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Fungal Bioremediation of Arsenic: An organic procedure to eliminate the rare but potent venom of generations

Dolly Tyagi[1], Sonu Tyagi[2], Ravi Kant Narayan[3]
[1] M. Tech, Deenbandhu Chotu Ram University of Science and Technology, Murthal, Sonepat, Haryana, India
[2] Senior Resident, Department of Anatomy, PGIMS, Rohtak, Haryana, India
[3] Senior Resident, Department of Anatomy, All India Institute of Medical Sciences, Patna, Bihar, India

Abstract
The history is marked by the use of arsenic as a venom for generations. Though an advantageous element when utilised in controlled manner, it's exposure in excess has been found responsible for neurologic, dermatologic, and carcinogenic effects. World health organisation includes arsenic in the list of top 10 chemicals of major public health concern, and hence formulated a number of options to reduce levels of arsenic in drinking-water. Roots of widely dispersed plants and soil samples were collected from an arsenic contaminated industrial area in Delhi. Plant roots were cut into 1-2 mm pieces and laid on potato dextrose agar plates containing 20 µg /l chloramphenicol (PDAC) and 70 mg/l of either arsenate or arsenite. All arsenate tolerant isolates were separately inoculated onto PDAC plates with 700 mg/l arsenate. The same procedure was followed for the arsenite tolerant isolates. Isolates that did not grow were discarded whereas ones that grew were regarded as being tolerant to a high level of arsenic and were used for further testing. It wa observed that Aspergillus flavus, Aspergillus fumigatus and Aspergillus niger were most effective in arsenite removal and can be used on commercially for bioremediation of arsenic.

Keywords: arsenic, bioremediation, Aspergillus flavus, Aspergillus fumigatus and Aspergillus Niger

Introduction

Arsenic is one of those rare metal which is ubiquitously present in our environment. The Human history is marked by the notorious use of this rare element in changing the thrones of various empires, as Nero used it to murder Britannicus, in order to inherit the throne and become Emperor of Rome. Though the metal has been in use for medicinal purpose from the time of Hippocrates [1]. The early 19th century saw the use of arsenic in treating trypanosomiasis by Fowler's solution (1% potassium arsenite), formulated by Thomas Fowler. Later in 1910, a drug i. e. Arsphenamine, popularly known as Salvarsan or compound 606 was introduced as the first effective treatment for syphilis. The anti-syphilitic activity of this organo-arsenic compound was discovered by Sahachiro Hata in 1909 and the drug was first synthesized in 1907 in Paul Ehrlich's lab by Alfred Bertheim [2]. In the present time, arsenic is used in the treatment of wide variety of helminthic diseases and blood related disorders such as acute pro-myelocytic leukemia. Though an advantageous metal but be it known that both the acute and chronic exposure of arsenic can lead to a variety of neurologic, dermatologic, and carcinogenic effects, such as peripheral neuropathy, diabetes, ischemic heart disease, melanosis, keratosis, and impairment of liver functions [3].

The ubiquitous presence of arsenic is seen in the earth's crust as ores such as arsenopyrite (grey arsenic, FeAsS), realgar or sandarach (red arsenic, AsS), orpiment (yellow arsenic, As_2S_3), and arsenolite, an oxidation product of arsenic sulphides (white arsenic, As_2O_3). The toxicity of inorganic arsenic compounds are more than organic compounds [4].

The major source of exposure to arsenic is via drinking water, followed by exposure to industrial processed goods such as glass, pigments, textiles, paper, metal adhesives, wood preservatives and ammunition, processed animal hides, pesticides, feed additives and tobacco. World health organisation includes arsenic in the list of top 10 chemicals of major public health concern, and hence formulated a number of options to reduce levels of arsenic in drinking-water. These are

a) Substitute high-arsenic sources, such as groundwater, with low-arsenic, microbiologically safe sources such as rain water and treated surface water.
b) Discriminate between high-arsenic and low-arsenic sources and educate the masses to reduce the exposure of the same.
c) Install arsenic removal systems – either centralized or domestic – and ensure the appropriate disposal of the removed arsenic [4].

