Journal of Crop and Weed 7(2): 249-252 (2011)

Biovolatilization and bioaccumulation of pentavalent arsenic by fungal strains isolated from intensively cultivated soil

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NAIP on Arsenic in food chain: Cause, Effect and Mitigation
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Received:24.03.2011, Revised: 31.08.2011, Accepted : 29.09.2011

Key words: Arsenate, bio-accumulation, bio-volatilization, fungi, removal

Arsenic has been recognized as an environmental toxicant and public health hazardous element in several countries throughout the world. Inorganic arsenic of geological origin is found in groundwater used for drinking purposes in several parts of the world such as Bangladesh and West Bengal, India (Ademola *et al.* 2009). Chronic exposure to high levels of arsenic and its compounds, especially in drinking water can have several carcinogenic, mutagenic and teratogenic implications (WHO. 2001).The accumulation of arsenic in agricultural soils occurs from continuous addition of contaminated irrigation water and consequently in food chain results in deteoriation in the quality of agricultural products and eventually damage of human health. It ultimately spreads among humans and animals in the form of a deadly disease namely arsenocosis. Bioaccumulation is a mechanism of immobilisation of the metal(loid)s by biomass, while biovolatilisation majorly occurs by biomethylation (Paez-Espino *et al.* 2009). Over a century ago, arsenic biovolatilisation by a fungal strain *Scopulariopsis brevicaulis* was first obtained (Gosio *et al.* 1892). The fungus S. brevicaulis could produce trimethylarsine (TMA) from arsenite (Chalenger *et al.* 1935). Recently, more microorganisms capable of conducting these processes have also been found. Carrasco *et al.* 2005 suggested that Sinorhizobium meliloti, which is resistant to arsenic, was able to bioaccumulate up to 3 times the amount of arsenic levels as non-resistant strains. Phaeolus schweinitzii, Scopulariopsis brevicaulis, Neosartorya fischeri have all been shown to be capable of arsenic bioaccumulation and biovolatilization (Cernansky *et al* 2007). However, very little information is available about fungi that are highly capable of arsenic bioaccumulation and biovolatilization. The intracellular uptake of metal ions from a substrate into living cells, otherwise known as bioaccumulation may lead to the biological removal of metals by fungi (Paez-Espino *et al.* 2009). Such accumulation of heavy metals by fungal biomass may be particularly relevant because of its potential low cost application in bioremediation and recovery of metals. Although bioremediation of heavy metal using fungi is widely examined (Aksu *et al.* 1999, Gadd *et al.* 2000, Lo *et al.* 1999, Ramsay *et al.* 1999) much less is known about the bioaccumulation by viable indigenous fungi from agricultural soil. The main objective of this work was to measure the bioaccumulation and biovolatilisation of As by two isolated As-resistant fungi under laboratory conditions towards arsenate (As^V) and its arsenic accumulation potential, and consequently to explore its potential for use as a bioremediation agent at arsenic-contaminated sites.

Isolation and identification of arsenic-resistant fungal strains and screening for higher arsenic tolerance

Soil sample was collected from arsenic contaminated intensively cultivated agricultural field (N 23°00′27.4″ and E 088°36′03.7″) in the district of Nadia, West Bengal, India during the *Boro* season of 2009. 1 g of soil sample was mixed with 10 ml of sterile distilled water and shake for half hour and left for sedimentation. Then 0.1 ml of the supernatant solution was spread on PDA plates containing 50 mg l^{-1} of arsenate. Sodium arsenate used as the source of pentavalent arsenic. All plates were incubated at 27^0 C for 48-72 hour. Fungi growing on these plates were further enriched on potato dextrose agar (PDA) plates for isolation of single colonies.The isolated fungi from the previous experiment were, therefore, screened for their abilities to tolerate highest level of arsenic. All arsenate tolerant isolates were separately inoculated onto PDA plates with 100 mg l^{-1} arsenate. All plates were incubated at 27°C for 3 days. In order to find out the arsenic tolerance limit of the isolated 10 fungal strains the cultures were grown in 50 ml potato dextrose broth, containing different concentration of arsenate (25-1000 mg l^{-1}) in 250 ml conical flask and were incubated in 27°C ^{0}C for 3 days. Selected fungi having higher resistance ability were subjected for morphological identification. Slides of hyphae, conidiophores, and conidia were prepared by wet mounting with lacto-fuchsin and examined by viewing at 1000X magnification using a compound microscope (model no: CH20iBIMS, Olympus India Pvt Limited). Size and color of fungal colonies on PDA were also recorded.

