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# ISOLATION, CHARACTERIZATION, AND POTENTIAL APPLICATIO N OF ARSENITE-OXIDIZING HYDROGENOPHAGA PSEUDOFLAVA FOR THE TIGRIS RIVER WASTEWATER BIOREMEDIATION

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**Abstract:**

The Tigris River is one of the most significant rivers in the Middle East, flowing through several countries and playing a vital role in the region's history and development. Trace element contamination in water bodies like the Tigris River can have significant effects on the bacteria present in the ecosystem. Bacteria play crucial roles in nutrient cycling, organic matter decomposition, and overall ecosystem health. This study aimed to ISOLATED from metal-contaminated Tigris River, the bacterium Hydrogenophaga pseudoflava has been demonstrated to be resistant to a wide range of heavy metals. This discovery points to a potential use for the bacteria in the bioremediation of industrial effluents. As(III) concentrations as high as 3 mg ml-1 were tolerated by H. pseudoflava, as were Cu2+ concentrations of 0.7 mg ml-1, Hg2+ concentrations of 0.4 mg ml-1, Ni2+ concentrations of 0.4 mg ml-1, and Cr6+ concentrations of 0.5 mg ml-1. H. pseudoflava flourished best at a pH 7 and 30°C temperature range. After optimum incubation for 24, 48, and 72 hours, H. pseudoflava was able to oxidize 42% (42 g mg-1 of protein), 78% (78 g mg-1 of protein), and 95% (95 g mg-1 of protein) of As(III) from the medium. Arsenite oxidizing capability of H. pseudoflava suggests its potential application in the biological treatment of arsenic-contaminated wastewaters.

**Keywords:** Arsenic, wastewater, Hydrogenophaga pseudoflava bioremediation.

## INTRODUCTION

Arsenic is at the top of the superfund list because it is the most widely distributed harmful element in the environment. In many nations throughout the world, arsenic pollution of drinking water is a major municipal health issue (Liu et al., 2009). Hughes (2002) and Shi et al. (2004) both confirm that arsenic may cause cancer in humans. Arsenic poisoning manifests as skin lesions and mucous membrane damage, as well as harm to the gastrointestinal, respiratory, cardiovascular, and nervous systems. Cancers of the skin, liver, and lungs have also been linked to it (Wang et al., 2001). Therefore, the World Health Organization suggests a temporary arsenic limit of 10 ppb in drinking water.

The findings of genotoxicity experiments showed that arsenic is mutagenic, since it causes chromosomal abnormalities and sister chromatid exchanges when it is present during DNA replication. DNA damage, changes in DNA methylation, downregulation of p53 and telomerase (Chou et al., 2001; Wang et al., 2001), oxidative stress, cell proliferation induction, and activation of transcription factors (Wu et al., 1999) are all possible outcomes. DNA damage caused by arsenic has been demonstrated (Matsui et al., 1999) to occur as a result of the creation of reactive oxygen species.

Some of the traditional techniques for removing metals from industrial effluents include chemical precipitation, oxidation or reduction, ion exchange, filtration, electrochemical treatment, reverse osmosis, membrane technologies, and evaporation recovery (Kuan et al., 2009). When the metals in solution are on the order of 1 to 100 mg l-1, these procedures may be inefficient or prohibitively costly (Nourbakhsh et al., 1994). As a result, the creation of a cutting-edge, economically viable, and ecologically sound method for purifying water of metals is urgently required.

Despite the presence of large amounts of heavy metal, several microbial species are able to thrive (Rehman et al., 2007). Anderson and Cook (2004) detailed the cultivation of bacteria able to tolerate high concentrations of arsenic

species (up to 100 mM arsenate or up to 20 mM arsenite), including *Aeromonas, Exiguobacterium, Acinetobacter, Bacillus, and Pseudomonas*. The genus Acidithiobacillus, the genus Bacillus, the genus Deinococcus, the genus *Desulfito bacterium,* and the genus Pseudomonas (Ahmed and Rehman, 2009; Dopson et al., 2001). Since heavy metals are so prevalent in the environment, microorganisms have developed strategies to survive in their presence (White and Gadd, 1986). Isolation and characterization of arsenic-resistant bacteria capable of oxidizing arsenite from a contaminated environment, and assessment of their use for metal detoxification and environmental bioremediation.

