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Arsenic Accumulation and Biosorption by Arsenic Resistant Bacillus Subtilis Isolated from The Soil

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Abstract

Environmental contamination with heavy metals, especially arsenic (As) is one of the major problems in recent years. The present study demonstrates the effective removal and accumulation of arsenic by a soil bacterium called *Bacillus subtilis* strain A-7. The isolate was found to be highly resistant to arsenic with the Minimum Inhibitory Concentration value of 500 μg/ml. The removal studies indicated a significant decrease in the metal concentration in the growth medium by the bacterium which was successfully adsorbed in the cell wall with the few parts accumulated inside the cells. The FTIR and SEM results reflected that the bacteria was quite active in quickly adsorbing the arsenic without any significant damage caused to the cells. This suggests the adsorption and accumulation efficiency of the *Bacillus subtilis* as the main mechanism in dealing with this toxic metalloid. Such arsenic resistant bacterium can be effectively applied for the bioremediation of contaminated soil and water.

Keywords: Biosorption, Arsenic contamination, Arsenic resistant bacteria, Biomass, Bioremediation

1. Introduction

Metals form an integral part of the earth's crust and are mostly present as insoluble precipitates and minerals. Natural processes like rainfall leaching, weathering, surface runoff and microbial leaching introduces a large concentrations of metal ions in ground water and other water bodies, thereby making their way into the ecosystem. Nevertheless, the greater load of metal ions enters the environment as a result of anthropogenic activities such as mining, use of pesticides and metal refining [1]. Metalloids especially arsenic (As) is potentially toxic and is considered as a carcinogen. It exists in two common redox forms: the less soluble arsenate (As^{+5}) and the more soluble arsenite (As^{+3}). One of the most toxic and prevalent forms of arsenic is the arsenic trioxide (As_2O_3), which is readily absorbed from the lungs and skin. The prolong exposure of arsenic may cause severe health problems like nausea, vomiting, diarrhea, gastrointestinal hemorrhage, cerebral edema, tachycardia, dysrhythmias, hypovolemic shock, coma, cancer and even death [2]. This makes it very important to understand the removal strategies of this toxic metal from the soil and water bodies to make them fit for the survival of living organisms. Many of the physicochemical processes like precipitation, chelation, ion exchange, chemical treatment etc. have been reported but have not proved to be effective. The use of microbes in the biosorption of these metals seems to be a good approach in cleaning of the contaminated sites and their recovery.

Microorganisms possess different mechanisms to detoxify arsenic which includes biotransformation, biosorption, exopolysaccharide secretion, bioaccumulation, and oxido-reduction reactions. A variety of

bacteria is known today which are not only resistant to arsenic but have evolved efficient mechanisms in dealing with it [3, 4]. The advantages that is provided by the bacteria makes them suitable for the treatment of contaminates sites. This includes quick growth, easy to harvest and comfortable application.

Biosorption is a quick and easy method which typically involves the bacterial cells in mediating the transport of the metal ion to the cell surface. This process may involve the temporary or permanent binding of metals on to the cell surface with the help of different processes like adsorption, ion exchange, coordination, complexation, chelation and microprecipitation. The mechanism involved is the electrical boning between the anionic groups of the cell wall and cationic metals, although Van der Waals forces, covalent bonding and redox interactions can also be involved. However, the permanent accumulation of metals by the bacterial cell is an irreversible process which is largely used in the cleaning of the contaminated sites [5, 6].

Microorganisms can accumulate essential and nonessential metal ions in their cell walls because of the presence of macromolecules which have an abundance of charged functional groups such as carboxylate, amine, imidazole, sulfhydryl, sulphate, hydroxyl and phosphate. This facilitates the polyfunctional metal binding sites for both cationic and anionic metal complexes to the cell surface [7]. The absorption, adsorption and desorption of metals is largely dependent on the environmental factors such as pH, presence of competing ligands, temperature, dose and time [8]. The employment of microbial bioremediation using bacteria and fungi which exhibits superior biosorption and bio-accumulation capabilities has emerged as a viable solution for the detoxification of heavy metals. The temporary binding of metals using microbes is fast, reversible and can also be used for the metal recovery. This indicates that microbial-based solutions are sustainable, rapid and offer cost-effective methods for reducing the toxicity of arsenic.

