

Significance of bio-treatment by acid washing for enlargement of arsenic desorption in indigenous arsenic-resistant bacteria from gold mine

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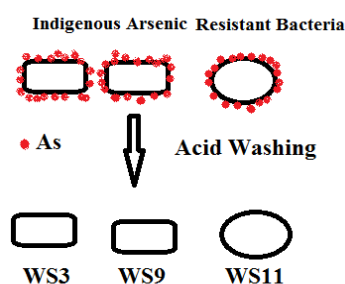
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Graphical abstract



Abstract

Mining activities can lead to the generation of large quantities of heavy metal, specifically arsenic which is released from a gold mine, causing widespread contamination of the ecosystem. Removal of carcinogenic and toxic arsenic from wastewater is essential for the safety of water that may be used for irrigation or drinking. In this study, three different of indigenous arsenic resistant bacterial strains were isolated from gold mine environment, *Bacillus thuringiensis* strain WS3, *Pseudomonas stutzeri* strain WS9, and *Micrococcus yunnanensis* strain WS11. WS9, WS3, and WS11 reached stationary phase after eight, ten, and seven hours, respectively, at 37 °C when grown in LB with arsenic. Gram staining showed WS9 as gram-negative rods, WS3 as gram-positive rods, and WS11 as gram-positive cocci. From the Silver nitrate test, WS3 and WS11 reduced As (V) to As (III) while WS9 oxidized As (III) to As (V). The desorption of arsenic using acid washing and parameters affecting the desorption of arsenic such as acid concentration, time, adsorbent dosage, and different volume of acid solution were investigated. The batch experiments were carried out using bacterial biomass cultured in LB with 2 mM arsenite (III) and 5 mM arsenate (V). Optimum conditions for desorption arsenic were determined, being 1 M acid concentration at 37 °C and 2 hours of contact with (50 mg) bacterial biomass in 100 ml acid solution. The removal of arsenite and arsenate increased after acid washing of bacterial biomass of the three strains. Consequently, desorption of arsenic using acid washing is essential for biomass regeneration.

Keywords: Acid washing, desorption, arsenic, biomass, bio-treatment, goldmine.

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INTRODUCTION

Arsenic (As) has an atomic number of 33 and it comprises around 0.0005% of the crust of the earth. It has an atomic weight of (74.9216) and it melts at 817 °C and boils at 613 °C at 28 atm. Furthermore, it has a silver-grey brittle crystalline colour and vapours at 372 °C in pressure of 1 mm Hg with a specific gravity of 5.73. It also exists in the oxidation states (+5, +3, 0, -3) (Wu *et al.*, 2010). Arsenic can be found in the environment as arsenious acids ($H_3AsO_3^{2+}$, H_3AsO_4 , H_3AsO_5). Moreover, As (V) is like a soft acid and can form a complex with sulfides. On the other hand, As (III) is a firm acid which forms a compound with nitrogen and oxides (Mohan and Pittman, 2007). More than 100 million people universally, mainly in Bangladesh, India, China, Taiwan, Thailand, Chili, and Romania are exposed to dangerous levels of arsenic (Singh *et al.*, 2007). Therefore, the removal of heavy metals such as arsenic from wastewater and water are important to protect a public health (Zaini *et al.*, 2011).

Microbes have co-habit with different metals from initial history. Thus, microorganisms have been effectively used to remove heavy metal such as Arsenic from wastewater in a variety of patterns. Consequently, from a functional concept, metals can be divided into three groups: (i) non-toxic and essential such as Mg and Ca, (ii) harmful at high concentrations and essential in low concentration such as Zn, Mo, Cu, Ni, Co, Fe, and Mn, and (iii) toxic even in low concentration such as Cd, Hg, and As. In addition, interaction with metals relies on the specific metal and its chemical speciation (Valls and De Lorenzo, 2002). Bio-treatment has received great attention in the recent years due to its low cost and high capacities. The purpose of this study was to propose a suitable conventional pre-treatment

technology of indigenous arsenic resistant bacterial biomass to increase arsenic removal efficiency from wastewater.