The third option can be availed via procedures such as arsenic removal include oxidation, coagulation-precipitation, absorption, ion exchange, and membrane techniques. One of the recent methods to remove the arsenic or other contaminants of soil, water, and air by converting them to benign product is known as bioremediation. According to the EPA (Environmental Protection Agency, USA), bioremediation is a "treatment that uses naturally occurring organisms to break down hazardous substances into less toxic or non-toxic substances". When the contaminated material is treated at the site of production, it is termed as in-situ bioremediation while treating the same after removal is known as ex-situ bioremediation. Phyto-remediation, phyto-extraction, phyto-stabilization, phyto-volatilization, rhizo-filtration are some of the

bioremediation related technologies [3].

Bioremediation may occur on its own (natural attenuation or intrinsic bioremediation) or may only effectively occur through the addition of fertilizers, oxygen, etc., that help encourage the growth of the pollution-eating microbes within the medium (bio-stimulation). Microorganisms used to perform the function of bioremediation are known as bio-remediators. Microorganisms that can be used as bio-remediators are bacteria, fungi, yeast and algae. These microbes while surviving in environment having arsenic, have evolved their biochemical mechanisms to use arsenic either as an electron acceptor for anaerobic respiration, or as an electron donor to support chemoautotrophic fixation of CO_2 (carbon dioxide) into cell carbon [3]. The white rot fungi have the ability to transform the pollutants from the contaminants in soil through ligninolytic enzymes [5].

The present study aims to evaluate the impact of different fungus such as Aspergillus flavus, Aspergillus fumigatus and Aspergillus niger in bioremediation of arsenic and also formulate a protocol for the same.

Materials and Methods

Collection and isolation of fungi

Roots of widely dispersed plants and soil samples were collected from an arsenic contaminated industrial area in Delhi. The samples were stored in sterile plastic bags and transported to the laboratory in Delhi within 3 hr. Approximately half of the samples were assigned to media containing arsenate; the rest to media with arsenite. The sodium salts of arsenate and arsenite were used throughout this study. Plant roots were cut into 1-2 mm pieces and laid on potato dextrose agar plates containing 20 µg /l chloramphenicol (PDAC) and 70 mg/l of either arsenate or arsenite. For soil samples, 1 g of sample was mixed with 10 ml of sterile distilled water and left for sedimentation. Then 0.1 ml of the supernatant solution was spread on PDAC plates containing 70 µg/l of either arsenate or arsenite. All plates were incubated at 27℃ for 48-72 hour. Fungi growing on these plates were regarded as having at least a low level of arsenic tolerance and were restreaked on potato dextrose agar (PDA) plates for isolation of single colonies. All fungal isolates are currently maintained by Stem Fort Regenerative Medical Centre, Rohini, New Delhi as stock cultures on PDA slants and stored in a refrigerator. Cultures were sub cultured every 3-6 months.

Screening for high arsenic tolerance

Preliminary research indicated an average arsenic concentration in soil of approximately 700 mg/l. The isolated fungi from the previous experiment were, therefore, screened for their abilities to tolerate this level of arsenic. All arsenate tolerant isolates were separately inoculated onto PDAC plates with 700 mg/l arsenate. The same procedure was followed for the arsenite tolerant isolates. There were 3 replicates per isolate. All plates were incubated at 27℃ for 7 days. Isolates that did not grow were discarded whereas ones that grew were regarded as being tolerant to a high level of arsenic and were used for further testing.

Screening for efficient arsenic removal

The fungal isolates that were found to be tolerant of arsenic at 700 mg/l in the previous experiment were tested for their ability to remove arsenic from liquid medium. Two 8 mm disks were cut with a sterile cork borer from the margin of PDA plates and inoculated into 250 ml Erlenmeyer flasks containing 50 ml potato dextrose broth plus 20 µg/l chloramphenicol (PDBC) and 10 mg/l of either arsenate or arsenite. There were 3 replicates per isolate. Uninoculated flasks (no fungus) were used as controls. All flasks were incubated at 27℃ on a rotary shaker set at 150 rpm. After 5 days of incubation the medium in each flask was filtered through a 0.45 mm millipore filter. Each filtrate of media with arsenite added was analysed for arsenite level by hydride generation atomic absorption spectrophotometry (HG-AAS). The samples were digested with nitric acid. For determination of arsenate concentrations, KI (5% w/v) and ascorbic acid (5% w/v) were added to the sample to transform arsenate to arsenite. The arsenite level was then determined by HG-AAS. The arsenate concentration of the filtrate was assumed to equal the arsenite level determined by HG-AAS.

