dx.doi.org/10.22093/wwj.2021.314968.3196

Original Paper

Journal of Water and Wastewater, Vol. 32, No. 5, pp: 61-69

New Isolated Extremophiles Arsenic Oxidizing Bacteria for the Removal of Arsenic from High- and Low-COD Media

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(Received Nov. 13, 2021 Accepted Dec. 21, 2021)

To cite this article:
Fatemi, A. Z., Tabatabaei, M., Derakhshandeh, A., Borghei, S. M., Noshadi, M. 2022. "New isolated extremophiles arsenic oxidizing bacteria for the removal of arsenic from high- and low-COD media" Journal of Water and Wastewater, 32(5), 61-69. Doi: 10.22093/wwj.2021.314968.3196.

Abstract

There is an urgent need for the removal of arsenic (As) from groundwater and wastewater as it is a very hazardous heavy metal for human and environmental health. In this research, As-resistant and oxidizing bacteria were isolated from the Maharloo Lake (27 km southeast of Shiraz city) and identified to a great extent. Three isolated bacillus-shaped strains (called F5, F6 and F7) tolerated up to 1 M $AsNaO_2$, grew up to 3.5 M NaCl and pH 12, and consumed NaSCN and $Na_2S_2O_3$. The molecular analysis confirmed the originality of the strains to a high extent. The As absorption rate by these bacteria was measured by the atomic absorption method, and their effect was examined on a water sample from the south of Kerman city (Iran) and a synthetic wastewater sample with a chemical oxygen demand (COD) of about 180,000 kg/m^3 that was able to absorb high levels of arsenic.

Keywords: Extremophile, Arsenic, Industrial Wastewater, Alkaliphilic, Maharloo Lake.

1. Introduction

Arsenic (As, atomic number 33) is a chemical that can combine with some other chemicals such as sulfur (Arsenic and Compounds, 2001). A variety of allotropes observed for As are often gray in the industry (Irgolic, 1982).

The presence of As in the environment causes many problems for human health. This metal can enter the body through the skin, digestive tract, and respiratory system and induce short-time effects in the individual

and irreversible consequences in the next generation (Banaaraghi et al., 2010).

It is found in the soil as insoluble sulfide and sulfur compounds, such as Tennantite, Realgar, Lollingite, and Arsenopyrite (Elangovan and Chalakh, 2006).

Even though it is naturally present in the earth's crust, its levels have increased by anthropogenic activities, including excessive use of pesticides, herbicides, additives for wood preservation, and pharmaceuticals, leading to environmental contamination and toxicity (Mandal and Suzuki, 2002).

The most common natural forms are arsenic [V], arsenate and arsenic [III], and arsenite. Arsenite can bind to sulfhydryl and dithiol groups in proteins (Adeniji, 2004).

Arsenate has the ability to bind to phosphorus and can block oxidative phosphorylation. As can cause lung, kidney, liver, respiratory tract, and prostate cancers (Preiss et al., 2015).

The World Health Organization has determined an As level of 0.01 mg/l in drinking water, which is followed by most industrial countries. Accordingly, As removal is one of the most critical and essential issues for human communities. Arsenate and arsenite are both toxic but the latter is far more toxic, and both can induce cellular damage in the human body (Achour et al., 2007).

The arsenate ion and inorganic phosphate are structurally similar, hence, arsenate can enter the cell membrane through the phosphate transfer system and inhibit phosphorylation-demanding metabolic reactions and ATP synthesis (Shrestha et al., 2008).

The common techniques, such as chemical precipitation, chemical reduction/oxidation, ion exchange, filtration, and reverse osmosis, which are used for the removal of heavy metal ions from dilute solutions, are typically expensive and inefficient (Nriagu, 2002).

Therefore, there is currently a tendency toward biofiltration, in particular, the use of microorganisms that are both highly potent and are new genotypically and phenotypically. The first report on As oxidation by heterotrophic and autotrophic bacteria was published in 1918 (Paul et al., 2015).