To the best of our knowledge, there are no reports to date on optimising the pre-treatment of the biomass of indigenous arsenic-resistant bacteria used for the bioremediation of As (III) and As (V). Therefore, in the present study, we focused on characterising arsenic-resistant bacteria isolated from laden tailing dam sludge in goldmine and enhancing their ability to remove arsenic from aqueous solution through acid treatment. In addition, the desorption of As (III) and As (V) from the biomass of indigenous arsenic-resistant bacteria was studied and optimised at different acid concentrations, contact time, bacterial biomass dosage (mg), and hydrochloric acid solution volume (mL).

EXPERIMENTAL

Culture medium

Luria Bertani (LB) was made by mixing (5 g) yeast extract, (10 g) sodium chloride, and (10 g) Tryptone in (950 ml) dH₂O with the pH adjusted to pH 7.0 and the volume continued to 1 litre. After that, the mixture was autoclaved for 20 min at 121 °C. For solid LB, 1.5% agar powder was added to the LB broth before autoclaving.

Analysis of arsenite

The modified molybdenum blue method was used to measure the concentration of As (III) and As (V) in the solutions (Cummings *et al.*, 1999). The remaining arsenic in the solution was calculated from the slope of the standard curve. The standard curve for arsenite (III) and arsenate (V) was made by plotting absorbance at 865 nm against

the concentrations of As (III) and As (I–10 ppm) (Altowayti, Algaifi, et al., 2019).

Bacterial strains

Bacillus thuringiensis strain WS3, *Pseudomonas stutzeri* strain WS9, and *Micrococcus yunnanensis* strain WS11 were isolated from gold mine environment and submitted to the NCBI gene bank under accession numbers (MF099871), (MF106176), and (MF107934), respectively.

Growth profile of arsenic-resistant bacteria (WS3, WS9, and WS11).

Growth profiles of WS3 and WS9 were determined in LB medium containing 2 mM arsenite whilst that for WS11 in LB containing 5 mM. Isolates (10 v/v) were cultured in the respective media at 37 °C with shaking at 150 rpm for 15 hr. The growth of the bacteria was measured spectrophotometrically at OD₆₀₀ nm at hourly intervals. LB broth without inoculum was used as a control.

Gram staining and cell morphology

Gram staining was observed under a light microscope. Gram-positive bacteria were stained purple whereas Gram-negative bacteria were stained pink.

Silver nitrate test

The isolated bacteria (WS3, WS9, and WS1) were cultured on 0.1 Trypticase Soy Agar incorporated with 1 mM As (III) or As (V) for 24 hours at 37 °C. Then, the agar plates were flooded with 0.1 M AgNO₃. Brownish precipitates revealed the presence of arsenate (silver orthoarsenate) in the medium while yellow precipitates (silver orthoarsenite) revealed the presence of arsenite in the medium (Simeonova et al., 2004).

Preparation of bacterial biomass of WS3, WS9 and WS11

Isolates WS3 and WS9 were grown in LB medium with 2 mM As (III) and WS11 was grown in LB medium with 5 mM As (V) until the maximum growth was reached. Then, the biomass was centrifuged at 10000 rpm for 15 min and washed two times with ultra-pure water. Afterward, the biomass was dried out for 15 h at 70 °C and crushed to small units using a mortar and pestle.

Desorption of arsenic

1) Effect of hydrochloric acid concentration

Hydrochloric acid has been confirmed to be efficient for desorption of arsenic and regenerated biomass. The experiments were carried out at different concentrations of acid solution (0.2, 0.4, 0.6, 0.8, 1, 1.2, and 1.4 M) to determine the optimum acid concentration for biomass washing. 100 ml of the acid solution was added to 100 mg of each strain in the 100 ml conical flask. The solution was then mixed at 37 °C for 5 h. Then, the biomass in the acid solution was separated by centrifuging at 10,000 rpm at 4 °C for 15 min before the extracted arsenic was analyzed.