Selection of a fungal isolate for efficient removal of both arsenate and Arsenite

The fungal isolates that removed the greatest amount of arsenate or arsenite were further tested to select the best one for removing both these arsenic compounds from liquid medium. To do this, the 5 most efficient isolates for arsenite removal from the previous experiment were inoculated (8 mm disk) into 50 ml PDBC with 10 mg/l arsenate. The two most efficient isolates for arsenate removal were treated in a similar manner but with arsenite added to PDBC. There were 3 replicates for each experiment. Controls consisted of flasks with either arsenite or arsenate but with no fungal inocula. All flasks were incubated at 27℃ for 5 days on a rotary shaker (150 rpm). The medium in each flask was then filtered through a 0.45 mm millipore filter. Filtrates were analysed for arsenite and arsenate concentration by HG-AAS as described previously.

Identification of selected fungal isolate

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. Size and color of fungal colonies on PDA were also recorded.

After morphological identification of fungus, we found out that three fungal strains i.e Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger were most efficient in arsenic removal therefore further studies involved the use of these strains only.

Temperature and pH effects on arsenic removal

Environmental factors, i. e. pH and temperature, were tested to observe their influence on arsenic removal by the selected fungi. The levels of pH and temperature used were those expected to be found in the field. PDBC with 10 mg l-1 of arsenate or arsenite was adjusted to pH 5, 7 or 9 with lactic acid and NaOH prior to inoculation with an 8 mm disk of the selected isolate. There were 4 sets of experiments as follows:

1. Arsenate with pH 5, 7 and 9 at 27℃,
2. Similar to (1) but at 37℃,
3. Arsenite with pH 5, 7 and 9 at 27℃ and
4. Similar to (3) but at 37℃.

The low and the high temperatures approximated those of shaded and sunny positions, respectively. There were 3

replicates for each experiment. All flasks were incubated for 5 days on a rotary shaker (150 rpm). After that, the culture media were separately filtered through 0.45 mm millipore filters. Filtrates and fungal biomass were analysed for arsenite and arsenate by HG-AAS as described previously.

Effect of arsenic concentration on fungal growth
Inoculum (8 mm disk) from a 14-day-old culture of the selected isolate was inoculated on to the center of PDA plates containing different concentrations i. e, 0, 10, 100, and 1,000 mg l-1, of arsenate or arsenite. There were 3 replicates for each experiment. All plates were incubated at 27°C for 14 days. Colony diameter was measured every 2 days during the incubation period.

Effect of inoculum source on arsenic removal
Mycelium inoculum of the selected fungus was obtained from 3 sources ie, from a PDA plate (8 mm disk), from a 5 day old 50 ml PDB culture (fungal biomass), and from an autoclaved 5 days old 50 ml PDB culture (fungal biomass). The inoculum from each group was separately inoculated into 50 ml PDBC with 10 mg/l of either arsenate or arsenite. There were 3 replicates for each treatment. All flasks were incubated at 27℃ on a rotary shaker at 150 rpm for 5 days. At the end of the experiment, 20 ml of the medium was withdrawn from each experimental flask. The samples were analysed for arsenite and arsenate concentrations by HG-AAS as described previously. The biomass was separated from the remaining medium (30 ml) by filtration and washed three times with deionized distilled water. The three washing solutions of each biomass were mixed together and were analysed for arsenite or arsenate concentration by HG-AAS. The washed biomass was then dried for 3 nights at 80℃, digested with concentrated nitric acid, and analysed for arsenic concentration by graphite furnace atomic absorption spectrometry (GF-AAS).