This study is aimed to investigate 1) the arsenic resistance of the bacterial isolates, 2) arsenic removal and accumulation of the bacteria in the growth medium, and 3) effect of environmental parameters on biosorption capacity of the isolate.

2. Materials and methods

2.1. Preparation of arsenic stock solution

The reagents used in the experiment were all analytical grade (AR) and ultrapure water was used for the preparation of stocks and reagents. Stock solution (1000 mg/l) of disodium hydrogen arsenate heptahydrate (Na2HAsO4. 7H2O) was prepared in deionized water, filter sterilized and stored in dark at room temperature. Working solutions of required concentrations were freshly prepared by diluting the stock solution.

2.2.Isolation, characterization and identification of arsenic resistant bacteria

The selective isolation of arsenic resistant bacteria was followed using enrichment culture method. Soil samples were collected, sieved and used in the experiment. For the isolation procedures, 1g soil was inoculated in the Erlenmeyer flask containing Luria Bertani (LB) broth (30 ml) supplemented with different concentrations (100-500 μg/ml) of arsenate and incubated for 48 h at 120 rpm and 37 °C. The flasks showing turbidity were selected and spread (0.1 ml) on Nutrient Agar Medium (NAM), followed by incubation at 37 °C for 48 h. The colonies appearing on the plates were selected and streaked for the purity on the NAM having same concentration of sodium arsenate.

The isolated bacteria were tested for their resistance against arsenic by the agar plate method. Minimum Inhibitory Concentration (MIC) was determined by growing the bacteria on NAM amended with increasing concentrations of sodium arsenate. After the incubation period of 3 days, the lowest

concentration required to prevent the visible growth of bacterial isolates was checked and marked as their MICs.

The bacteria showing the highest level of MIC was selected for the further studies and was identified on the basis of colony morphology, microscopic characteristics, biochemical analysis and 16S rRNA sequencing.

2.3.Arsenic sorption experiments

The efficiency of the bacterial isolate to grow and remove arsenic was determined in the LB broth supplemented with 100 µg/ml concentration of sodium arsenate. The medium devoid of arsenic served as control. The experiment was conducted in three sets, each for the test and control. The flasks containing LB broth was inoculated with fresh bacterial culture and incubated at 37 °C for 48 h. Growth was determined by observing the turbidity and taking the optical density at 600 nm using spectrophotometer at an interval of 6 h. Arsenic removal assays were carried out at the given time intervals by harvesting the samples and collecting the pellet by centrifugation at 10,000 rpm for 10 min. The cell pellet was digested using aquaregia and accumulation of arsenic in the cells was determined. The amount of arsenic removed from the growth medium was estimated in the cell free supernatant using the following formula:

$$R\ \% = \frac{\mathrm{I-F}}{I}\ x\ 100$$

Where, R is the removal capacity of the bacterial isolate, I is the initial concentration and F is the final concentration

2.4.Effect of temperature and pH on arsenic biosorption

To understand the effect of pH on the removal of arsenic, the bacterial isolates were grown in the medium supplemented with 100 µg/ml of arsenic and adjusted to different pH (5-11), followed with the incubated at 37 °C for 48 h. For the study of temperature effects on the arsenic biosorption, the cell culture was incubated at 4, 25, 28, 33, 37, 40 and 60 °C for 48 h. The optimum pH and temperature required for the growth of the isolates was determined by recording the optical density at 600 nm using spectrophotometer.

2.5.FTIR and SEM analysis of adsorbed arsenic in the bacterial cell

The effect of arsenic on the surface morphology of bacterial cells were analysed using Fourier transform infrared (FTIR) spectroscopy and Scanning Electron Microscopy (SEM). For the FTIR, the bacterial cell pellet was mixed with KBr in a ratio of 1:100 and the prepared sample was then analysed. For SEM, the dehydrated cell pellet was mixed coated with a conductive material, Gold using an instrument called a sputter coater and observed under microscope.