2) Effect of contact time

To study the influence of contact time on desorption of arsenic from bacterial biomass, every 100 mg of biomass was mixed with 100 mL acid solution at an optimum concentration in 100 mL conical flask for different washing durations (1, 1.5, 2, 2.5, 3 hours). The acid solution and the bacterial biomass were separated by centrifugation at 10,000 rpm for 15 min at 4 °C and the arsenic removed was examined.

3) Effect of bacterial biomass dosage (mg)

The dosage of bacterial biomass (mg) is an essential factor in biomass washing. The optimum hydrochloric concentration and time were fixed. The dosage of 20, 30, 40, 50, 60, and 70 mg biomass for each strain WS3, WS9, and WS11 were added separately to 100 ml acid solution at optimum mixing time. Then, the acid solution and the bacterial biomass were separated by centrifuging at 10000 rpm for 15 min at 4 °C and the arsenic desorbed was calculated.

4) Effect of volume (mL) of hydrochloric acid solution

The volume (ml) of the acid solution is a significant element in biomass washing. In this experiment, the optimum parameters of hydrochloric acid concentration and time specified in previous tests were fixed to optimum biomass dosage. Based on 50 mg of biomass, the volumes of 25, 50, 75, 100, 125, and 150 ml of acid solution were applied. Then, the acid solution and the bacterial biomass were separated by centrifugation at 10000 rpm for 15 min at 4 °C and the arsenic in the solution was measured.

Adsorption of arsenic

The biomass (50 mg) for the three strains WS3, WS9, and WS11 was separately subjected to acid washing using 100 ml of 1 M HCl for 2 hours then centrifuged at 10000 rpm at 4 °C for 15 min and washed twice with ultra-pure water to remove excess acid. Finally, the washed biomass was dried at 70 °C for 15 hr and used for adsorption of As (III) and As (V) (Bahari et al., 2013). The biomass (5 mg) for WS3, WS9, and WS11 was separately mixed with As (III) and As (V) (8 ppm) in 100 ml conical flask containing 10 ml of the arsenic solution before and after acid washing. All tests were done in triplicate. The biomass was removed from the mixture by centrifugation for 15 min at 10000 rpm and 4 °C. The Arsenic remaining in the solution was examined.

RESULTS AND DISCUSSION

Growth profile

It is very important to know the growth profile of bacteria to get the maximum biomass of the cell which can be achieved in the late exponential phase to carry out the next experiment. The growth profiles of WS3, WS9, and WS11 as presented in Fig. 1 were obtained by monitoring changes in optical density (OD₆₀₀) for 12 h at 37°C. From Fig. 1, the maximum growth for both WS3 and WS11 were after 10 h and 7 h and it was 8 h for WS9 in the LB medium with the highest OD₆₀₀ measured which were (2.429), (2.493), and (2.198), respectively. Nonetheless, the growth profiles confirmed these strains have the ability to be cultivated in the lab using LB medium with arsenite or arsenate since these bacteria were previously isolated from an arsenic contaminated environment. Bacteria that can tolerate high concentrations of arsenic species are widespread in environment and have been successfully isolated (Anderson and Cook, 2004; Escalante et al., 2009; Pepi et al., 2007; Suresh et al., 2004). It has been reported that bacteria capable of resisting toxic heavy metals such as arsenic possess a protein known as DPS (DNA protection during starvation) which allows them to tolerate stressful conditions such as high concentrations of heavy metals (Chiancone and Ceci, 2010; Martinez and Kolter, 1997).