## MATERIALS AND METHODS

### Sample collection and analysis

Samples of industrial effluent were taken from (Insert Site Name Here) in screw-capped, sterile vials. Wastewater was tested for many physicochemical characteristics in accordance with APHA (1989), including temperature, pH, dissolved oxygen, and arsenic content.

### Isolation of arsenite resistant bacteria

To isolate arsenic-resistant bacteria, 100 µl of wastewater sample was spread on Luria-Bertani (LB) agar plates with 100 µg As(III) ml-1. LB agar plates were prepared by dissolving 1 g NaCl, 1 g tryptone, and 0.5 g yeast extract in 100 ml distilled water, pH adjusted to 7-7.2, and adding 1.5 g agar to a 250-ml fla For 15 minutes at 121 degrees, the medium was autoclaved. Incubation at 30°C for 24 hours produced bacterial colonies. As (III)'s impact on bacterial growth was examined in acetate minimal medium (Pattanapipitpaisal et al., 2001) with (g/l): NH4Cl, 1.0; CaCl2.H2O, 0.001; MgSO4.7H2O, 0.2; FeSO4.7H2O, 0.001; sodium acetate, 5; yeast extract, 0.5; K2HPO4, 0.5 (pH 7) supplemented with NaH2AsO3 (100 µg ml-1). The temperature stayed at 30°C for 24 hours. This procedure was performed with 0.1, 0.2, 0.3, and 3 mg ml-1 arsenite to determine the bacterium's MIC. The experiments were repeated to verify dependability.

Simeonova et al. (2004) describe the AgNO3 arsenite-oxidizing bacteria test. Agar plates with arsenite-resistant bacterial isolates were flooded with 0.1 M sodium nitrite (AgNO3) after 48 hours at 30 degrees Celsius. The medium contained a brownish arsenate precipitate (Lett et al., 2001).

### Bacterial Isolate Identification

Molecular identification was performed using Carozzi et al. (1991) genomic DNA extraction. According to Rehman et al. (2007), primers RS-1 (5'-AAACTCAAATGAATTGACGG-3') and RS-3 (5'-ACGGGCGGTGTGTAC-3') were used to amplify the 16S rRNA gene by PCR. The PCR was completed after a 5-minute denaturation at 94 degrees Celsius, 35 cycles of annealing at 55 degrees Celsius for 1 minute, extension at 72 degrees Celsius for 2 minutes, and a 5-minute extension at 72 degrees Celsius. The gel-cut 0.5kb PCR product was cloned into the pTZ57R/T vector. A fermentas purification kit (#K0513) purified 16S rRNA gene amplicons, as shown by electrophoresis on 1% agarose gels. The genetic study was done using a Beckman Coulter Inc. Model CEQ-800 Genetic study System in Fullerton, California. 16S rRNA gene sequences were compared to GenBank sequences to find the closest match.

### Determination of optimum growth conditions

Both temperature and pH were taken into account to ensure the bacteria thrived. Five milliliters of LB broth were introduced to three autoclaved test tubes in a four-set design to determine the optimal temperature for overnight growth of a bacterial isolate culture. The four groups of tubes were incubated at temperatures of 25, 30, 37, and 45°C. Their absorbance at 600 nm was determined using a LAMBDA 650 UV/Vis spectrophotometer (PerkinElmer, USA) after 12 hours of incubation. In order to find the ideal pH, 9 sets of 3 test tubes each were made using LB broth with pH adjusted to 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0, and then autoclaved. Twenty microliters of a newly prepared culture of the bacteria was used to inoculate these tubes. Their absorbance at 600 nm was determined after 12 hours of incubation.