As oxidation can be involved in the growth mechanisms of heterotrophic bacteria, this role is more important than their detoxification. Under standard conditions, As oxidation is a thermodynamic ion-exchange reaction and can provide sufficient energy for the growth of chemolithotrophic microorganisms (Green, 1919).

Extremophilic bacteria are microorganisms with a high ability to tolerate harsh conditions and environmental stresses. They inhabit craters, ice rocks, acidic and alkaline lakes, hypersaline environments, oceans, and heights; therefore, they have high adaptability to harsh environmental conditions. Bacteria play an important role in the As biochemical cycles in the environment (Sani and Rathinam, 2018).

Extremophiles include animals, plants, insects, fungi, and bacteria. Extremophilic bacteria are divided into acidophilic (growing in a low pH), alkaliphilic (optimum growth in a high pH), halophilic (growing at a high salt concentration), thermophilic (optimum growth at > 80°C), psychrophilic (optimum growth at low temperatures), barophilic (optimum growth at high pressures), oligotrophic (growing in environments with minimal nutrients), endolithic (growing on rock and rocky spaces), and xerophilic (optimum growth in arid regions) groups. Moreover, there are other groups of microorganisms that can tolerate more severe conditions than the mentioned ecological environments (Dumorné et al., 2017).

Due to their special features, these microorganisms are appropriate choices for researchers in industrial applications (Upadhyay et al., 2018; Yang and Rosen, 2016).

Since these bacteria live in unnatural environments, including volcanoes, the Dead Sea and generally salt lakes, heavy metal-contaminated soils, and Arctic ices, they have strongly acclimatized to these environments by changing their metabolism based on living in such conditions and can provide their vital and nutritional requirements (Cavalca et al., 2013; Dey et al., 2016).

Alkaliphiles are a class of extremophiles that can survive in alkaline pH (8-11), with optimum growth at pH 9 (Sorokin et al., 2013).

They grow in saturated salt lakes, insect intestines, hydrothermal vents, deep-sea sediments, and carbonate-rich soils (Preiss et al., 2015).

As can exist in different oxidation states with a variety of toxicities and solubility levels. To survive in environments containing heavy metal ions, bacteria have different mechanisms to resist this constraint (Sowmya et al., 2014).

These bacteria either use As in their metabolism or utilize it as the final electron acceptor in the aerobic respiration pathway (Lee et al., 2005).

These microorganisms are widely dispersed in the environment, and heavy metal-contaminated soils and waters are the best sites for the isolation of these strains (Shrestha et al., 2008; Yang and Rosen, 2016).

In this research, As- and S-oxidizing bacteria were isolated and identified from the water and sediments of Maharloo Lake. This lake, also known as the Salt Lake, is a seasonal salty lake located 27 km Southeast of Shiraz, Iran. It is rich in potassium salt and contains considerable amounts of other salts. One of its origins is a dry river in the city of Shiraz that supplies water to the lake bed in intense rainfalls and water overflow. The lake water usually evaporates in summer and completely exposes its white bed due to the presence of salts. In midsummer, the color of the lake is seen as reddish dark

 dx.doi.org/10.22093/wwj.2021.314968.3196

pink due to a high evaporation rate, high salt concentrations, and as a result of tides.

2. Materials and Methods

Samples were isolated from the surface sediments of the Maharloo lake. The pH of the samples was in the range of 9.4-10.5.

2.1. Culture medium composition

A final Na ion concentration of 1 M was used in the culture medium with a pH of 11. The culture medium composition and the amounts of the ingredients were as: Na_2Co_3 (30 g/l), NaCl (60 g/l), $NaHCO_3$ (8 g/l), K_2HPO_4 (1 g/l), and KNO_3 (0.5 g/l). $AsNaO_2$ as an energy source was added to the culture medium. Organic carbon was not added at the first stage of isolation.

The sediment (1%) was added to the medium and then incubated at 120 rpm at 37 °C for 7 days. 10 × serial dilutions were prepared from the liquid medium and inoculated onto the agar-containing media for isolation of colonies.

To prepare the agar containing solid medium, 4% agar solution was first autoclaved and then added to this basal salty alkaline medium due to the alkalinity of the agar medium. The permissible level of adding Na ion to this medium is 2 M, and a greater level leads to its deposition in the medium. This culture medium provides the conditions for the isolation of Asoxidizing halophilic bacteria.

