods for treating this harmful arsenic are inefficient, costly and not eco-friendly. So a global thrust was felt in searching of an alternative. Hence bioremediation is attaining an important procedure for abatement of metal pollution due to its low cost and high efficacy. Earlier Samal et al.[1] reported that among different algal strain blue green algal species *Oscillatoria- Lyngbya* mixed culture showed maximum efficiency in removing 64% Arsenic (V) and 60% Arsenic(III) after 21 days incubation from 0.1 mg/l arsenic (III) enriched medium. Similarly bacterial cultures also efficient in arsenic removal as reported by a group of researchers, they investigated As(V) reduction characteristics of two different bacteria named *Pseudomonas stutzeri* and *Bacillus cereus* and found 500ppb As(V) was completely reduced to As(III) by *Bacillus cereus* and *Pseudomonas stutzeri* in 114h and 120h respectively[2]. Among different microorganisms used in bioremediation fungi are considered as most effective species for metal removal from metal contaminated sites due to their survivability in higher concentration of metals. Evidence for internal absorption and the mechanism used by *A. niger* to detoxify environmental copper and zinc has been observed by earlier researchers [3]. Potential of filamentous fungi in bioremediation of heavy metals containing industrial effluents and waste waters has been increasingly reported from different parts of the world [4,5,6,7].

As far Kolkata is significantly less arsenic contaminated area, so we isolated fungal strains from some pollution stressed sites around peri urban areas of Kolkata. Our attempt was to screen out some Arsenic tolerant fungal strains which could be used in remediation of arsenic pollution to some extent in near future.

II. Materials and Methods:

2.1 Isolation of fungal strains:

Fungal strains were isolated from soils of five different sites of Kolkata; namely

- (i) Waste dumping site of Dhapa areas of Kolkata;
- (ii) Tannery waste dump sites along Eastern metropolitan bypass areas of Kolkata;
- (iii) Contaminated site within the area of Central Bus terminus, Esplanade area, Kolkata ;
- (iv) Waste dump sites within the premises of Calcutta medical college hospital, Kolkata.

The fungal strains were isolated through standard dilution plating methods [8] in Czapek-Dox(CD) agar media. Identically Czapecdox medium was prepared (glucose -2gm, NaNO₃ – 2.5gm, KCl -0.5gm, MgSO₄.7H₂O - 0.5gm, FeSO₄ – 0.5gm, ZnSO₄, 7H₂O – 0.5gm, KH₂PO₄ – 1gm and agar powder 15gms per liter). (APHA, 1992)

The species were purified by streaking repeatedly on the same medium and were identified on basis of macroscopic (colony character, morphology, colour, texture, shape, diameter and appearance of colony) and microscopic (septation in mycelium, presence of specific reproductive structures, shape and structure of conidia and conidiospore etc) characteristic using fungal identification manuals [9,10].

2.2 Growth optimization

Isolated strains were grown in sterilized CD broth medium for establishment of the optimum temperature, pH that supports the exuberant growth of those species. The pH of the medium was adjusted using dilute HCL or NaOH. The temperature ranges were varied from 20°C- 40°C, and pH was from 3 to 9.

2.3 Antibiotic sensitivity Assessment

Antibiotic (antifungal) sensitivity of each metal tolerant fungal strains were measured using cup assay method [11]. The antibiotics used for this assay were Nystatin, Griseofulvin and Kanamycin.

2.4 Arsenic and another heavy metal tolerance assessment:

To explore the tolerance of the isolates to the As, each freshly prepared growth medium were amended with selected metal concentrations varies from 50mgL⁻¹ to 5000mgL⁻¹. After 5 days of incubation, the extent of colony growth (in diameter) were compared with the control one (i.e. growth of fungi in medium without metal). All the experiments were carried out in triplicate.

For other heavy metals (Cd, Pb, Hg, Cr and Zn) resistance of the fungal isolates were determined by conventional cup assay method[11]. Selected metal tolerant fungal strains were grown separately in CD medium supplemented with diverse concentration (sub-lethal to lethal) of each heavy metal separately. Then cups were poured with various other metallic solutions (Cd, Pb, Hg, Cr and Zn). For each test triplicates were made and incubated for 5days. Zone of inhibition of fungal growth around the cup was examined and measured in each case separately

2.5 Arsenic Removal potential of isolated strains

To study the arsenic removal capacity the selected fungal strains were grown in Czapek Dox broth (25ml) with Sodium arsenite (NaAsO₃) concentration 20, 100 and 1000 ppm for 3, 6 and 9 days with continuous shaking incubation at 30°C and pH 7. Fungal biomass was harvested by filtration through 0.22µ Millipore filter and the residual arsenic concentration of the broth was measured by FI-HG-AAS (Perkin Elmer Analyst-400) following standard procedure. All results are represented as the mean of triplicates.