### Effect of arsenite on bacterial growth

We measured the growth of a bacterial isolate in acetate minimum medium supplemented with 0, 0.5, 1, 2, and 3 mg ml-1 of arsenite. One set of five flasks containing 50 ml medium was used for the bacterial isolate; the flasks were autoclaved, and 20 l of the new inoculums were added. The cultures were kept in a 30°C incubator with a 100 rpm shaker. At 0, 4, 8, 12, 16, 20, 24, 28, 32, and 36 hours, a sample of the culture was obtained to determine the absorbance at 600 nm.

### Resistance to heavy metal ions

Stock solutions of 10 mgml-1 of several metal salts (cadmium chloride, copper sulphate, potassium dichromate, mercuric chloride, and nickel chloride) were used to determine the bacterial isolates' cross heavy metal resistance. To test for cross-resistance, we used a minimum medium consisting of acetate and progressively higher concentrations of each metal, starting at 100 g ml-1. Twenty microliters of an overnight bacterial culture were added to a medium and metal ion-filled culture flask for a 24-hour incubation at 30 degrees Celsius. Optical density at 600 nm was used to evaluate development.

### Arsenic oxidation by bacterial isolate

As (III) at a concentration of 100 g ml-1 was added to the acetate minimum medium to test the bacterial isolate's capacity to oxidize arsenite. As (III) was also grown in a medium devoid of bacteria but having the same concentration as the treatment medium (100 g ml-1). After 24 hours incubation at 30 degrees Celsius, the cells were collected by centrifugation at 14,000 revolutions per minute, washed twice in 50 millimeters of phosphate buffer (pH 7), and resuspended in 5 milliliters of the same buffer. Sonication (Heilscher Ultrasonic Processors UP 400, S) was

used to disrupt the cells at 4°C for 15 seconds before they were centrifuged at 14000 rpm for 1 hour, also at 4°C. The membrane fraction is the pellet after being resuspended in 50 mM phosphate buffer (pH 7), while the soluble fraction is the supernatant. To measure arsenite oxidase activity, we added 2 mM phenazine methosulfate (PMS) to 50 mM phosphate buffer (pH 7) containing 200 M As(III) at a suitable time period (Anderson et al.,1992). After hydrolyzing the cells in 0.4 N NaOH at 100°C for 10 minutes, the protein content was calculated using the Lowry technique (1951). The gold standard was bovine serum albumin.

**Figure 1.** Shows 0.1 M AgNO3 was added to an agar plate enriched with NaH2AsO3 (100 g ml-1), a brownish precipitate appeared in which H. pseudoflava had been growing..

### Statistical analysis

All tests were performed in duplicate and data was collected. It was common practice to keep at least three individual flasks going at once for a single treatment. The average and standard deviation of every set of three measurements were determined.

## RESULTS

### Physicochemical characteristics of wastewater

Arsenic-resistant bacteria were identified after the physicochemical properties of industrial waste water were determined. Various samples showed a temperature spread of 28°C to 35°C, a pH spread of 6.4–8.0, and a dissolved oxygen distribution of 0–1., 0.42 ± 0.03 and 1.20 ± 0.01 mg $l^{-1}$ and As ranged between 0.80 ± 0.04 and 1.92± 0.03 µg $ml^{-1}$.

### Isolation of arsenic-oxidizing bacterium

Isolation of single colonies on plates with high concentrations of As(III) resulted in the initial collection of six bacterial isolates when the medium contained 100 g As(III) ml-1. Only one bacterial isolate that was tested was resistant to As(III) at 3 mg ml-1, hence it was chosen for future research. Only one bacterial isolate out of several showed the capacity to oxidize arsenite into arsenate when tested with AgNO3 on agar plates. The reaction between AgNO3 and arsenite or arsenate ions was used as the basis for the silver nitrate test. Bright yellow precipitate is produced by the interaction with As(III), while brownish precipitate is produced by the interaction with As(V) (Krumova et al., 2008). High arsenite oxidizing potential was observed in *H. pseudoflava* isolated from Indus- trial wastewater in this investigation (Figure 1).