After the specification of the colonies, 1% yeast extract was added to the agar-containing solid medium to isolate facultative chemolithotrophic bacteria.

2.2. Qualitative testing

The isolated colonies were separately cultured on the agar containing medium and 2 ml of 1.5% AgNO3 solution was added to the colonies after the growth of bacteria. After 2 h, brown-colored plates were introduced as As oxidizing ones.

Due to possible color interference and detection error because of a high salt concentration in the culture medium, qualitative testing was done in 96-wells ELISA microplates (Simeonova et al., 2004).

To do this, a liquid culture was first prepared from the bacteria and then centrifuged in 5000 rpm for 15 min. Wells were washed twice with 3% NaCl solution.

The Tris-HCl buffer in a pH range of 7-10 was added to the microplates to optimize the pH. The washed bacteria were seeded into the microplate wells; 6 ppm of $AsNaO_2$ was poured into wells, and incubated for 48 h. This was followed by adding 1.5% AgNO3 to each well. As oxidizing bacteria were then isolated by colorimetry and a standard color protocol.

After 48 h, bacteria from individual microplate wells were cultured in solid plates to examine their viability.

2.3. Biochemical tests

The sugar use, motility, indole, MR-VP, hydrolyzing of gelatin, nitrate, oxidase, and catalase tests were conducted here. Besides, different concentrations (1-2 mm) of $AsNaO_2$ ions were included in the culture medium. The strains were also examined in terms of resistance to potassium dichromate ($K_2Cr_2O_7$), sodium sulfide (Na_2S), and potassium thiocyanate (KSCN).

2.4. Amplification and sequencing of *16S rRNA gene*

For the molecular analysis, DNA was extracted by the phenol-chloroform method (Salto and Mivra, 1963; Saitou and Nei, 1987).

The *16S rRNA* gene was replicated by polymerase chain reaction (PCR) using specific primers: 5′-AGAGTTTGATCCTGGCTCAG-3′ (forward) and 5′-ACGGCTACCTTGTTACGACTT-3′ (reverse). The PCR product was sequenced by Macrogen Company (South Korea) and the result was analyzed online by NCBI and using the Bioedit software.

2.5. As absorption test by bacteria

As absorption rate was examined in the selected strains, which were individually cultured in a medium containing 10 ppm of $AsNaO_2$, and after incubation, centrifuged at 5000 rpm for 15 min. As level in the supernatant was measured by the atomic absorption method and compared with that before inoculation and the bacterial culture. The bacterial consortium was also examined in terms of As absorption by the method mentioned above.

2.6. Sonication for ensuring the uptake of arsenic by bacteria

An experiment was performed as follows to prove the uptake of arsenic by bacteria from the medium. A liquid bacterial culture medium was first prepared. Additionally, 100 ml of the medium containing 7 ppm of sodium arsenite was poured into 500 ml flasks, to which a mixture of three bacteria with a volume equal to 1% of the culture medium volume was added. The procedure was repeated three times (A1, A2, and A3) to ensure the results. Further, the samples were incubated at 120 rpm for 5 days at 37 °C.

Then, 25 ml of each sample was centrifuged at 5000 rpm for 15 min at ambient temperature, and supernatant was used for atomic absorption analysis. The residual sediment bearing bacterial mass was washed three times with 3% NaCl solution, and washed cells were prepared for sonication. Following sonication at 14 × 30 s-pulses for the complete cell lysis, the centrifuge was carried out at 5000 rpm for 15 min at ambient temperature, and the supernatant was applied for atomic absorption analysis.

To ensure the complete cell lysis, the sediment was plated onto the solid culture medium.

2.7. Synthetic industrial wastewater

A combination of various mineral salts was prepared similar to industrial wastewater in terms of As sulfur compounds and sugar-free 5% beer without alcohol was added to increase chemical oxygen demand (COD) up to 180,000 Kg/m^3. COD was measured by a COD meter. The bacterial consortium sample was cultured therein, followed by examining the absorbance level.

