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Bull Environ Contam Toxicol (2014) 92:369–373
DOI 10.1007/s00128-014-1212-y

Arsenate Resistant *Penicillium coffeae*: A Potential Fungus for Soil Bioremediation

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Received: 26 July 2013 / Accepted: 17 January 2014 / Published online: 29 January 2014
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Abstract Bioremediation is an effective method for the treatment of major metal contaminated sites. Fungi were isolated from soil samples collected from different arsenate contaminated areas across India. An isolate, *Penicillium coffeae*, exhibited resistance to arsenate up to 500 mM. Results indicated that pretreatment of biomass with alkali (NaOH) enhanced the percentage of adsorption to 66.8 % as compared to that of live and untreated dead biomass whose adsorption was 22.9 % and 60.2 % respectively. The physiological parameters evaluated in this study may help pilot studies aimed at bioremediation of arsenate contaminated effluents using arsenate resistant fungus *P. coffeae*.

Keywords Bioremediation · Heavy metal · Arsenate · Biosorption · Dead biomass

Increased industrialization and human activities introduce novel toxic chemicals into the environment which disrupt the natural biogeochemical cycles (Alluri et al. 2007) in the environment. Heavy metals are of major public health concern (Wei et al. 2010) as they are released in the discharge of effluents by a number of industrial processes such as electroplating, tanning, pulp processing and steel manufacturing. The heavy metals include arsenate, lead, mercury, silicon, chromium and copper.

Arsenate occurs naturally in a wide range of minerals. Widespread use of arsenate in copper smelting industries, metallurgical activities, pigments and insecticides are the major sources of arsenate in soil and natural waters (Maheswari and Murugesan 2011). Those who are affected with arsenate show symptoms such as eczema of skin/mucous membrane, hyperkeratosis of palms and soles, warts, encephalopathy, neuropathy, cardiovascular disorders, respiratory problems, leukemia, cancers (Al-Sabbak et al. 2012). Removal of arsenate from the effluents is very important considering environmental, economical and health problems they can pose. Several chemical and physical methods have been devised and employed for the removal of heavy metals such as ion exchange, precipitation, evaporation, reverse osmosis, sorption. Recent development in biotechnology has established many potential methods of remediation such as biosorption using microorganisms like fungi, algae, and bacteria (Bishnoi and Garima 2005). A biosorption process has many advantages over conventional processes of heavy metal removal such as low cost, high efficiency, less energy consumption, minimization of toxic sludge, no additional nutrient requirement, regeneration of biosorbent and possibility of metal recovery (Ahalya et al. 2007). Due to their ubiquitous presence, fungi have elicited interest in their use to remove heavy metals in the natural environment (Bishnoi and Garima 2005). Both biologically active and inactive fungal biomass have the capacity to adsorb metal ions (Yan and Viraraghavan 2000). Fungi can thus solubilize, transform and uptake the metal species (Sulaymon et al. 2013). This study reports the ability of a fungal strain, *Penicillium coffeae*, isolated from arsenate contaminated environments and its potential use in biosorption of arsenate. In addition, critical parameters influencing biosorption of arsenate by *P. coffeae* such as, temperature, pH, biomass, pretreatment with alkali have also been elucidated.

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Materials and Methods

One hundred soil samples were collected from different arsenate contaminated areas across India. These soil samples were screened on potato dextrose agar (PDA) medium amended with 2 mM arsenate for the initial isolation of arsenate resistant fungi.

Fungi were isolated from different soil samples by serial dilution and standard spread plate technique. The plates were incubated at 28°C for 6 days. Fungal growth on the plates as determined by visual observation of the colony size was assessed to have at least a low level tolerance to arsenate. Identification of fungi was done by a combination of visualizing colony morphology and microscopic examination. Fungal isolates were maintained on PDA slants as pure cultures (Maheswari and Murugesan 2011). Identification of the fungal isolate chosen for further study was confirmed by sequencing genes coding for ribosomal RNA as described below.