2.6. Statistical analysis

Experiments were performed in triplicate and data were expressed as the mean ± standard deviation (n = 3). The data were analysed and compared by one-way analysis of variance (ANOVA) using SPSS.

3. Results and Discussion

All together 37 indigenous bacterial strains were isolated from the soil which showed resistance to varying concentrations of arsenic. The resistance showed by the bacterial isolates was in the range of 250-500 µg/ml. Among the isolated bacteria, one strain (A-7) showed exceptional ability to withstand the higher concentration of arsenic (500 µg/ml) and was selected for the study. Initial colony morphology and biochemical characterization showed that the bacteria belong to the genus Bacillus. The selected bacterial isolate was Gram positive, rod shape, spore forming, non-motile and obligate aerobe. The detailed characteristics of the isolates are given in table 1.

Table 1: Characterization of the isolate A-7

Characteristics/ Tests	Results
Gram staining	Gram +
Shape	Rod
Spore formation	+
Colour of the colony	White
Elevation	Flat
Motility	-
Catalase	+
VP test	+
Citrate utilization	+
Indole production	+
Carbohydrate fermentation	+
Oxidase test	+
Starch hydrolysis	+
Urease	-
Phenyl- alanine deaminase	-
Gelatine hydrolysis	-
Nitrate reductase	-
IMViC test	-

Taxonomic identity of the bacterial isolates was ascertained by analysing the 16S rRNA gene sequences. Phylogenetic analysis revealed close lineage of the isolate with *Bacillus subtilis*. Arsenic resistance have been previously reported by this genera in many studies [9, 10], indicating the remarkable ability of the *Bacillus* to grow and survive in diverse environments.

Table 2: Bacterial strains showing significant similarity with A-7

S. No.	Closest Neighbour	Percent Similarity	Accession Number
1.	*Bacillus subtilis* strain HAS31	100	OP727615.1
2.	*Bacillus subtilis* strain FJYA24	100	CP173415.1
3.	*Bacillus subtilis* strain SSM	100	PQ579888.1
4.	*Bacillus subtilis* strain cel	100	CP172966.1
5.	*Bacillus subtilis* strain M51	100	PQ394939.1
6.	*Bacillus subtilis* strain 45	100	PQ441872.1
7.	*Bacillus subtilis* strain BF12B1	100	CP162517.1
8.	*Bacillus subtilis* strain B13	100	PQ565634.1
9.	*Bacillus subtilis* strain B54	100	PQ565638.1
10.	*Bacillus halotolerans* strain W3	100	PQ221917.1
11.	*Bacillus subtilis* strain N33	100	CP163458.1
12.	*Bacillus subtilis* strain S2	100	PQ225877.1
13.	*Bacillus halotolerans* strain S6	100	PQ225943.1

14.	*Bacillus subtilis* strain VNMKV 1	100	PQ270622.1
15.	*Bacillus subtilis* strain J1	100	CP126973.1

Study of the growth kinetics and arsenic removal efficiency of the bacterial isolates showed that the strain was able to comfortably grow in the presence of 100 µg/ml of arsenic, reaching to its exponential phase within 24 h of incubation (Figure 1). However, this was a little late when compared to the control cells which attained their exponential phase in 16 hours of growth. This can be attributed to the toxicity of arsenic to most cells including bacteria [11, 12, 13]. The results also showed that the growth of the isolates was directly proportional to the arsenic removed from the growth medium. With the beginning of the lag phase, the bacteria was able to remove small amount of arsenic from the medium. The possible reason might be the adjustment of the cells which takes place in this phase to the new environment. During the exponential phase, the bacterium reached its maximum growth rate which also helped in maximum removal of arsenic. At the end of the experiment, around 98% of arsenic was successfully removed from the growth medium (Figure 2). The arsenic removed was accumulated inside the bacterial cells to a significant level, showing the accumulating capacity of the isolate (Figure 3). These results are in line with the previous studies which shows that bacteria can accumulate arsenic inside their cells [14, 15, 16].