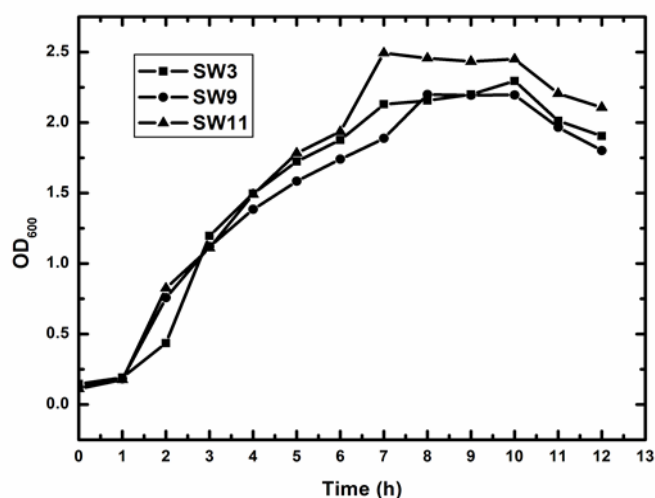


Fig. 1 Growth Profile of Arsenic-Resistant Bacteria (WS3, WS9, WS11)

Gram staining

Gram staining is used to distinguish between bacteria according to different constituents of their cell wall. The Gram stain procedure

differentiates between Gram-negative and Gram-positive groups by colouring these cells pink or violet. Consequently, WS3 and WS11 were found to be gram-positive (thick layer of peptidoglycan, 90% of the cell wall), rod and cocci shape, respectively. Whilst, WS9 gram-negative (thin layer of peptidoglycan, 10% of the cell wall; high lipid content), rod-shape bacteria as shown in Fig. 2.

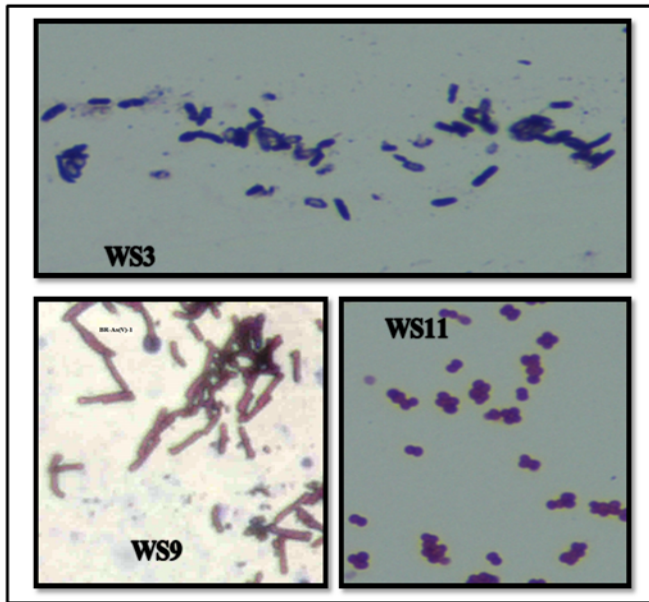


Fig. 2 Gram staining and Cell Morphology at 100× magnifications WS3 and WS11: Gram-positive. WS9: Gram-negative.

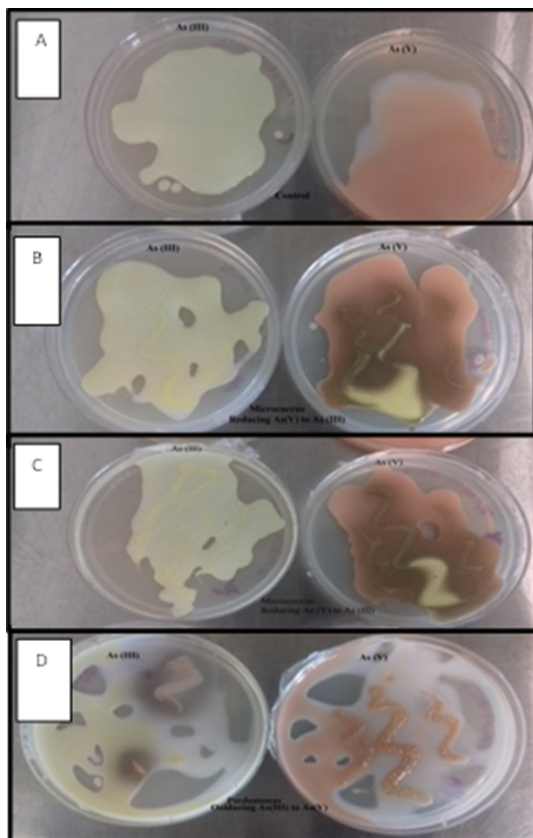


Fig. 3 A: control, B: WS3: Reducing bacteria, C: WS11: Reducing bacteria, D: WS9: Oxidising bacteria.