The fungal culture was grown in PDB for 7 days. The mycelium was harvested and lyophilized. The lyophilized mycelium was powdered and added with 500 µL TE buffer [100 mM tris (pH 8.0), 10 mM EDTA, 2 % SDS], 50–100 µg of Proteinase K and incubated for 30–60 min at 55–60°C. The salt concentration was adjusted to 1.4 M with 5 M NaCl (140 µL) and 1/10th volume of 10 % CTAB, incubated for 30 min at 0°C. The reaction mixture was centrifuged for 10 min at 10,000 rpm. The supernatant was precipitated with ammonium acetate followed by 0.55 volume of isopropanol, centrifuged for 10 min at 10,000 rpm (Moller et al. 1992). The pellet was washed twice with cold 70 % ethanol, air dried and resuspended in 50 µL of RNase free water. Agarose gel electrophoresis was performed to confirm the presence of DNA. The isolated genomic DNA was subjected to Polymerase Chain Reaction (PCR) (Lewis et al. 2010) using ITS1 as forward primer and ITS4 as reverse primer (Kendall and Paul 2005). The purified PCR product was sequenced by the Dye Deoxy Terminator cycle sequencing kit (Qiagen Inc). The obtained sequence was compiled and compared with the available sequence data from Gen Bank in National Center for Biotechnology Information (NCBI), United States of America (http://www.ncbi.nlm.nih.gov/genbank/). Phylogenetic analysis was conducted using the program MEGA 4 and consensus tree was constructed (Tamura et al. 2007).

The arsenate (2 mM) tolerant isolates selected after the preliminary isolation were further screened for high arsenate tolerance by inoculating onto the PDA plates incorporated with increasing concentrations of arsenate (10–700 mM, sodium arsenate dibasic heptahydrate, A6756, Sigma). Plates were incubated at 28°C for 6 days (Visoottiviseth and Ponviroj 2001). Those isolates showing growth on these plates were considered to be tolerant to a high concentration of arsenate and were used for further study (Kumar et al. 2011) and one such isolate *P. coffeae* was chosen for further evaluation in liquid medium.

The effect of pH of the medium on the growth of *P. coffeae* as well as adsorption of arsenate was tested using PDA medium adjusted to pH 5.0–9.0. Fungal discs (6 mm) were placed on the PDA adjusted to different pH levels. The plates were incubated at 28°C for 6 days and growth of the fungi was assessed by measuring the diameter of the colony (Say et al. 2003).

The isolates which showed tolerance to a high concentration of arsenate were further tested for their arsenate tolerance in liquid medium. Spore suspension of the selected isolate was prepared and transferred into 3 Erlenmeyer flasks containing 100 mL of potato dextrose broth incorporated with arsenate of 300, 400 and 500 mM respectively. Uninoculated medium containing 300, 400 and 500 mM of arsenate served as controls. The flasks were incubated at 28°C on a rotary shaker at 120 rpm for 6 days. After the incubation period, contents of the flasks were filtered through a membrane filter (0.45 µm, Millipore). The filtrate was analyzed for the arsenate concentration by Hydride Generation Atomic Absorption Spectrophotometry (HG-AAS, AA-7000, Shimadzu) (Visoottiviseth and Ponviroj 2001). Experiments were conducted in duplicates and results were expressed as residual arsenate present in the filtrate as compared to controls.

Further the effect of dead and pretreated fungal biomass on arsenate adsorption was tested. The fungal biomass was prepared by growing the organism in fresh potato dextrose broth, incubated at 28°C for 6 days. After the incubation period, the fungal mat was harvested by filtration using muslin cloth, washed thoroughly with deionized (Millipore water) water and further.

1. Dried overnight at 60°C and ground using a mortar and pestle (dead biomass).
2. Boiled for 15 min in 500 mL of 0.5 N sodium hydroxide solution, washed with deionized water and dried (pretreated biomass) (Yan and Viraraghavan 2000).