Kinetics of arsenic removal
In this experiment, the effects of arsenic on fungal growth and media pH were monitored and removal and uptake of arsenic from the medium by fungal cells was measured. Inoculum (8 mm disk) of the selected isolate was added to PDBC containing 10 mg l-1 arsenite. The fungus was similarly inoculated to PDBC but with 10 mg/l arsenate. Each arsenic treatment had 3 replicates. This assay had both positive controls (no arsenic but with fungal inoculum) and negative controls (with arsenic but no fungal inoculum). All flasks were incubated at 27℃ on a rotary shaker set at 150 rpm. The pH was measured and a sample was taken from each flask at different exposure times: 0, 2, 4, 6, 8, 10 and 14 days. The samples were filtered through 0.45 mm filters. Filtrates were analysed for arsenic concentration using HG-AAS. Biomass remaining on filters was dried for 3 days at 80℃, weighed and digested with concentrated nitric acid for determination of arsenic concentration by GFAAS. To determine if arsenate was transformed to arsenite by the fungus, the arsenite concentration was first measured in the media of treatments that initially contained only arsenate by HG-AAS. Potassium iodide and ascorbic acid were then added to the media to reduce any remaining arsenate to arsenite and the arsenite concentration was determined again by HG-AAS. The arsenate in the medium was then calculated as the difference in arsenite concentrations before and after KI/ascorbic acid reduction. For the treatments with arsenite containing media, the filtrates were analysed for arsenite only.

Results & Observations

Fungal isolates
Of all the samples, 20 isolates could tolerate 70 mg/l arsenite and 15 could tolerate 70 mg/l arsenate.

High arsenic-tolerant isolates
Of the 20 summer isolates that tolerated arsenite at 70 mg/l, 18 also tolerated arsenite at 700 mg/l. Similarly, 11 of the 15 isolates that tolerated arsenate at 70 mg/l could also tolerate the same compound at 700 mg/l.

Arsenic removal by isolates
The 10 most efficient isolates for removing arsenic from liquid media are shown in Tables 3 and 4. The two isolates that removed the most arsenite (A4 & A10) and the two that removed the most arsenate (B5 and B6) were chosen for further tests to determine their ability to remove the alternate compound (Table 5). Isolate A10 appeared to be the most efficient of all isolates for removing both arsenite and arsenate from liquid medium (i. e. approximately 30% of arsenite and 40% of arsenate removed from the liquid media during the 5-day incubation period).

Description and identification of the fungal isolate
This isolate had septate hyphae and grew fast at 27℃, attaining a diameter of 5-6 cm on PDA within 7 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 colour change began at the center of the colony then spread outward. Conidiophores were single (mononematous) consisting of a single stipe terminating in phialides (monoverticillate). Conidiophores were hyaline and smooth-walled. Phialides were flask-shaped. Conidia were arranged in long chains, globose and rough walled. Based on microscopic morphology isolate with maximum efficiency of arsenate and arsenite were identified as a species of Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger.

Temperature and pH effects on arsenic removal
Isolate A10 grew only at one of the two temperatures tested (i. e. 27℃). There was no growth at 37℃. Consequently, the concentration of arsenite/ arsenate in liquid media (10 mg/l) at 37℃ did not decrease over the 5-day incubation period at any pH value. At 27℃, the highest percent removal of arsenite by the isolate was found at pH 5.0, followed by pH 7.0. The lowest percent removal was obtained at pH 9.0. The percent removal of arsenate decreased with increasing pH in a similar manner with arsenate. For both arsenite and arsenate treatments, percent removal by the selected strain at pH 5.0 was not significantly different to that at pH 7 (Fig. 1 - a & b). By the fifth day of incubation at 27℃, the media containing arsenite and arsenate with an initial pH of 5.0 was more viscous than it was at the start of the experiment and the pH of the medium had decreased to ~ pH 3.0-3.8. Growth of the fungal isolate, based on the observed amount of fungal mycelia, was better at an initial pH of 5 than at other pH values. Our results, therefore, indicate that a pH of 5.0 and a temperature of 27℃ was the most suitable for both fungal growth and arsenic removal.