Silver nitrate test

All strains were grown on 0.1 × TSA incorporated with 1 mM As (III) or As (V) for 2 days before the plates were flooded with 0.1 M

AgNO₃ solution. From Fig. 3, WS3 and WS11 are As-reducing bacteria due to the formation of yellow precipitates which indicate the presence of As (III), while WS9 is As-oxidizing bacteria because the brownish precipitates show the presence of As (V). Therefore, it was concluded that the isolates WS3 and WS11 are capable of producing arsenate reductase enzymes (cytoplasmic arsenate reductase and a membrane-bound or periplasmic respiratory arsenate reductase) while WS9 have the ability to produce arsenite oxidase enzyme (periplasmic arsenite oxidase). Many arsenate-reducing bacteria (or arsenate reducers) capable of reducing As (V) to As (III) have also been identified and classified as *Wolinella Bacillus*, *Sulfurospirillum*, *Desulfomicrobium*, *Citrobacter*, and *Clostridium* (Fan et al., 2008; Silver and Phung, 2005) while arsenite-oxidizing bacteria (or arsenite oxidizers) have been isolated from arsenic contaminated environments and classified as *Pseudomonas*, *Thermus*, *Hydrogenophaga*, *Alcaligenes*, *Achromobacter*, and *Agrobacterium*, among others (Oremland et al., 2002; Salmassi et al., 2002). In general, As(III) is more hazardous to organisms and human than As(V) (Hughes, 2002).

Desorption of arsenic from bacterial biomass by acid washing

1) Effect of hydrochloric acid concentration

Arsenic desorption resulted from bacterial biomass at different concentrations of hydrochloric acid are shown in Fig. 4. With the rise of the acid concentration to 1 M HCl, arsenic in the solution increases to 3.001, 2.820, and 3.271 ppm for WS3, WS9, and WS11, respectively. In addition, desorption of arsenic from biomass may be due to these reasons. On the biomass, the arsenic mainly adsorbs in the form of arsenic-oxygen anions with a negative charge. Firstly, chloride ions compete with arsenic-oxygen, thus releasing the adsorbed arsenic. Secondly, in the acid washing tests, with the increase of the solution acidity, desorption of arsenic has been improved due to acidolysis, making adsorbed arsenic removed from the surface of the biomass. Thirdly, hydrochloric acid makes competitive effects with arsenic-oxygen anions, which make arsenic-oxygen anions formerly complexed with hydrogen of acid (Liu et al., 2014).

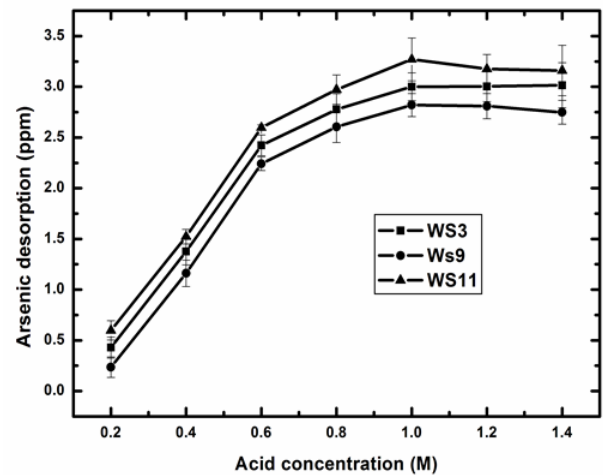


Fig. 4 Arsenic desorption from biomass using different concentrations of acid.