### Bacterial identification

To validate the species of this local isolate, its amplified 16S rRNA gene fragment (500 bp) was uploaded to the NCBI (National Center for Biotechnology Information) database for comparison with other DNA sequences. According to the results of the BLAST search, this gene is 98% similar to those found in H. pseudoflava. GenBank accession number EU729357 represents the submission of the nucleotide sequences encoding for the 16S rRNA gene of H. pseudoflava. Similar strains of Pseudomonas alcaliphila (strain Q1-3 EU 144361.1; gi/157649155; 100% similarity), *H. pseudoflava* (strain SF1 FJ600733.1; gi/222431627; 100% similarity), and Pseudomonas sp. (gi/222431647; 100% similarity) were also found. Figure 2 shows a dendrogram depicting the percentage of Pseudomonas species with which the bacterial isolate has characteristics.

### Conditions ideal for development

An arsenic-resistant bacterial isolate thrived best at 30 degrees Celsius. At a pH of 7, H. pseudoflava thrived. Different concentrations of As (III) (zero, half a milligram, one milligram, two milligrams, and three milligrams) were used to analyze the shape of the growth curve. In the presence of 1, 2, and 3 mg arsenite ml-1, the growth pattern of H. pseudoflava was substantially different from control, and the growth rate of H. pseudoflava was slower. The development trend is seen in Figure 3.

### Multiple metal resistances

*Up to 3 mg ml-1 of As(III), H. pseudoflava showed no ill effects. The resistance of H. pseudoflava to various heavy*

*metals as Cd, Cr(VI), Cu, Hg, and Ni was also tested. Cd2+ toxicity was not a problem for H. pseudoflava* (100µg ml$^{-1}$), $Cu^{2+}$ (700 µg ml$^{-1}$), $Cr^{6+}$ (500 µg ml$^{-1}$), $Hg^{2+}$ (400 µg ml$^{-1}$) and $Ni^{2+}$ (300 µg ml$^{-1}$).