III. Results and Discussion:

Almost twenty different fungal strains were isolated from soil samples of four different sites of Kolkata. Two arsenic tolerant strains ASC1 and ASB3 (isolated from Dhapa soil) were more arsenic resistant than other eighteen species, so we have carried out all our experiments with these two strains. The strains were identified as *Aspergillus flavus* (ASC1) and *A.niger* (ASB3) considering their colony color, colony shape, conidiospore color etc.

With increase in pH from 3 to 7 the biomass yield was found to enhance in both cases (ASC1 and ASB3) being maximum at pH 7, further increase in pH caused decrease in biomass. Similarly maximum mycelial weight were obtained at 30°C temperature in both cases (Table 1 & Table-2).Temperature 30°C and pH 7, both these physical condition showed maximum growth in terms of mycelial weight so all further experiments were carried out maintaining these growth conditions.

Table-1 Optimisation of pH for growth of two isolated fungi (in terms of mycelia weight) after 5days of incubation

Strain Name	Mycelial weight(g) (Mean ±SD)			
	pH 3	pH 5	pH 7	pH 9
ASC1 (<i>Aspergillus flavus</i>)	1.3±0.2	1.57±0.3	1.89±0.52	1.26±0.63
ASB3 (<i>A.niger</i>)	1.5±0.78	1.87±0.54	2.1±0.21	1.7±0.45

Table-2 Optimisation of temperature for growth of two isolated fungi(in terms of mycelia weight) after 5days of incubation

Strain Name	Mycelial weight(g) (Mean ±SD)		
	Temp.20°C	Temp.30°C	Temp.40°C
ASC1 (<i>Aspergillus flavus</i>)	1.15±0.25	1.95±0.45	1.01±0.32
ASB3 (<i>A.niger</i>)	1.3±0.20	2.01±0.35	1.69±0.56

Three different antibiotics (Nystatin, Griseofulvin and Kanamycin) were used to evaluate the antibiotic sensitivity of those two strains. ASB3 is found to be more resistant towards Griseofulvin and Kanamycin than ASC1, while against Nystatin both the strains showed similar range of tolerance (Table 3).

Table 3 Antibiotic sensitivity assessment of both isolated strains

Strains	Antibiotics					
	Nystatin		Griseofulvin		Kanamycin	
	Maximum growing concentration (ppm)	Minimum inhibitory concentration (MIC) (ppm)	Maximum growing concentration (ppm)	Minimum inhibitory concentration (MIC) (ppm)	Maximum growing concentration (ppm)	Minimum inhibitory concentration (MIC) (ppm)
AsC1	60	80	60	80	60	80
AsB3	60	80	80	100	80	100

Our results depict that ASC1 is more arsenic tolerant than ASB3, as Minimum Inhibitory Concentration (MIC) of arsenic for ASC1 was estimated to be around 2000 ppm, where as for ASB3 it is 1200ppm. Similarly ASC1 is found to be resistant against Cd, Pb and Cr, while ASB3 is resistant for Hg, and Zn (Table 4)

Table-4 Metal tolerance assessment of isolated strains

Strain Name	Minimum Inhibitory Concentration (MIC) in ppm					
	As	Cd	Pb	Hg	Zn	Cr
ASC1 (<i>Aspergillus flavus</i>)	2000	500	700	55	650	1000
ASB3 (<i>A. niger</i>)	1200	450	625	60	750	900

Similar trend of results were obtained by Gautam et al.[12] where they reported maximum growth of fungi at neutral range of pH. It is reported that temperature is considered as the most important growth factor for fungi [13]. Our results are in tune with the work of Santos & Linardi [14] who have reported earlier that common incubation temperature for the growth of fungi such as *A. niger*, *Fusarium* sp., *Penicillium* sp. and *Graphium* sp. was taken as 30°C. Actually this range of temperatures that encourage growth makes this isolate suitable for use in bioremediation in tropical climates.

Our results depicted that ASC1 is capable of removing more arsenic than ASB3 (Fig 1 & Fig2). In case of both the strains maximum removal efficacy were obtained at 6th day of incubation, with further increase in incubation time (i.e from 6th day to 9th day) a saturation was observed in removal efficacy for both of the cases(i.e. for both strains). Both the strains showed their maximum removal (70%-76%) efficiency when grown in 100ppm concentration of Arsenic. Further increase in metal concentration i.e from 100ppm to 1000ppm a significant decrease in removal percentage was noticed.