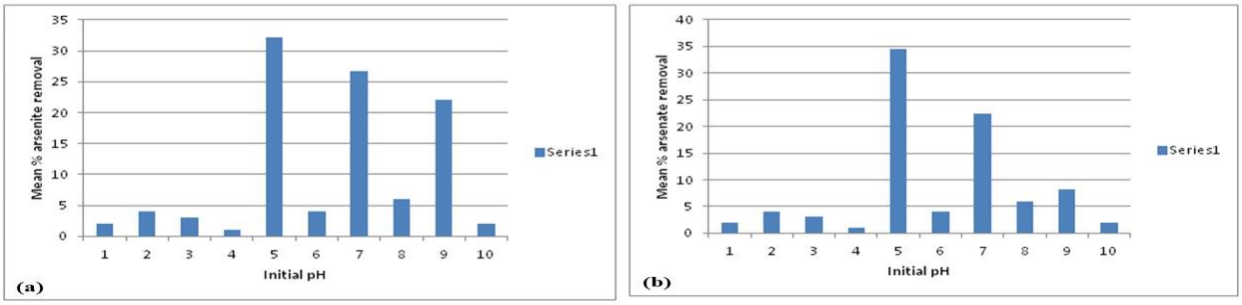

Figure- 1 (a & b) Arsenite (a) and arsenate (b) removal from liquid media at different initial pH values by the fungal isolate, A10, after 5 days incubation at 27°C. Bars indicate SD (three replicates). Columns with different letters are significantly different (t-test, α= 0.05).

Arsenic concentration effects on fungal growth

There was little effect of 10 mg/l and 100 mg/l concentrations of both arsenite and arsenate on fungal colony diameter when compared with that of the controls as represented by Figure 2 (a & b) but inhibition of growth occurred at the highest concentration of 1,000 mg/l for both arsenite and arsenate. This was most obvious at day 10 at which time radial colony diameters were approximately 76% those of the controls.

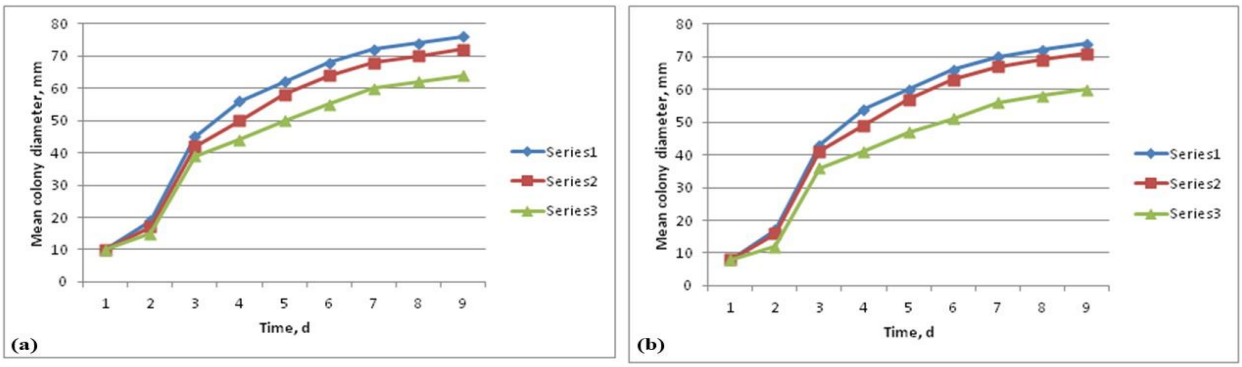

Figure- 2 (a & b) The graph represent the mean growth of the fungus isolate i.e (*Aspergillus flavous, Aspergillus fumigatus, Aspergillus niger)* A10 at different concentrations of arsenite (a) and arsenate (b) on PDA plates over a 14 days incubation period at 27°C. Bars indicate SD (3 replications) and, when not shown, were smaller than the symbol.