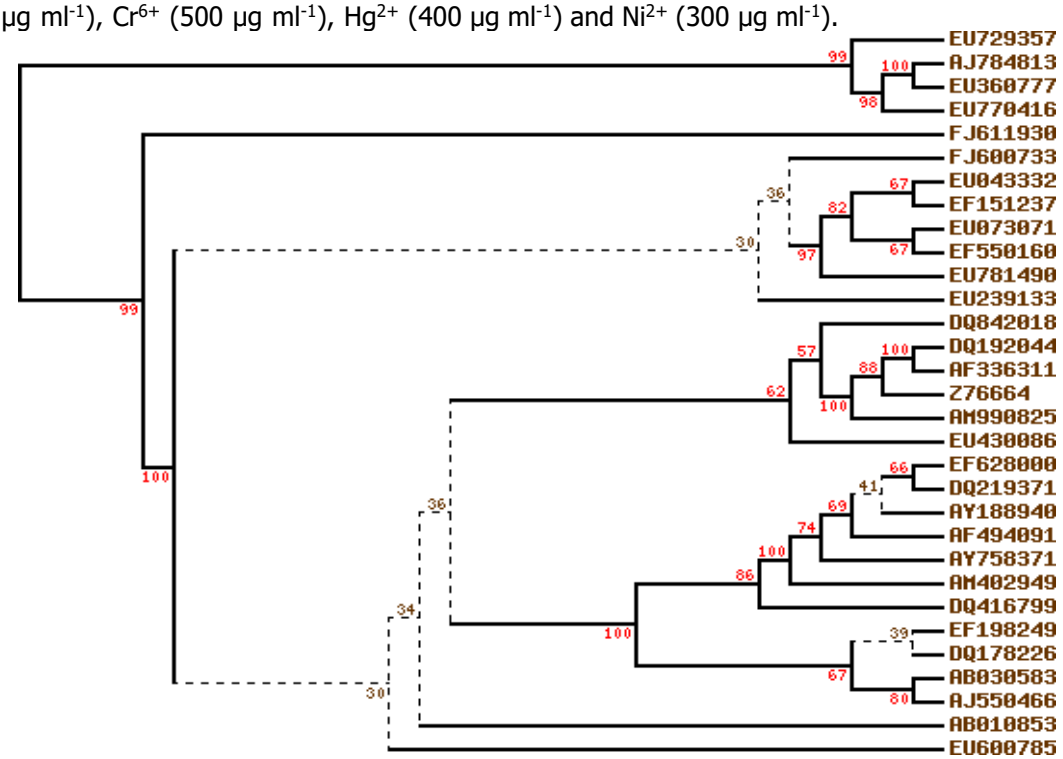


**Figure 2.** Dendrogram showing the similarity of *H. pseudoflava* with other close members of *Pseudomonas* sp.

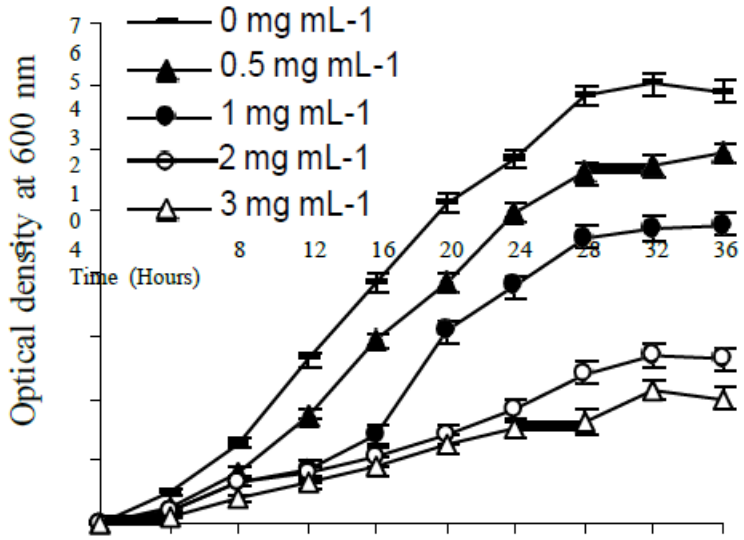


**Figure 3.** Growth of H. pseudoflava cells in acetate minimum media following exposure to several doses of arsenite (0.50, 1.20, 2.40, and 3.40 mg l-1) at 30 degrees Celsius.

## Arsenite-oxidation ability of *H. pseudoflava*

The ability of the bacterial isolate to oxidize arsenite was measured in crude cell extracts. After 24, 48, and 72 hours, *H. pseudoflava* were able to oxidize 42%, 78%, and 95% of the As(III) in the medium, respectively (42 g mg-1 of protein, 78 g mg-1 of protein, and 95 g mg-1 of protein). When comparing arsenite-free and As(III)-treated cultures, the absence of arsenite significantly reduced arsenite oxidase activity..