Bhar et al.[4] worked with *Aspergillus*, *Rhizopus* and *Penicillium* to show the rate of removal of As(III) and As(V) by above mentioned fungal strains and they found *Penicillium* with maximum removal efficiency and removal increases with increase of time interval which coincides with our results that with increasing incubation time (3rd day to 6th day) arsenic removal efficacies also enhanced upto a saturation time(9thday).

Ridvan et al.[15] also worked with *Penicillium purpurogenum* to show bioadsorbtion capacity of heavy metal Pb, Cd, As and Hg and found metal accumulation Pb(II)>Cd(II)>Hg(II)>As(III)

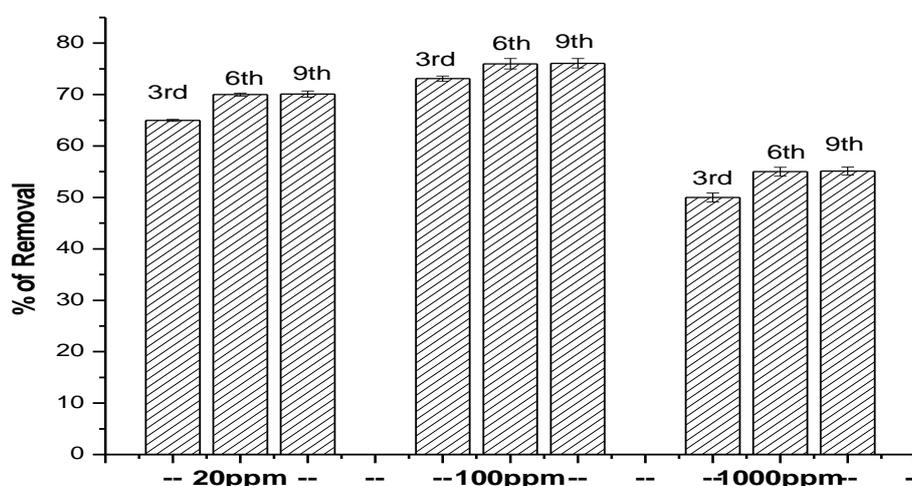


Fig-1 Arsenic removal (%) of ASC1(*A. flavus*) from 20ppm,100ppm and 1000ppm Arsenic enriched media after 3rd day, 6th day and 9th day of incubation period

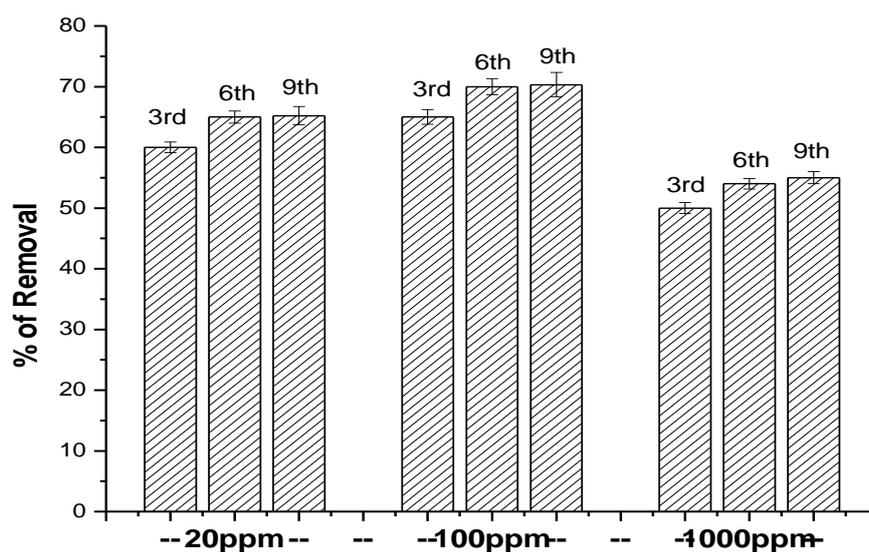


Fig-2 Arsenic removal (%) of ASB3(*A.niger*) from 20ppm, 100ppm and 1000ppm Arsenic enriched media after 3rd day, 6th day and 9th day of incubation period

Our results are in tune with the findings of a group of researchers, who reported arsenic removal potential of *Aspergillus candidus* and *A. flavus* respectively[16,17]. Similarly Srivastava et al.[18] detailed arsenic removal potential of *Trichoderma* and *Aspergillus* sp, isolated from arsenic contaminated sites of West Bengal. Almost ten species they identified which are tolerant upto 5000mg/L of arsenic.

IV. Conclusion:

These two isolated arsenic tolerant fungal strains (ASC1 and ASB3) could remove a significant amount of arsenic from different arsenic enriched media in laboratory condition. In near future these two fungal strains will be effective in arsenic removal planning from arsenic contaminated sites. The study revealed that a cost effective model of bioremediation of arsenic can be developed in a pilot scale.

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