2) Effect of contact time

Washing time is an essential procedure factor in the bioremediation. The experiment examined the effect of time did in the optimum of acid concentration stated in the previous experiment. From Fig. 5, the rates of arsenic desorption increased rapidly within the first hour, then began to be linear, and attained maximum values of 3.241, 2.998, and 3.434 ppm for WS3, WS9, and WS11, respectively. The desorption efficiency was enhanced significantly by increasing the washing time (Alam et al., 2001). After the acid was added to the bacterial biomass, a series of complex physical and chemical reactions occur, such as precipitation, ion exchange, and competitive adsorption (Tokunaga and Hakuta, 2002).

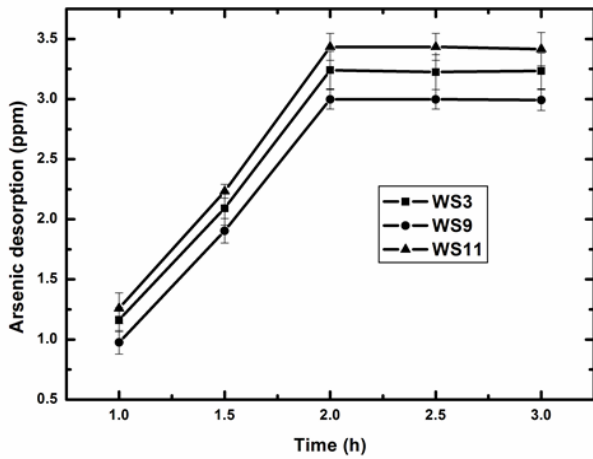


Fig. 5 Arsenic desorption of biomass at various time (h).

3) Effect of bacterial biomass dosage (mg)

The results of desorption of arsenic at different biomass dosages are shown in Fig. 6. The optimum conditions determined in the previous experiment were applied. The optimum biomass dosage was 50 mg with arsenic desorption efficiency of 3.514, 3.299, and 3.606 ppm for all three strains WS3, WS9, and WS11, respectively. When the biomass dosage increases, the arsenic desorption also increases until certain amount after that the arsenic extracted will decrease. After 50 mg of biomass, the arsenic desorption decreases due to the conglomeration of biomass and the acid solution does not have more contact area with biomass particles.

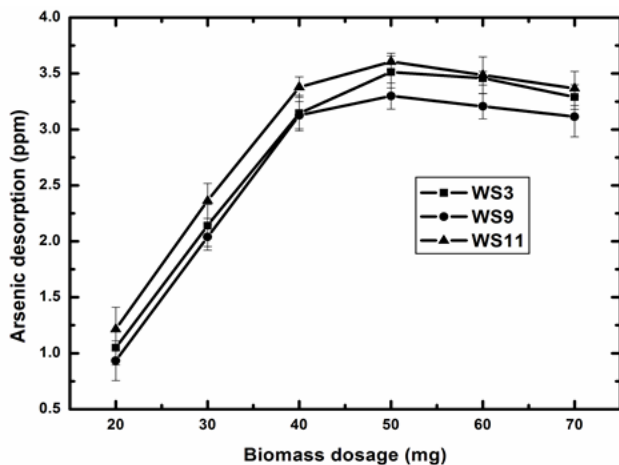


Fig. 6 Arsenic desorption of biomass using different adsorbent dosage (mg).

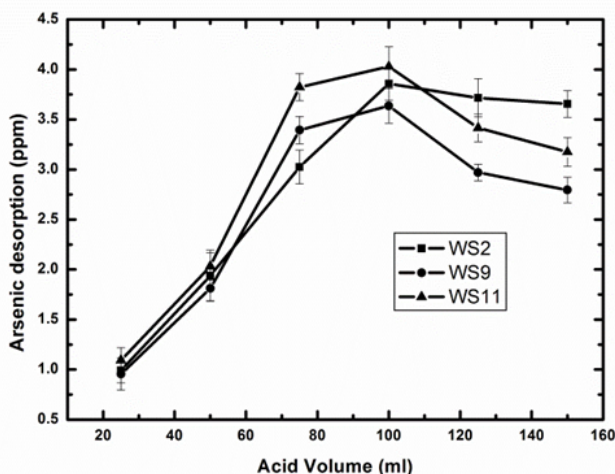


Fig. 7 Arsenic desorption of biomass using the different volume of acid (ml).