## DISCUSSION

Bacteria, fungi, ciliates, algae, mosses, macrophytes, and higher plants all have different ways of getting rid of heavy metals in water (Holan and Volesky, 1994; Pattanapipitpaisal et al., 2002; Rehman et al., 2007, 2008). Metals are reacted with in numerous ways by cells, including biosorption by cell biomass, active cell movement, binding by cytosolic molecules, and entrapment within cellular capsules,

**Table 1.** Arsenite resistance in different microorganisms.

| Microorganisms | Arsenite (mM) | Reference |
|---|---|---|
| *Aeromonas* sp. CA1, *Exiguobacterium* sp. WK6 | 2 0 | Anderson and Cook (2004) |
| *Corynebacterium glutamicum* | 60 | Mateos et al. (2006) |
| *Aeromonas*, *Bacillus*, *Pseudomonas* | 16.6 | Pepi et al. (2007) |
| *Pseudomonas putida* | 26 | Chang et al. (2007) |
| *Thiomonas* sp. | 2.6 | Duquesne et al. (2008) |
| *Pseudomonas* sp. | 0.5 | Valenzuela et al. (2009) |
| *Hydrogenophaga pseudoflava* | 40 | This study |

Formation of protein-DNA adducts (Zhitkovitch and Costa, 1992), stress protein activation (Ballatori, 1994), precipitation, and oxidation-reduction reactions (Lovely and Coates, 1997).
Based on the findings of the 16S rDNA sequence analysis, it was established that the arsenic-resistant bacterium found in this study was *H. pseudoflava*.
Several research teams have also identified arsenite-resistant bacteria from industrial effluents (Valenzuela et al., 2009). At a dosage of 3 mg ml-1, H. pseudoflava was shown to be extremely resistant to arsenite in this investigation (Table 1). At concentrations of 300, 700, 100, 400, and 500 g ml-1, *H. pseudoflava* demonstrated resistance against Ni2+, Cu2+, Cd2+, Hg2, and Cr6+, respectively. Metal concentrations were inversely related to resistance, with Cu2+ > Cr6+ > Hg2+ > Ni2+ > Cd2+.
Microbes with genetic determinants that give resistance to hazardous metals are selected and maintained in environments with these metals. Rosen (1999) found that many different types of bacteria had genes that confer resistance to inorganic arsenic, especially arsenate (As-(V)) and arsenite (As(III)). These chromosomal genes are responsible for the alternate substrate absorption of inorganic arsenic. These resistance determinants have been the subject of molecular study thanks to their frequent identification on plasmids in bacteria (Silver and Phung, 2005).
Both Gram-positive and -negative bacteria are resistant to arsenic species because of the ars operon, which mediates energy-dependent efflux of arsenate or arsenite from the cell (Cervantes et al., 1994; Ji and Silver, 1992a). Arsenite oxidase, an enzyme found in the periplasm, oxidizes As-(III), which may serve as a detoxifying step that permits microbes to withstand greater arsenite concentrations. Different bacterial strains, such as those described by Mukhopadhyay et al. (2002), Chang et al. (2007), Duquesne et al. (2008), and Valenzuela et al. (2009), have been shown to be capable of oxidizing As (III) to As(V). After 24, 48, and 72 hours of exposure to the media, H. pseudoflava was able to oxidize 42, 78, and 95% of the As(III) present. When compared to As(III)-treated cultures, non-As(III)-treated cultures showed almost negligible arsenite oxidase activity. Bioremediation of arsenic-polluted waterways may benefit from the use of arsenite-oxidizing bacteria (Valenzuela et al., 2009), which may play a key role in arsenic oxidation and mobilization.

## CONCLUSION

Arsenite, a more mobile, more soluble, and poisonous form of arsenic, is often reported as being present in water. Arsenite, a hazardous form of arsenic, may be safely eliminated by oxidizing it into arsenate, a less soluble form of the element. High resistance to arsenic up to 3 mg ml-1 was observed in H. pseudoflava in this investigation. In addition, we found that the cells were resistant to Cu2+ (700 g ml-1), Hg2+ (400 g ml-1), Ni2+ (400 g ml-1), and Cr6+ (500 g ml-1). In a medium containing As(III), H. pseudoflava was able to oxidize 42, 78, and 95% of it after 24, 48, and 72 hours. Therefore, H. pseudoflava may be used to remediate arsenic-tainted industrial effluents.

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