Figure 1: Growth kinetics of arsenic resistant bacterium A-7

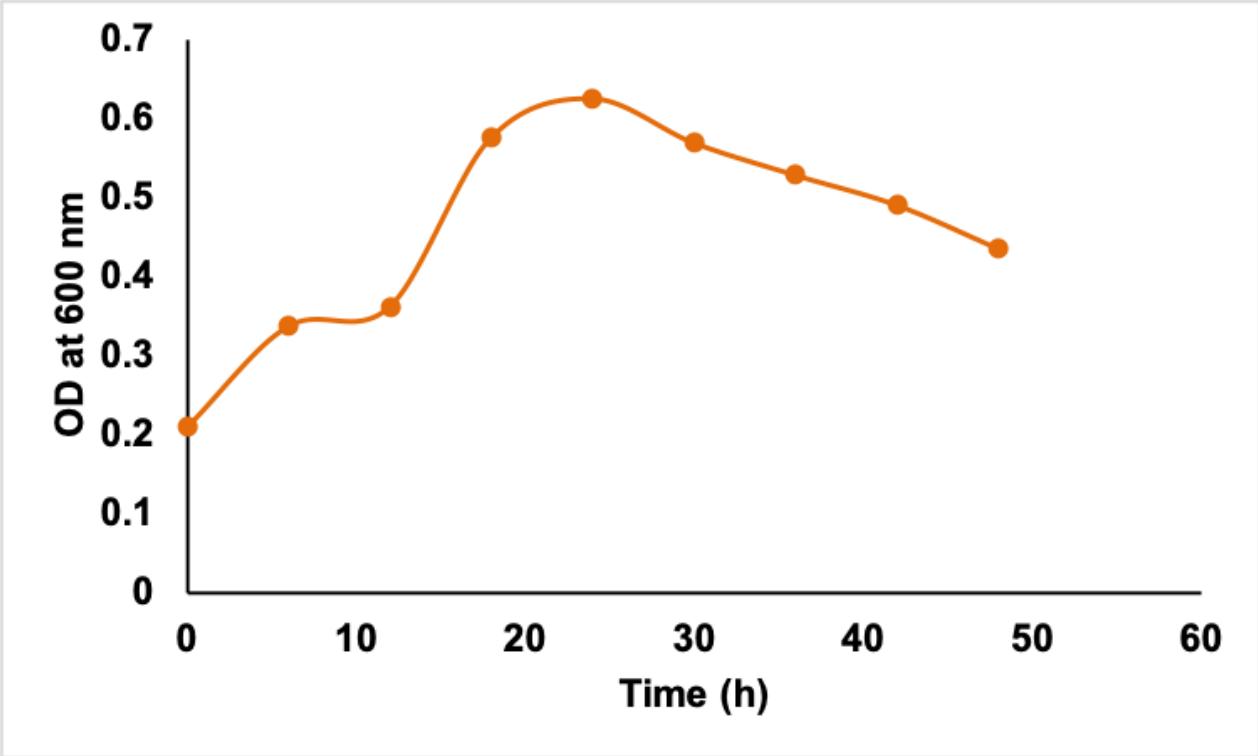


Figure 2: Arsenic removal efficiency of A-7

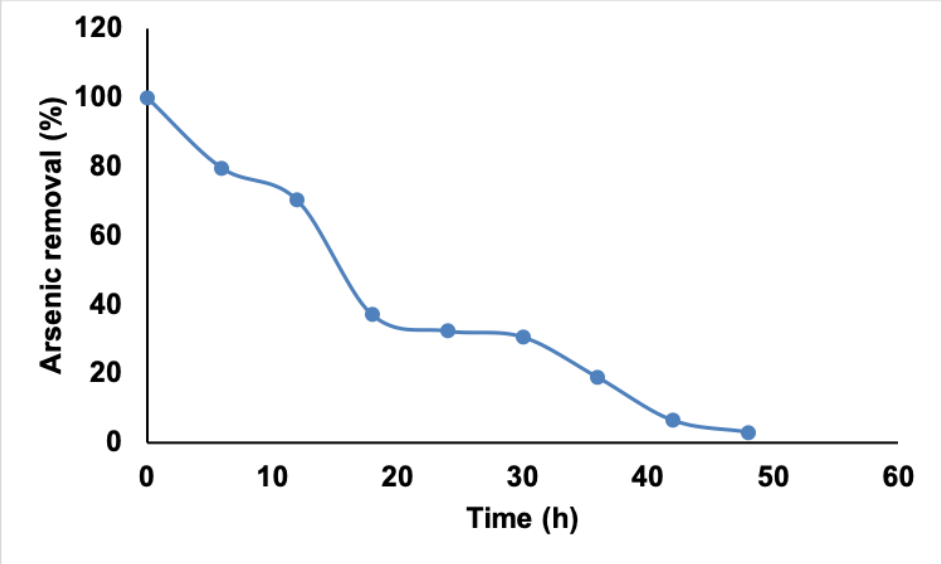
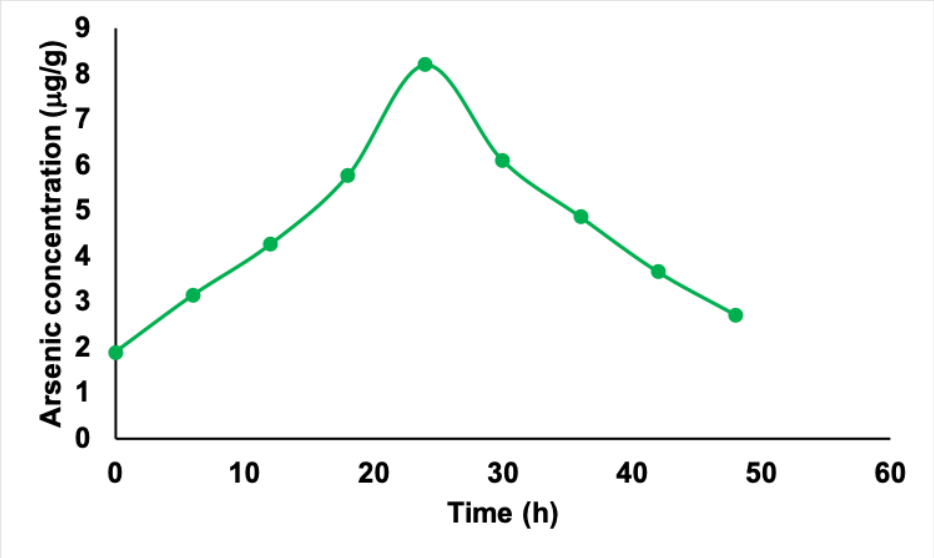


Figure 3: Arsenic accumulation by A-7



Removal of metals takes place in an optimum pH and temperature, which largely effects the growth of the bacteria and its capacity in accumulating metals inside the cell. The present study showed that the optimum pH required for the bacteria to grow and remove arsenic was 8. However, the organism was able to grow at pH 6-10, but the removal percent was higher at pH 8. Below pH 6 and above pH 10, the bacteria failed to show any sign of growth, Similarly, the optimum temperature noted for the growth of the isolate was 37 °C, which marked the highest removal of arsenic from the growth medium. The result is in line with the previous studies which indicate that pH and temperature are the key factors in promoting bacterial growth [17, 18].

The adsorption and accumulation of arsenic by the bacterial isolates was confirmed using FTIR analysis. Results showed that the arsenic loaded bacterial cells have a characteristics peak at 3500, 3000 with the most prominent one at 1600 cm^{-1}, attributing to the involvement of O-H, N-H and C-O-C bonds in arsenic binding (Figure 4). This also proves the efficiency of arsenic resistant bacterial isolate in the successful uptake of arsenic ions. The result very well corroborated with the previous findings [19, 20].

Study of the morphological properties through SEM showed that the bacteria exposed to arsenic was able to bind the anions on the cell surface, which gave a rough appearance to the cells (Figure 5). This happens when the metals forms a bond with the functional group present on the cell surface. After successful binding of the metal ions, they were then transported inside with the help of transporter proteins. This can be seen in the micrographs where clearly some of the cells have increased in size, possibly due to the accumulation of arsenic inside the cells. Arsenic accumulation is a common process which is adopted by the microorganisms to reduce their toxic effects. Previous studies have shown that many bacteria belonging to different genus can successfully bind metals ions and accumulate them inside the cell [21, 22].