4) Effect of hydrochloric acid solution volume (mL)

The optimum concentration of acid solution volume was 100 ml with arsenic desorbed 3.858, 3.637, and 4.030 ppm for WS3, WS9, and WS11, respectively, as shown in Fig. 7. With the increase in acid volume, arsenic desorption increases until certain volume after which no further increase in arsenic removal is observed.

Table 1 Summary of experimental parameters for arsenic desorption from bacterial biomass and optimal values highlight in bold font.

Experiments	1	2	3	4	5	6
HCl concentration (M)	0.2	0.4	0.6	1	1.2	1.4
contact time (h)	1	1.5	2	2.5	3	-
biomass dosage (mg)	20	30	40	50	60	70
HCl Volume (mL)	25	50	75	100	125	150

Removal of arsenite and arsenate before and after acid washing by bacterial biomass

Although many have studied arsenic removal by bacterial biomass for water treatment purposes, only a few have explained pre-treatment of biomass for the sorption reaction. Adsorption of heavy metal by microbial biomass occurs generally due to the different charges between functional groups on the microbe’s surface and the heavy metals ions. Consequently, arsenic is difficult to be adsorbed by microbial biomass because, in a neutral environment, arsenite (III) is present as a neutral oxide (H_3AsO_3) while arsenate (V) is present as negatively charged oxide ions ($H_2AsO_4^-$, $HAsO_4^{2-}$). For the highest arsenic adsorption by bacterial biomass, the cell wall of the bacteria must be activated physically, such as in heat processing, or chemically such as in acid washing (Kapoor *et al.*, 1999; Loukidou *et al.*, 2003). In this study, 5 mg of bacterial biomass of WS3, WS9, and WS11 activated by hydrochloric acid washing enhanced the arsenite removal percentage from 33% to 69%, 30 to 65%, and 38% to 73%, respectively, as shown in Table 2. Moreover, WS11 has the highest arsenite (III) removal (5.857 ppm) after acid washing while the WS9 has the lowest ability for removing arsenite (5.221 ppm) as shown in Table 2.

Table 2 Removing of As (III) (ppm) by bacterial biomass.

Bacteria Strain	Before Acid washing			After acid washing		
	Ce	Removal As (III)	Removal As (III) %	Ce	Removal As (III)	Removal As (III) %
WS3	5.339	2.661	33	2.457	5.543	69
WS9	5.619	2.381	30	2.779	5.221	65
WS11	4.961	3.039	38	2.143	5.857	73

On the other hand, the removal percentage of arsenate (V) increased from 43%, 42%, 44% to 74%, 70%, 78% after acid washing as shown in Table 3 for the three strains WS3, WS9 and WS11, respectively. Likewise, WS11 showed the highest arsenate (V) removal (5.857 ppm) after acid washing whereas WS9 was least able to remove arsenate (V) (5.623 ppm) as shown in Table 3.

Table 3 Removing of As (V)(ppm) by bacterial biomass.

Bacteria Strain	Before Acid washing			After acid washing		
	Ce (ppm)	Removal As (V)	Removal As (V) %	Ce (ppm)	Removal As (V)	Removal As (V) %
WS3	4.598	3.402	43	2.092	5.908	74
WS9	4.645	3.355	42	2.377	5.623	70
WS11	4.479	3.521	44	1.784	6.216	78

The quantity of As (III) (mg) removed per unit of bacterial biomass (g) q_e was calculated using this equation (Altowayti, Allozy, et al., 2019; Haris et al., 2018):

$$q_e = \frac{(C_i - C_e)V}{m}$$

where

C_e : Equilibrium concentration (mg)

C_i : Initial concentration (mg)

m: Bacterial biomass (g)

V: Volume of solution (L)

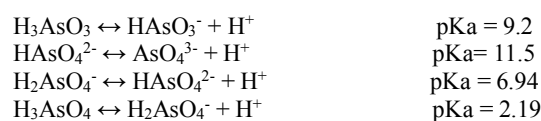
The maximal arsenite (III) loading capacity (q_e) for the WS2, WS9, and WS11 were (11.085), (10.443) and (11.715) mg arsenite/ g bacteria biomass, respectively. Moreover, the ability of one unit of bacterial biomass (g) to remove a quantity of As (V) (mg) were (11.817), (11.245) and (12.431) mg arsenate/ g bacteria biomass for the WS2, WS9, and WS11, respectively.