A water sample containing about 40 ppm of As was also taken from a village in the south of Kerman province. A basal mineral culture medium was prepared using this water sample instead of distilled water. No organic compound was added to this mineral composition and it had very low COD. The bacterial consortium was added to this culture and the absorbance was measured as described above. The consortium in the mineral medium with 1% yeast extract was incubated at 120 rpm at 37 °C for 10 days. Samples were taken every day, centrifuged at 5000 rpm for 15 min, and the supernatant was examined for As levels. Initially, 10 ppm of $AsNaO_2$ was added to the culture medium.

2.8. A graph showing the amount of arsenic absorbed each day dependent on CFU

A group of three bacteria was cultivated and introduced to a medium containing a 16 ppm sodium arsenite mixture. To examine arsenic intake during ten days, the colony count technique was used to determine CFU daily.

Table 1. The results of biochemical tests of three isolates

Characteristic	F7	F5	F6
MR	-	-	-
VP	-	-	-
Indole	-	-	-
Motility	-	-	-
Nitrate reduction	+	+	-
Hydrolysis of Gelatin	-	-	-
Sugers fermentation:			
• Xylose	-	-	+
• Fructose	-	-	-
• Inolin	-	-	-
• Lactose	-	-	-
• Maltose	-	-	-
• Arabinose	-	-	+
• Saccharose	-	-	+
• D-Maltose	-	-	+
• Melezitoze	-	-	-
• Glocose	-	-	-
• Galactose	-	-	-
• Melibiose	-	-	-
Oxidase	+	+	+
Catalase	+	+	+
Consumption of:			
✓ Na_2S	+	+	+
✓ KSCN	+	+	+
✓ Na_3AsO_4	+	+	+
✓$NaAsO_2$	+	+	+
pH	7-12	7-12	7-12
NaCl	3-20%	3-20%	3-20%
Shape	Rod	Rod	Rod
Gram staining	Positive	Positive	Positive

3. Results

A total of 150 isolated bacterial strains initially tolerated up to 200 mM of As, and three strains could tolerate up to 600 mM of As. All the 150 strains could grow in culture media containing $AsNaO_2$ and NaH_2AsO_4. Three isolated strains were facultative chemolithotrophic bacteria, i.e., they were grown well in a medium containing organic carbon, and the incubation time was lowered from 7 days to 48 h.

Three isolated strains, named F1, F2, and F3, could grow up to 600 mM of $AsNaO_2$ and all were bacillus-shaped, Gram-positive, and non-motile, capable of growing in a medium with 3.5 mM of NaCl and pH 12 (Table 1).

3.1. Molecular analysis and homology with similar strains

F1 shows 95.74% homology with *Bacillus cellulosilyticus*. F2 has 94.71% homology with *Bacillus cellulosilyticus*. F3 displays 98% homology with *Saipaludibacillus aurantiacus* (Fig. 1).

3.2. Qualitative oxidation test on microplates

The bacterial consortium presented a darker brown color and higher oxidation is definitely expected for the combination of three bacteria. The bacterial oxidation rate and viability were higher in the Tris-HCl buffer with a pH of 9.

As expected, the bacteria grew better in microplates as they were alkaliphiles (Fig. 2).

3.3. Arsenic absorption test by individual bacteria and theirconsortium

Table 2 compares individual bacteria and their consortium, indicating that the consortium generally presented much better results.

3.4. Synthetic industrial wastewater

The bacterial consortium grew excellently in this high COD and the atomic absorption analysis indicated that about half of the added As was absorbed by the bacterial inoculum.