De-ionized water incorporated with different concentrations of arsenate (300, 400, 500 mM; Sathishkumar et al. 2008) was used for the experiment. The obtained biomass (after the pretreatment) was added to the respective flasks at a biomass rate of 2 g/L. The flasks were kept at 28°C for 24 h in a rotary shaker at 120 rpm. After 24 h, the solution was filtered through a millipore filter (0.45 µm). Each filtrate was analyzed for the concentration of arsenate by HG-AAS (Visoottiviseth and Ponviroj 2001).

Statistical analysis was performed on all the experiments done in duplicate and experimental data was subjected to

the analysis of variance, Post Hoc and Scheffe test ($p = 0.05$) using SPSS software (version 11.5).

Results and Discussion

During the first round of screening with 2 mM of arsenate in the solid medium, most of the isolates (80 % of the isolates) showed resistance, whereas in the second level of screening (10–100 mM) twenty-eight fungal isolates showed tolerance up to 100 mM of arsenate (Fig. 1). During the third round of screening three isolates were found to be tolerant up to 200 mM of arsenate (Table 1) and during the final round of screening only one isolate was resistant to 700 mM of arsenate. The isolate was identified as *P. coffeae* by nucleotide sequencing (Fig. 2). The nucleotide sequence was submitted to the NCBI Genbank database under the accession number KF924258 and selected for further study. The efficiency of *P. coffeae* in resisting arsenate was compared with that of other fungi reported previously. Kumar et al. (2011) identified five isolates from arsenate contaminated agricultural soil which can resist arsenate as high as 103 mg/L which is higher than that of our findings . However Say et al. (2003) isolated a fungus, *Penicillium purpurogenum,* which can resist arsenate as high as 35.6 mg/g of arsenate which is much lower than that of our findings.

Microbial biosorption has potential in treatment of metal containing effluents from industrial wastes and the present

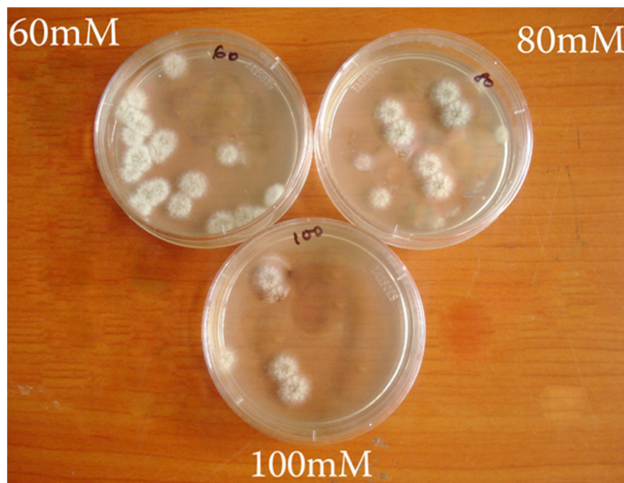

Fig. 1 Isolation of arsenate tolerant fungi from soil samples

Table 1 Fungi resistant to different concentrations of arsenate (200–500 mM)

Organisms	Concentration of arsenate in mM			
	200	300	400	500
Aspergillus niger. 3	+	–	–	–
Aspergillus sp. 2	–	–	–	–
Aspergillus. 3	–	–	–	–
Pencillium sp. 3	–	–	–	–
Pencillium sp. 4	+	–	–	–
Fusarium spp. 4	–	–	–	–
Fusarium sp. 6	–	–	–	–
Penicillium sp. 5	–	–	–	–
Aspergillus niger	–	–	–	–
Penicillium sp. 6	+	+	+	+
Penicillium sp. 7	–	–	–	–
Aspergillus Niger	–	–	–	–
Helminthosporium sp.	–	–	–	–
Aspergillus sp. 4	–	–	–	–
Cladosporium sp. 2	–	–	–	–