Bioaccumulation and biovolatilisation of arsenic by arsenic-resistant fungus

Fungal bioaccumulation and biovolatilisation of arsenate were conducted using modified methods

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(Shiming *et al.* 2010). Erlenmeyer flasks containing 50 ml of PDA medium were inoculated with 0.1 ml spore suspension prepared from pure culture of AsF-02 and AsF-08. These two strains showed higher arsenic resistance in PDA medium. After incubation for 5 days on a shaker at 27°C with 150 rpm, the compact fungal biomass and culture medium were separated by centrifugation. To further verify the capability of different fungal strains to biovolatilise arsenic under laboratory conditions, an experiment based on the modified method of Edvantoro *et al.* 2004 was performed. Filter papers were soaked in mercury solution for 3 hour and then dried in the dark. Erlenmeyer flasks containing 50 ml liquid PDA media with or without 50 mg l^{-1} of arsenate were inoculated with 0.1 ml spore suspension of AsF-02 and AsF-08, respectively and incubate for 3 days. The arsenic concentration of fungal biomass, filtered media and arsenic in filter papers were then analyzed using the following method.

The fungal biomass weighted to 0.1 g and the trapped filter papers were digested with tri-acid mixture (HNO_3: H_2SO_4: $HClO_4$: 10:1:4, v/v) until a clear solution was obtained. These digested were adequate filtered by using Whatman No. 42 filter paper. 10 ml of the filtrate was taken and in 50 ml volumetric flask, 5 ml of concentrated HCl and 1 ml of mixed reagent [5% KI (w/v) + 5% Ascorbic acid (w/v)] were added to it, kept for 45 minutes to ensure complete reaction and the volume was made up to 50 ml. Same procedure followed in case of filtered media without digest it. The total arsenic content in the solution was determined by using atomic absorption spectrophotometer (AAS), Perkin Elmer AAnalyst 200 coupled with Flow Injection Analysis System (FIAS 400) where the carrier solution was 10% v/v HCl, following Olsen method (McLare*n et al.*, 1998). A set of standard solutions of 2.5, 5, 10, and 20 mg l^{-1} arsenic were used for calibration.

Isolation and identification of arsenic-resistant fungal strains and screening for high arsenic tolerance

Ten pure fungal isolates were obtained through enrichment culture in PDA medium and the arsenic tolerance capacity of those isolates were estimated with different concentration of arsenate in PDA medium after 3 days of incubation (Table 1). It was observed that all the isolates could grow and withstand the arsenic toxicity upto 500 mg l^{-1} of arsenate. Two isolates could grow in media containing 700 mg l^{-1} of arsenate (AsF-02 and AsF-08). They were selected for further study. In general, microbial ability to grow at high metal concentration is found coupled with a variety of specific mechanism of resistance and environmental factors. Mechanisms of resistance by microorganism include microbial surface sorption, enzymatic transformation, precipitation by oxidation/reduction reaction, and biosynthesis of metal binding proteins or extracellular polymers, whereas environmental factors may include the surrounding pH and redox potential, metal speciation, soil particulates, and soluble organic matters. Those isolated microorganisms might have the similar arsenic resistance mechanisms with different degrees.

Table 1: Arsenate tolerance capacity of the fungal isolates after 3 days of incubation

Strain	Concentration of arsenate in mg l^{-1}											
	25	50	100	200	300	400	500	600	700	800	900	1000
AsF-01	+	+	+	+	+	+	+	-	-	-	-	-
AsF-02	+	+	+	+	+	+	+	+	+	-	-	-
AsF-03	+	+	+	+	+	+	+	-	-	-	-	-
AsF-04	+	+	+	+	+	+	+	-	-	-	-	-
AsF-05	+	+	+	+	+	+	+	-	-	-	-	-
AsF-06	+	+	+	+	+	+	+	-	-	-	-	-
AsF--07	+	+	+	+	+	+	+	-	-	-	-	-
AsF-08	+	+	+	+	+	+	+	+	+	-	-	-
AsF-09	+	+	+	+	+	+	+	-	-	-	-	-
AsF-10	+	+	+	+	+	+	+	-	-	-	-	-

Note: '+' indicates growth appeared, '-' indicates growth not appeared

AsF-02 had septate hyphae and grew fast at 27° C, attaining a diameter of 5-6 cm on PDA within 3 days of incubation. The colony consisted of a velvety layer of conidiophores that were initially white but changed progressively to light green and then dark green or grey-green as incubation continued. The Conidiophores were single (monoverticillate) and smooth walled. Conidia were arranged in long chains. In case of AsF-08 colonies were yellow consisting of a dense felt of conidiophores hyphae were septate and show dichotomous. Based on microscopic morphology, AsF-02 was identified as a species of *Penicillium and* AsF-08 as *Aspergillus.*