Inoculum source effects on arsenic removal

The percent removal of arsenic compounds from liquid medium was higher with agar culture
inoculum than with liquid culture inoculum or with autoclaved inoculum (Table 1). Approximately 25% of arsenate and 22% of arsenite was removed from media inoculated with cells from PDA plate colonies. In contrast, living cells from PDB liquid culture removed approximately 15% and 14%, respectively, of arsenite and arsenate. For autoclaved cells, the percent arsenite and arsenate removal was very low at approximately 4% and 2%, respectively. Cells from the border of fungal colonies cultured on PDA plates were, therefore, used as inoculum in subsequent experiments.

Table 1: Arsenic removal and uptake from PDBC media of the fungus isolate, A10, by cells from PDA fungal colonies, cells from 5-d old PDB cultures, and autoclave-killed cells from 5-d old PDB cultures. The media were incubated at 27℃ for 5 days

	Mean (±SD) Arsenic (As) concentration (mg/l) in the filtered medium			mean (±SD) % As removal from the filtered medium	Mean Arsenic (As) weight (±SD) (µg) in the filtered medium			
	Control	Treatment	Removed	Medium	Removal*	In cells	In washing solution	Lost
PDA cultured cells								
As(III)	9.34±0.05	7.32±0.47	2.02±0.47	21.70±5.01	101.00±23.44	38.39±4.67	35.47±4.84	27.14
As (V)	8.15±0.09	6.14±0.02	2.01±0.16	24.70±2.02	100.50±15.23	39.97±1.11	34.71±8.49	25.82
Living 5-d old PDB cultured cells								
As(III)	9.86±0.06	8.44±0.25	1.42±0.25	14.36±2.57	71.00±12.67	17.46±3.23	21.18±4.05	32.36
As(V)	10.3±0.04	8.46±0.38	1.85±0.38	17.91±3.69	92.00±19.01	24.61±5.63	23.43±0.04	43.94
Autoclave-killed 5-d old PDB cultured cells								
As(III)	9.96±0.03	9.77±0.23	0.20±0.23	1.96±2.34				
As(V)	10.2±0.01	9.81±0.22	0.45±0.22	4.34±2.14				

Kinetics of arsenic removal by the fungus A10

Growth of A10 was not markedly affected by 10 mg/l of

either arsenite or arsenate during the first 6 days of incubation (Fig 3a, 4a). By the eighth day, however, the dry weight of the fungus was lower in arsenic-containing media than in control media and it remained lower until the end of the experiment. It is most likely that cell lysis occurred in the arsenic-treated media causing the observed differences between the treatments and controls. The pH of the medium decreased to pH 3.0-3.4 by the fourth day of incubation and remained steady until day eight in both arsenic-free and arsenic-containing media (Fig 3b, 4b). However, the pH of the arsenic- containing media increased soon afterwards in contrast to the pH of the arsenic-free media. The increase of pH observed in the arsenic-containing media may have resulted from cell lysis and the consequent release of cell contents, which have a pH of about 7.0. Arsenite accumulation in fungal cells increased sharply between the 2nd and 4th days of incubation, and reached a maximum by the 6th day (Fig 5a). The arsenite concentration in the cells then decreased in the days following incubation. A similar pattern of accumulation occurred for arsenate (Fig. 5b). After 2 days of incubation, about 15 % of arsenate was removed from the medium by the fungus. In addition, some arsenite (3 mg/l) could be detected in the medium that initially had only arsenate. This indicates that some arsenate was transformed to arsenite by the fungus. At day 4, 20.69 % of arsenate had been removed and the level of arsenite in the media had increased. At this time the concentration of arsenite that had been produced was equal to the amount of arsenate present in the medium. This indicates that all arsenate had completely transformed to arsenite by day 4. The results at day 6 were similar to those at day 4 in that about 21% of arsenate was removed from the medium and almost all the arsenate left in the medium had been transformed to arsenite. By day 8, the concentrations of arsenate and arsenite in the medium had both increased, possibly due to the occurrence of cell lysis (Fig 4a, 4b). Again, all arsenate in the medium had transformed to arsenite. From day 8 until the end of the experiment (day 14), concentrations of arsenate in the medium levelled off, indicating that no more arsenic was released into the medium. In addition, during this period all arsenate in the medium was completely transformed into arsenite by the fungus.