Moreover, several researchers also reported arsenic and other heavy metal removal through various bacterial biomass and other adsorbents. For instance, two strains known as *Bacillus circulans* and *Bacillus megaterium* could remove 34.5 and 32.0 mg of Cr(VI)/g dry weight, respectively, in 24 h when the initial concentration was 50 mg Cr(VI)/l (Srinath et al., 2002). Similarly, a different study reported 75% of the Cr (VI) ions were removed within 30 min of contact and maximum removal was obtained after 8 h by biomass of *Rhizopus nigricans* (Bai and Abraham, 2001). Furthermore, the maximum adsorption capacity of hematite nanoparticles and aggregates was (2899 ± 71.09 µg/g) for As (III) and (4122 ± 62.79 µg/g) for As(V) at equilibrium (Dickson et al., 2017). Likewise, a different study reported 60.21% of As (III) removal through two gram biomass of shelled Moringa Oleifera seed powder (Kumari et al., 2006). In another study, *Bacillus licheniformis* CC01 and *Acinetobacter calcoaceticus* FN 02, released 44% to 48% of the arsenic (Clausen, 2000). Also, in a study by Miyatake and Hayashi (2009), 0.386 mg of As (III) was removed by *Bacillus megaterium* strain UM-123. More recently, Miyatake and Hayashi (2011) reported 1.870 mg of As (III) was removed by *Bacillus cereus* strain W2.

As a result, the arsenic adsorption capacity (q_e mg/g) of the dried biomass of the three strains (WS3, WS9, WS11) after acid treatment was higher than that of the other adsorbents. Therefore, Pre-treatment of indigenous arsenic-resistant bacterial biomass by acid washing improves the capacity to remove As (III) and As (V) ions from aqueous solutions.

The increased removal of arsenite and arsenate by acid washing may be attributed to desorption of arsenic from the bacteria cell walls that may attach during the growth and increase the availability of the binding site. Loukidou et al. (2003) and Seki et al. (2005) identified the availability of different functional groups at different pH values for binding of metals. Hence, some biomass show a preference for specific heavy metals while others have broad range due to the fact that they do not exhibit any specific binding (Gupta et al., 2000). The desorption of arsenic occurred with a decrease in pH due to the

suitable ionic state of binding ligands and metal ion species (Miyatake and Hayashi, 2009; Wu, et al., 2010). The increase in pH will reduce gradually the rate of protonation. Therefore, at low pH (acidic conditions) arsenite (III) and arsenate (V) will be expelled from the surface of biomass due to increasing rate of protonation as shown in these equations:



Desorption of other heavy metals such as cadmium and lead using acid solutions has been reported in other studies (Chang et al., 1997; Chojnacka et al., 2005; Puranik and Paknikar, 1997).

CONCLUSION

Bio-treatment is an important method for the treatment of heavy metals such as arsenic from wastewater resulting from natural or anthropogenic sources. The highest desorption of arsenic from the bacterial biomass were 3.858, 3.637, and 4.030 ppm for WS3, WS9, and WS11, respectively, at optimum conditions. In this study, arsenic-resistant bacteria WS3, WS9, and WS11 showed different affinities and capacities to remove arsenite and arsenate from aqueous solution. Moreover, the ability of WS3, WS9, and WS11 to remove arsenite and arsenate increased after acid washing plausibly due to the removal of adsorbed arsenic from the cell wall. Consequently, acid washing of bacterial biomass as a pre-treatment step is highly recommended prior to arsenic removal from wastewater. Biomass regeneration is also important for the practical applicability of the biomass when wastewater treatment is considered.

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