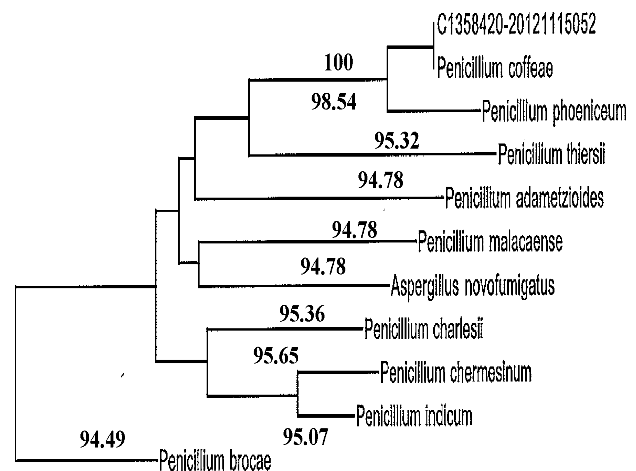

Fig. 2 Consensus phylogenetic tree generated for the nucleotide sequence of the *Penicillium coffeae*

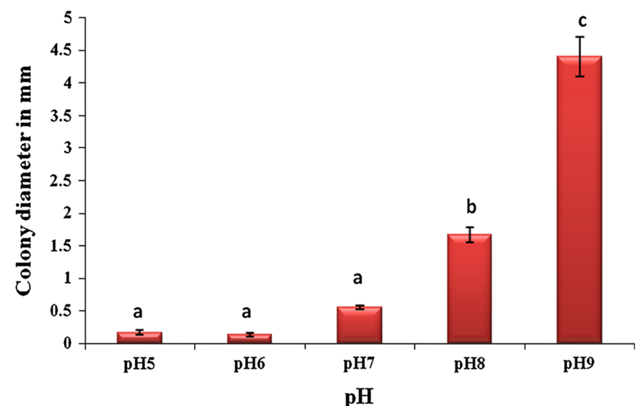

Fig. 3 Effect of pH on adsorption of arsenate by *Penicillium coffeae*. Letters *a*, *b* and *c* in the figure represent homogeneous subsets which provide grouping of means of values obtained. Groups that fall under different subsets (*a*, *b* and *c*) are significantly different from each other at $p = 0.05$

study demonstrated this ability of a fungal isolate that showed resistance to arsenate as high as 500 mM in a liquid medium. The isolate was capable of growing on agar medium with 700 mM of arsenate, however, adsorption in the liquid medium was observed to decline above 500 mM. The isolate was identified as *P. coffeae* (Fig. 2) which showed optimum growth and resistance to arsenate at pH 9.0.

The growth of *P. coffeae* colonies on PDA plates set at different pH was determined by measuring the colony diameter after growth for 6 days. There appeared to be a direct correlation between the increase in pH with growth of *P. coffeae*. This isolate showed maximum growth at pH 9.0 (Fig. 3) with 500 mM of arsenate. Adsorption of arsenate at high pH could be due to an increased negative charge on the cell surface which favored the electrochemical attraction and increased adsorption of the metal (Kang et al. 2007).

The isolate *P. coffeae* was found to be tolerant to arsenate up to 700 mM in solid medium. Hence, this isolate was further evaluated for its ability to adsorb arsenate in liquid medium as live, dead and treated biomass. Untreated (live and dead) and treated biomass was cultured in liquid medium containing 300, 400 and 500 mM of arsenate for 6 days at 28°C. After incubation, the mycelia were separated from the culture broth and the residual arsenate in the culture filtrate was analyzed. The results were compared to that of uninoculated controls. The controls showed adsorption values of 6.8 %, 10.4 % and 16.2 % at 300, 400 mM and 500 mM, respectively (Fig. 4). The percentage adsorption with live biomass ranged from 12 % to 23 %. Dead biomass showed significantly improved levels of adsorption as compared to live mycelia. The adsorption percentage by dead biomass ranged from 28.3 %, 16.18 % and 60.2 % at 300, 400 and 500 mM respectively (Fig. 4). Alkali treated mycelia showed the best adsorption (66.8 %) at 500 mM of arsenate. However the percent adsorption by pretreated biomass was determined to be 58.4 %, 54.5 % and 66.8 % at 300, 400 and 500 mM of arsenate, respectively. Pretreated mycelia showed the highest percentage of adsorption and may represent the optimal condition for maximum adsorption of arsenate by *P. coffeae* (Fig. 4).