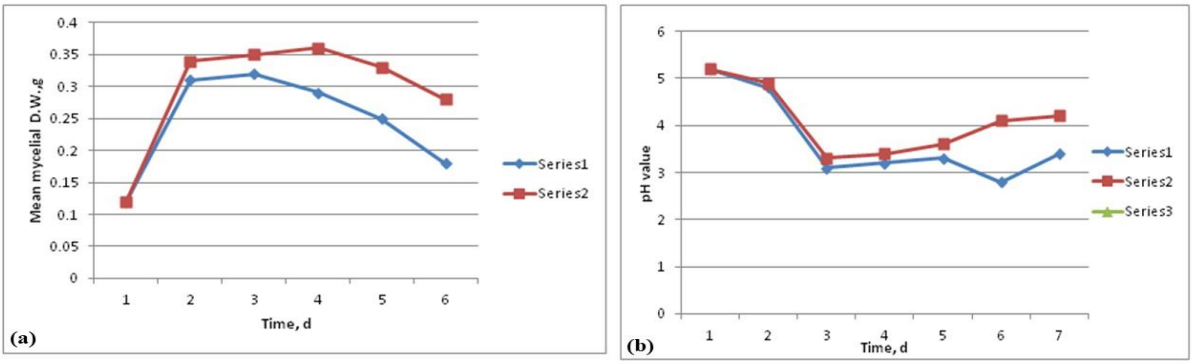

Figure- 3 (a & b) Change in mycelial dry weight (a) and pH (b) during growth of the selected fungus over a 14 d incubation period in PDBC media containing sodium arsenite and two PDBC control media (arsenite but no fungus, fungus but no arsenite). Cultures were incubated on a rotary shaker (150 rpm) at 27°C. Bars indicate SD (three replicates) and, when not shown, were smaller than the symbol.

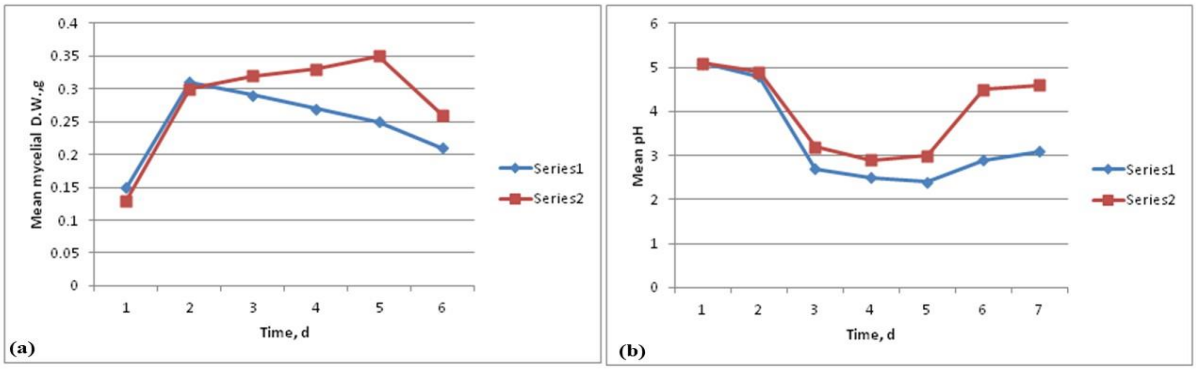

Figure- 4 (a & b) Change in mycelial dry weight (a) and pH (b) during growth of the selected fungus over a 14 days incubation period in PDBC media containing sodium arsenate. Two PDBC control media (arsenate but no fungus, fungus but no arsenate) were included in the experiment. Cultures were incubated on a rotary shaker (150 rpm) at 27°C. Bars indicate S.D. (three replicates) and, when not shown, were smaller than the symbol