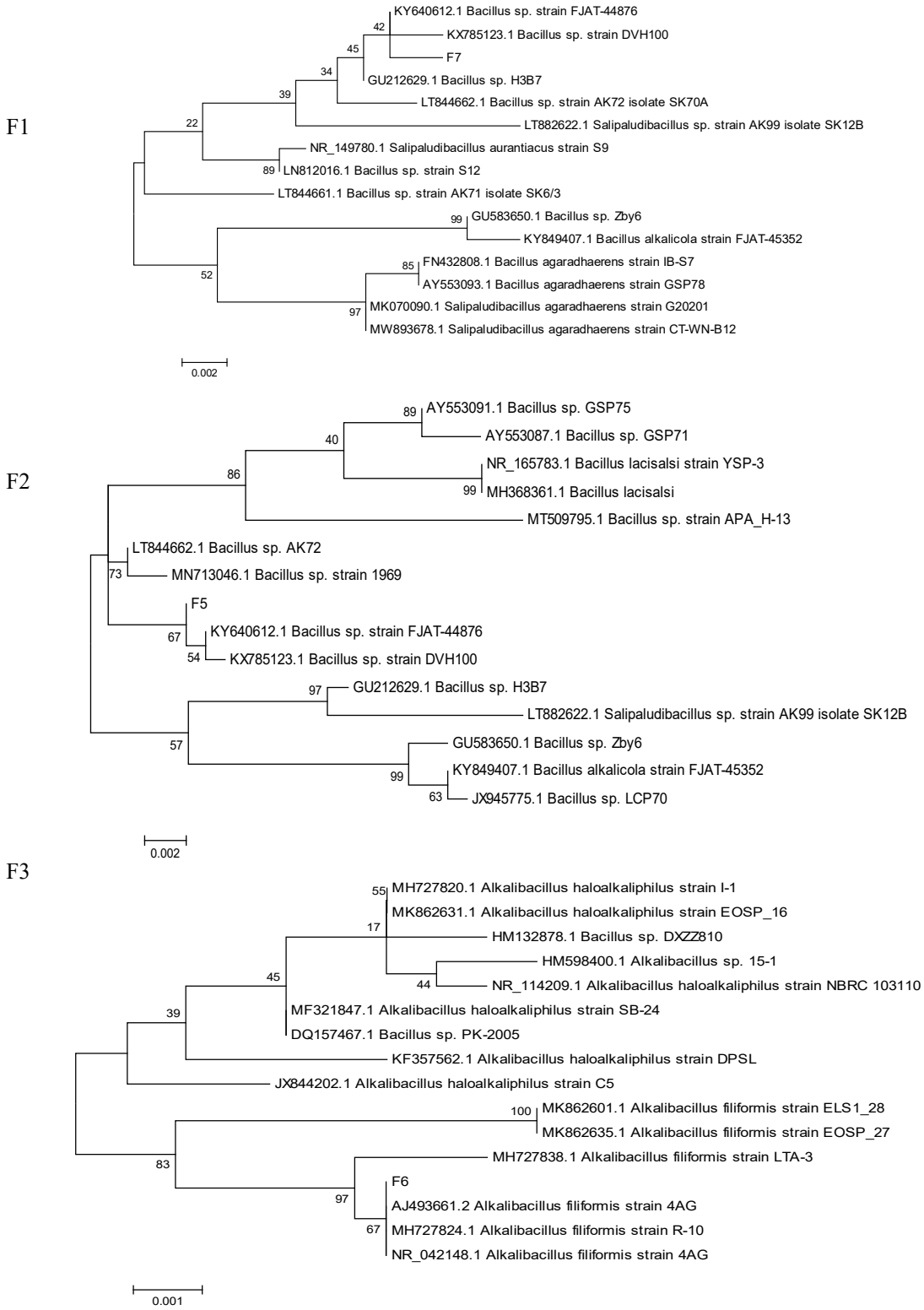

Fig. 1. Phylogenetic tree; phylogenetic analysis based on 16S rDNA sequences. All strains obtained in this study and sequences available in NCBI database were used after multiple alignment by clustal W. Distance and clustering with the neighbor-joining method were performed using the MEGA version 6 software package. Bootstrap values based on 1000 replication are given as percentage at the branching points. Bar, 0.02 nucleotide substitutions per nucleotide positions