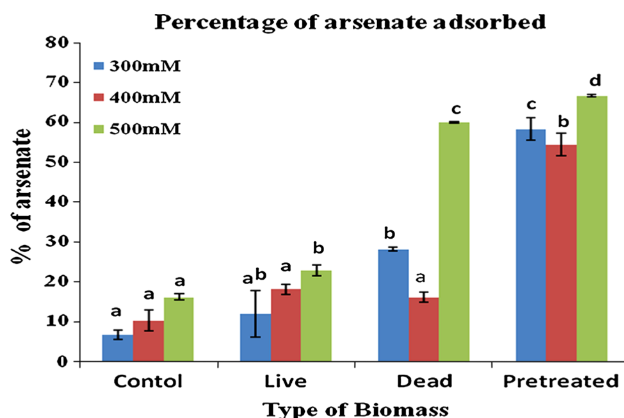

Fig. 4 Percentage of arsenate adsorbed by live, dead and pretreated biomass of *Penicillium coffeae*. Letters *a*, *b*, *c* and *d* in the figure represent homogeneous subsets which provide grouping of means of values obtained. Groups that fall under different subsets (*a*, *b*, *c* and *d*) are significantly different from each other at $p = 0.05$

The comparative study on arsenate tolerance by live, dead and pretreated biomass showed that the pretreatment of biomass with sodium hydroxide significantly increased the percentage of arsenate adsorption; this increase in adsorption could be due to the chemical modification of the cell wall components or exposure of metal binding sites present on the cell wall. NaOH removes amorphous polysaccharides, lipids and proteins from the cell wall that mask the reactive sites, thus generating space within the β glucan-chitin skeleton allowing metal ions to adsorb on the cell surface (El-Sayed and El-Morsy 2004). However the mechanism by which pretreated *P. coffeae* exhibits better metal resistance and adsorption is presently not known and requires further study.

Furthermore the dead biomass (heat dried at 60°C) showed a slight increase in adsorption of arsenate when compared to the live biomass. However in case of live biomass there may be transport of arsenate across the cell membrane causing intracellular arsenate accumulation and therefore altering the cell metabolism (Ahalya et al. 2007).

The ability of adsorption by the isolate reduced beyond certain concentration of arsenate (500 mM), which could be due to engaged groups such as carboxyl, hydroxyl and amide on the cell wall in the adsorption of arsenate resulting in attainment of saturation point (O'Connell et al. 2008). We also observed a considerable reduction in the adsorption efficiency of the organism when grown on arsenate free medium for a longer time. This finding was in accordance with Visoottiviseth and Ponviroj (2001).

This is the first report to show that *P. coffeae*, both live and dead, has the ability to adsorb arsenate at 500 mM, temperature of 28°C, at pH 9.0 and at growth period of 6.0 days. This report also gives an account of the efficacy of alkali (0.5 N NaOH) treated biomass of *P. coffeae* in arsenate adsorption and therefore is added to the list of arsenate adsorbers. Percentage of adsorption by pretreated biomass at 500 mM was 66.8 % as compared to that of live and untreated dead biomass whose adsorption percentage was 22.9 % and 60.2 % respectively. As a follow-up of the present study, the efficiency of dead cells of *P. coffeae* will be assessed in immobilized form as immobilization of cells not only improves the mechanical strength of native biomass, but also helps in reuse of the biomass for several rounds of adsorption and desorption (Narsi et al. 2007). Further investigation will also be carried out to assess the

adsorption efficiency of *P. coffeae* in a field study (Ahalya et al. 2007).

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