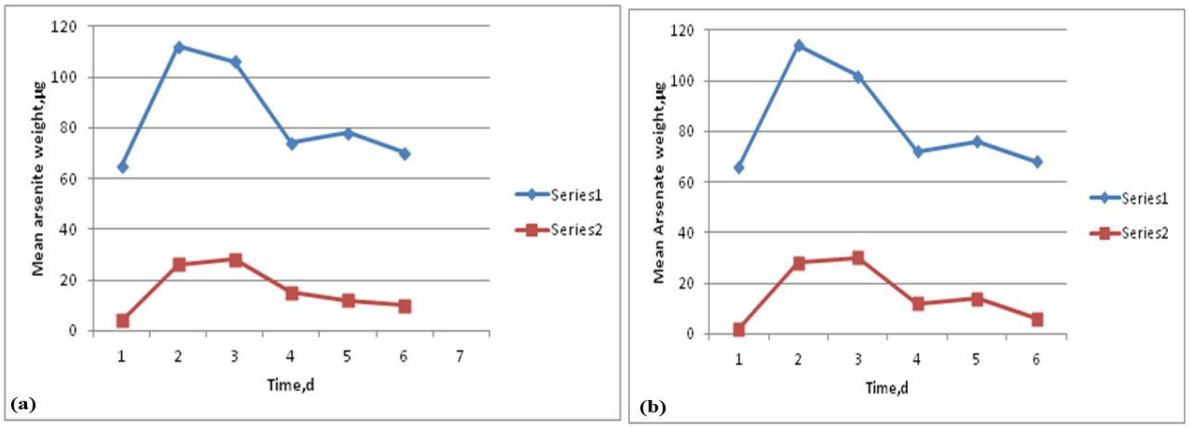

Figure- 5 (a & b) (a) Arsenite and (b) arsenate removal from PDBC medium and accumulation by mycelia of the fungal isolate A10 over a 14 days incubation period at 27°C. Bars indicate SD (three replicates).

Discussion

The soil isolate A4, A10, B6 not only took up and accumulated arsenite and arsenate in its cells but it also transformed arsenate to arsenite, which is the most toxic form of arsenic. On morphological identification of these three strains with maximum efficiency of arsenite and arsenate removal, these strains were characterised as A10 (*Aspergillus flavous*, *Aspergillus fumigatus*, *Aspergillus niger*). The mechanism as well as the resulting products of arsenic transformation by this fungus should be studied in detail. Till date, most of the attention to biosorption of toxic compounds by fungi has been for remediation of polluted environments. The processes contributing to fungal uptake and detoxification of toxic metals depend on an adequate energy source and are limited by toxicity thresholds. Once inside cells, metals may be preferentially stored in the cytosol in association with various metal binding polypeptides, e. g. metallothioneins or phytochelatins, or they become localized in organelles, such as vacuoles. Fungal cell wall is rigid and can tolerate and detoxify metal ions by active uptake, extracellular and intracellular precipitation, and valence transformation, with many absorbing heavy metals into their mycelium and spores. The surface of their cell wall acts as a ligand for binding metal ions, resulting in the removal of metals [6]. Cox and Alexander reported that the mechanism of arsenic uptake by the yeast *Candida humicola* was metabolism-linked and that arsenic competed with phosphate in the medium. Species of *Penicillium*, such as *P. chrysogenum*, *P. brevicaule* and *P. notatum*, were reported to have the ability to transform arsenic compounds, but to the best of our knowledge, the uptake of arsenic compounds by *Penicillium* sp. has never been reported [7].

Cell age has been proposed as an important factor limiting the ability of fungi to accumulate toxic metals. For example, Zajic and Chiu found that Penicillium isolated from wastewater exhibited significant growth in media containing salts of uranium, platinum and titanium and that uranium uptake, in particular, was dependent on culture age. Five day-old cultures were twice as effective as 15- day-old cultures. The reasons for the change in toxic metal uptake pattern with culture age are not very clear [8]. A possible explanation is that changes in cell surface chemistry and morphology with age contribute to lower uptake. Similarly, in this study we also observed that if cultures were stored in arsenate-free media for a long time, the efficiency of the fungus in removing arsenate from media was lower than when the original screening tests were performed.

To conclude, this can be inferred from the findings of the present study that fungal bioremediation can be utilised as a viable option to eliminate arsenic exposure.

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