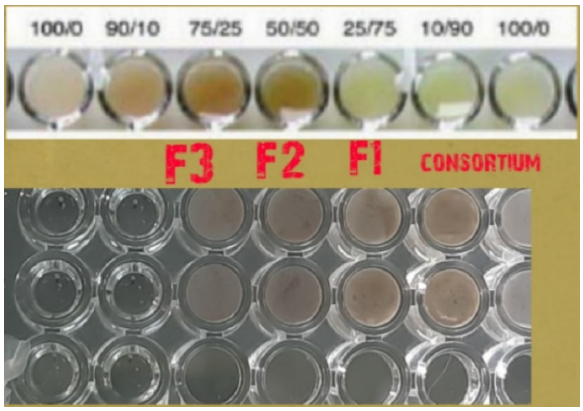

Fig. 2. Quantitative test for oxidation of sodium arsenite in microplates

Table 2. As removal rate (ppm)

Bacterial strain	F1	F2	F3	Consortium
As removal rate (ppm)	3.2	2.1	0.8	5

3.5. The water sample from the south of Kerman

With increasing incubation time, the absorption of arsenic by the bacterial consortium increased so that after 5 days' incubation and aeration, it showed the highest uptake (Table 3).

The bacterial consortium had the highest absorption on the fifth day of incubation and aeration, after which the bacteria entered the death phase due to fermentation in a batch medium (Table 4). By the 10^{th} day, the As level returned to the baseline value due to the disintegration of bacteria.

3.6. Sonication results

As shown, an average of about 5.2 ppm of arsenic remains, which indicates the uptake of 2.4 ppm of arsenic by bacterial mass (Table 5).

The results related to the atomic absorption of the sonicated solution are represented in Table 6, which

Table 3. As removal rate (ppb)

Bacterial strain	F1	F2	F3	Consortium
As removal rate (ppb)	20	30	30	Unmeasurable

Table 4. Arsenic absorption in 10 days

Sampling days	1	2	3	4	5	6	7	8	9	10
Removal rate (ppm)	1.6	1	1	5.2	0	0	0	1.4	1.5	1.6

Table 5. Summarizes the arsenic content of the samples after atomic absorption measurement

	Blank	A1	A2	A3
Arsenic content (ppm)	7.6	5.8	4.9	5

Table 6. Arsenic absorption by sonicated bacteria

	A1	A2	A3
Arsenic content (ppm)	1	0.5	1.5

demonstrates that around 1 ppm of arsenic was averagely absorbed by the bacterial mass of course, experimental and equipment errors are considered. In addition, a portion of lysed cells and arsenic may be lost during washing and three centrifuges. Further, no bacteria grew after the 72 h incubation on the solid medium in which the post-sonicated cells were cultured, which reflects the complete lysis of the cells.

3.7. A graph showing the amount of arsenic absorbed each day dependent on CFU

Atomic absorption spectrophotometry was used to determine the daily arsenic absorption levels, and then the maximum absorption has been determined to be five ppm on the fourth day. One of the bacteria in the culture media took over on the fifth day, and the quantity of arsenic in the medium began to rise. In this case, it meant that two additional bacteria had reached the death/lysis stage and that arsenic content had been discharged into the surroundings. The investigation was carried out in a batch context, which is important to mention (Fig. 3, Table 7).

4. Discussion

The designed living mechanism of extremophilic bacteria for survival in harsh environmental conditions makes them an excellent candidate for

Table 7. Daily intake of arsenic based on CFU

Time (h)	CFU	Arsenic absorption (ppm)
24	3×10^8	1.8
48	25×10^8	2
72	20×10^8	2
96	3×10^8	5
120	2×10^9	2
144	2×10^9	1
168	10^9	1
192	5×10^9	3
216	7×10^9	3
240	10^9	1

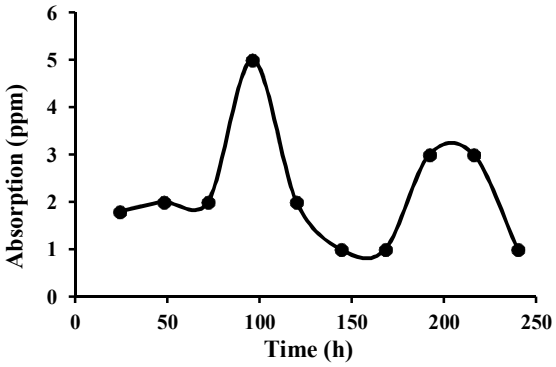

Fig. 3. Daily intake of arsenic based on CFU

industrial applications, with heavy metal contaminations, acidity, alkaliphilic conditions, and very high salinity.