Figure 4: FTIR analysis of the before (a) and after (b) arsenic sorption by the bacterial isolate

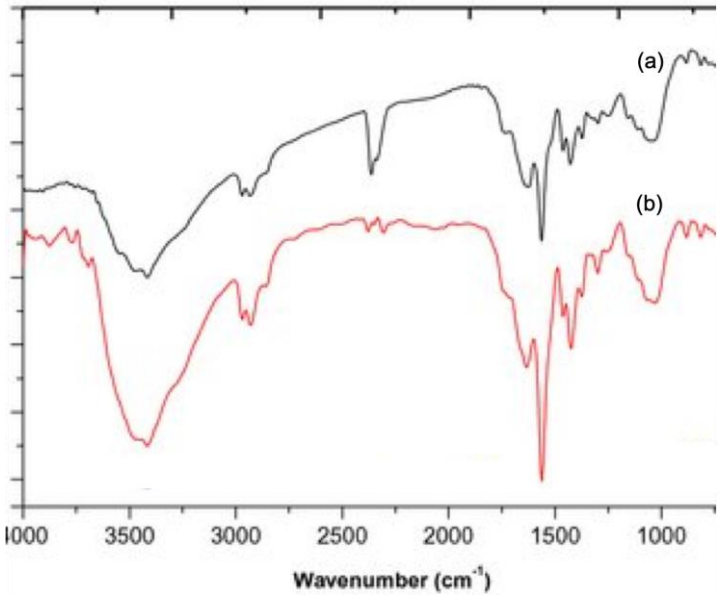
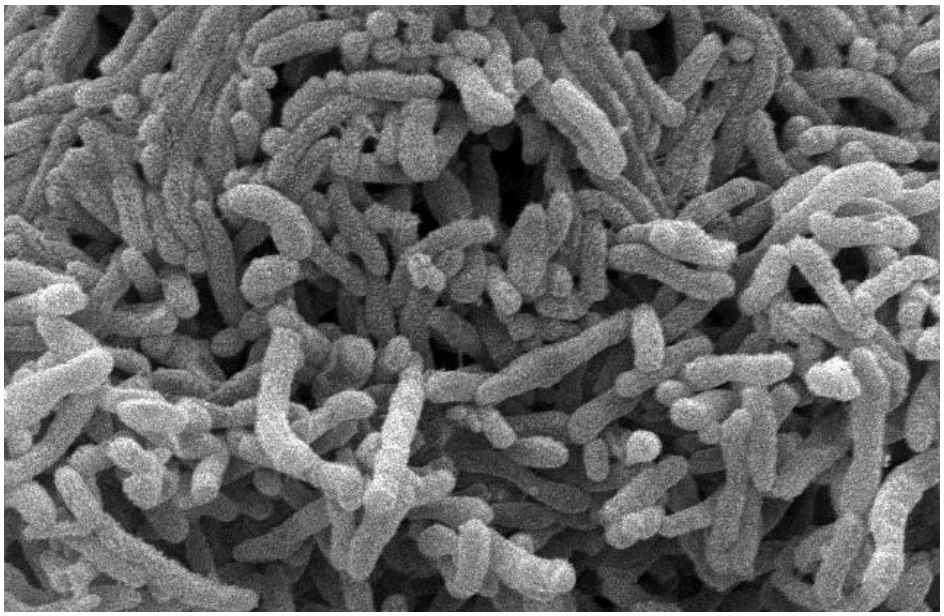


Figure 5: Scanning Electron Microscope of A-7 after accumulating arsenic



4. Conclusions

The study demonstrated that the soil bacterium A-7 has the ability to remove arsenic ions from the growth medium and can accumulate it inside the cell. The systematic removal study showed that the isolate was successful in removing 98 % of arsenic from the liquid medium in less than 48 h without impacting the efficiency of the cells. The arsenic resistant bacteria is an important candidate which can be successfully applied in the removal of arsenic from the contaminated water and in the recovery of the metals from the contaminated environment. The application of bacteria in metal removal needs further research in understanding the pathways utilized in response to the stress. A better understanding of the on-site treatment strategies of the arsenic contaminated ecosystem will open practical significance for the environmental remediation.

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