Bioaccumulation and biovolatilisation of arsenic by arsenic-resistant fungus

In the present experiment arsenic biovolatilization was studied in terms of biomass accumulation (g dry weight) and arsenic content (mg g^{-1}). The data showed (Table 2) that after cultivation for 5 days *Penicillium and Aspergillus* exhibited the arsenic accumulating efficiency of 14.3 µg g^{-1} and 11.2µg g^{-1} respectively. Arsenic bioaccumulation was higher in case of AsF-02 than in AsF-08. No As was observed in fungi used as controls which was cultivated in the absence of As. Arsenic resistant fungal species *Penicillium* was capable of volatilizing 25.8-43.9 µg arsenic in the culture system which contains 10 mg l^{-1} of arsenate (Shiming *et al.* 2010). In the present investigation *Aspergillus niger* showed the highest arsenic bioaccumulation ability (18.0 µg). In the current evaluation *Penicillium and Aspergillus* presented higher abilities of arsenic bioaccumulation and biovolatilization, but *Penicillium* sp was selected as elite one.

Table 2: Bioaccumulation and biovolatalization of arsenic by two arsenic resistant fungal isolates after a cultivation period of 5 days at 27° C. The initial arsenic content in the potato dextrose broth before cultivation was 50 mg l^{-1} of arsenate

Fungal strain	Dry weight of biomass (mg)	Efficiency of As captured in mycelium (µg g^{-1})	Initial arsenic content in the medium (mg l^{-1})	Amount remaining (mg l^{-1})	Amount remove (mg l^{-1})	% Removal
Penicillium sp	262.3	14.3	46.6	28.75	17.85	38.30
Aspergillus sp	308.2	11.2	47.9	33.2	14.7	30.68

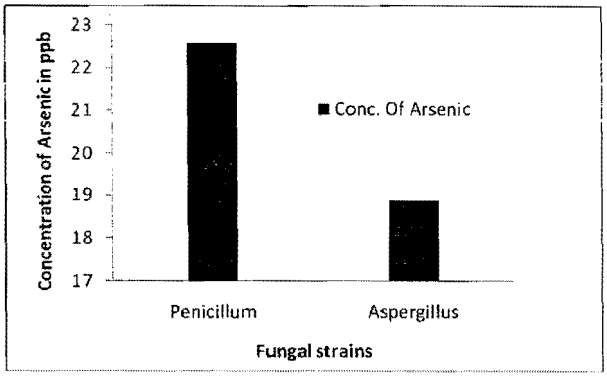

Fig.1. Concentration of volatile arsenic trapped in filter paper by two fungal isolates from a medium initially supplemented with 50 mg l^{-1} arsenate

The abilities of arsenic bioaccumulation and biovolatilization of various microbial strains were variable, and parameters such as the cultivation period, initial arsenic content, and other environmental factors may affect the efficiencies of accumulation and volatilization to some extent. Fungal strains, isolated from contaminated soils were assessed for their ability to produce arsine. After 10 days of cultivation, the amount of arsenic captured on the filter paper in case of *Penicillium* sp. and *Aspergillus* sp. were 22.6 ppb and 18.9 ppb respectively (Fig.1). This result indicated that

Penicillium and *Aspergillus* were capable of volatilizing arsenic from an environment heavily amended with it. The possible mechanism for arsenic trapping by mercury solution may be the absorption of arsine by mercury and production of a mercuric arsenide compounds. It is also used to test for the presence of arsenic, as recommended by the Pharmacopoeia. Recently, filter papers soaked in an HgNO3 solution have been developed to capture volatilised arsenide. The method adopted in this experiment would be ineffective in capturing all of the volatilized arsenic, because the arsenic-trapping process is largely affected by the diversity of the volatilised arsenide, the gas flow, undefined environmental conditions, among other factors. However, it was still feasible to certify the ability of arsenic biovolatilisation by the fungal strains. Further analyses of volatilized arsenide produced by *Penicillium* and *Aspergillus* using gas chromatography-mass spectrometry (GC-MS) or other techniques are required in the future. *Penicilliums* are known for their ability to methylate arsenic (Granchinho *et al* .2002). It was also reported that certain fungi belonging to the genera of *Penicillium* and *Aspergillus* could grow well on arsenical substrates without transforming arsenic compounds

(Kama et al. 1996). On the basis of these observations, microbial methylation with subsequent arsenic volatilisation may be developed as a remediation strategy of arsenic contaminated soils when a rapid removal is not a compulsory requirement.

In the present study, the As-resistant fungi *Penicillium* and *Aspergillus* are found to have the capability to accumulate and volatilize As in considerable amount from the environment suggesting their potential use in As removal from As contaminated soil environments.

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