These microorganisms can be algae, protozoa, archaea, or bacteria. In new approaches of biology and environmental engineering, there is an increasing interest in archaea and bacteria due to higher proliferation rates and limited nutritional needs. Another important point in the use of these microorganisms is related to semi-industrial and industrial phases and using bioreactors and fermenters. This is because they require severe growth conditions in terms of environmental factors, which in turn resolves the problem of fungal and other contaminants that are mostly bacilli spores, thereby spontaneously creating sterile conditions.

Upadhyay et al., published a review study on plant growth promoting microorganisms (PGPMs) including algae, fungi, and bacteria that promote the growth of plants. PGPMs can transform arsenic through methylation or other changes and reside in the rhizosphere of rice roots. The above changes prevent the absorption of arsenic by plants roots (Upadhyay et al., 2018).

Yang and Rosen, published a review study where arsM (encoding the arsenite (III) S-adenosylmethionine methyltransferase), arsl (encoding band lyse c-AS), and arsH (encoding methyl arsenite oxidase in bacteria and promotes the resistance of arsenic against bacteria) were investigated (Yang and Rosen, 2016).

Karn et al., used arsenic-oxidizing bacteria to transform arsenic (III) to Arsenic (V) in soil (ArsenicV has lower toxicity compared to Arsenic III). In addition, they used $FeCl_3$ to support the growth of bacteria (from pseudomonas and vibrioacromobacter strains) (Karn et al., 2017).

In the review study published by Cavalca et al., the potential impact of bacteria on the transformation of arsenic varieties and their role in water treatment was investigated (Cavalca et al., 2013).

Pazirandeh et al., studied the resorption of cadmium and mercury by investigating the expression of a metal binding motif in *E. coli* (Pazirandeh et al., 1998).

Bhakta et al. used their method to investigate the rate of absorption for arsenic and cadmium in *Exiguobacterium*, *Acinetobacter, Pseudomonas*, and *Planococcus* strains (Bhakta et al., 2014).

Numerous studies have been conducted on separating and identifying oxidizing and arsenic-resistant bacteria. However, extremophiles were investigated in the current study since they tolerate harsh environmental conditions. In addition, their application in the industry is more economical and practical since their growth environment prevents the growth of microorganisms that contaminate cultures. Furthermore, the unique characteristic of these bacteria is that they grow in very high and very low CODs and can absorb and oxidize arsenic.

In this study, 150 As oxidizing halophilic, alkaliphilic bacterial strains were isolated from the Maharloo Lake, three of which grew in 600 mM of $AsNaO_2$ and were identified as original in the preliminary molecular test. These bacteria could oxidize sulfur. Owing to these characteristics and high tolerance to heavy metals and salts, they are good candidates for industrial wastewater and Ro reject water with high pollution and excessive alkaline conditions. Since the bacteria had a high ability to grow in an elevated COD, they are excellent candidates as biological adsorbents in industrial, urban, and veterinary wastewaters, as well as generally all effluents with high COD.

5. Authors' contributions

This research was conducted as a part of a doctoral dissertation by Azadeh Zahra Fatemi, a Ph.D. candidate in the Faculty of Veterinary Medicine, Shiraz University, supervised by Mohammad Tabatabaei and Abdollah Derakhshandeh. First draft of themanuscript was written by AZF and edited and evaluated by MT. All the authors have read and approved the final version.

6. Acknowledgments

Most of this work was done in the Pathobiology Department of the Veterinary Faculty, University of Shiraz, and partly in the Biochemistry and Environmental Engineering Department of the Sharif University of Technology. The authors are grateful to all personnel in different departments of the two universities, in particular the biotechnological bacteriology laboratory (University of Shiraz) and the microbiology, water and wastewater, nanotechnology, and fermentation laboratories (Sharif University of Technology).

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