

*Full Length Research Paper*

# Investigation of bioremediation of arsenic by bacteria isolated from contaminated soil

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The aims of this study are isolating arsenite-resistant bacteria from arsenic contaminated soil and the investigation of arsenite bioremediation efficiency by the most resistant isolates. Isolation of arsenite-resistant bacteria and the minimum inhibitory concentration (MIC) were conducted by spread plate method and the agar dilution method on PHG-II agar plates supplemented with sodium arsenite respectively. The results showed that, 69 and 25% of arsenite resistant isolates were gram positive and negative bacilli, respectively. Its maximum MIC was 128 mM/L, which is related to such bacteria as *Bacillus macerans*, *Bacillus megaterimand* *Corynebacterium vitarumen*. There is a significant difference ( $P < 0.01$ ) between three isolates in arsenite removal potential and arsenite bioaccumulation. The maximum percentage of arsenite removal potential (92%) and arsenite bioaccumulation (36%) were related to *B. macerans*. The removal efficiency of arsenite for *B. macerans*, *C. (vitaromen)* and *B. megaterim* were 60, 43 and 38% after 48 h of growth, respectively, while after 144 h of *Bacillus macerans*, *Corynebacterium (vitaromen)* growth and 120 h of *Bacillus megaterim* growth were 92, 80 and 73% respectively. The results also were shown the highest percentage of arsenite in biomass (36%), arsenate from oxidation (27%) were related to *B. macerans*, *B. megaterium* and *B. megaterium*. These results express the probability of finding more arsenic accumulating bacteria from the contaminated soil environment and can be concluded that arsenic resistant and/or accumulating bacteria, such as *Bacillus* sp., are widespread in the polluted soils and are valuable candidates for bioremediation of arsenic contaminated ecosystems.

**Key words:** Arsenite, bacteria, bioremediation, MIC.

## INTRODUCTION

Human activities over the centuries has contaminated many areas of developing and developed countries (Evangelou et al., 2007). Soil contamination with heavy metals is one of the great problems of modern societies. Heavy metals periodically increase in the environment due to industrial activities and technology development. Increasing of these pollutants in the environment is considered as a serious threat to human and environmental health (Banaa Araghi et al., 2010). Unlike many organic contaminants disintegrated in the soil,

heavy metals are kept in the soil storage and according to their nature a group of pollutants are of most interest because of their danger and of course plenty of stability in most environments (Garbisu and Alkorta, 2001). Arsenic has long been an important environmental pollutant and in long term has been as a health risk to humans and other living organisms. In the past, arsenic compounds have been widely used in pesticides, herbicides and soil disinfectors, thus in some soils was in high concentrations (Pais and Jons, 1997). Arsenic is highly toxic metal element that annually threatens the health of millions of people in the world (Chen and Shao, 2009). Inorganic arsenic forms are more dangerous than other forms for human health and in terms of classification fall in cancer-causing ingredient (Andrews,

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2001). In recent decades following increasing environmental pollution by heavy metals, scientists attracted to biological purification methods. In most cases of cleaning the contaminated ecosystems with chemical methods involves heavy costs and irreparable damages (Brooks, 1995; Nwuche and ugoji, 2008). Therefore one appropriate method is using biological method. Generally population and microbial activities in soil and water contaminated with the presence of metal will be reduced and modified (Kelly et al., 1998). On the other hand resistant microorganisms have evolved mechanisms to tolerate the toxicity of heavy metals. Application of microorganisms for heavy metals remediation is considered as a natural, stable and economical solution. Previous researches have described the isolation and characterization of arsenic resistant bacteria from different environments and have indicated that these bacteria are able to grow chemolithotrophically with oxygen as an electron acceptor and As(III) as an electron donor (Duquesne et al., 2008; Santini et al., 2000). Arsenic-resistant bacteria play an important role in controlling the speciation and cycling of arsenic in the ecosystems (Inskeep et al., 2007). The aims of this study are isolating arsenite-resistant bacteria from arsenic contaminated soil and the investigation of arsenite bioremediation efficiency by high resistant isolates.

## MATERIALS AND METHODS

### Sampling

Soil samples were collected from the three different points of soil surface (0-20 cm) of the Research Farm (in Lavark, Najaf Abad) located in southwest of Isfahan, Iran. This soil was contaminated previously with arsenic through using of urban sewage sludge. The samples were mixed, transferred to the laboratory, passed through 2 mm sieve and used for physical-chemical and microbial analysis (Nwuche and ugoji, 2008).

### Arsenic measurement

In this study to measure arsenic the spectrophotometry method was used along with a reagent called Leuco malachite green (LMG). In this method arsenic reacts with Potassium iodate ( $KIO_3$ ) in the acidic environment and iodine will be released. Released iodine oxidizes LMG to MG and changes the color to the color of malachite green. Detection range of arsenic concentration in this method is 0.09-0.9 micro g/ml. The MG dye shows maximum absorption at 617 nm (Revanasiddappa et al., 2007).

### Arsenite measurement (As III)

Initially for the preparation of arsenite stock solution (1,000  $\mu$ g/ml), amount of 0.1734g  $NaAsO_2$  (sodium arsenite) resolved in 100 ml deionized distilled water. The standard solutions including 0.9-9.0  $\mu$ g of arsenite removed and poured in 10 ml volumetric balloons. Then 1 ml of Potassium iodate 1%, 0.5 ml of 1 M hydrochloric acid were added and the reaction mixture was shaken for 2 min. Then 0.5 ml of 0.05 LMG was added with shaking. Finally 2 ml acetat buffer (pH =4.5) was added and heated (40°C) in a water bath for 5

min, cooled and diluted with distilled water. After 5 min, absorbance of the dye was measured at 617 nm against the reagent blank. The concentration of arsenic (III) content was established by reference to the calibration graph (Revanasiddappa et al., 2007).

### Arsenate measurement (As V)

After filtering of this sample a certain volume will be removed, then 0.5 ml of 5% KI and 5 M HCL were added to the samples. All of the available arsenate were reduced to arsenite. In order to remove yellow to brown color which is due to the high amount of released iodine, the droplets of ascorbic acid were added (Pillai et al., 2000) then the amount of total arsenic in samples were measured by the method of arsenite measurement.

### Soil arsenic measurement

One gram of soil sample was placed in the nickel plate that already covered its bottom with NaOH. Heat the Nickel plate in order to NaOH be fully melted and mixed with soil (alkaline digestion). After cooling, the nickel plate was immersed in HCL (0.5 N) and waited in order to be digested slowly (Almond, 1953) then the amount of arsenite was measured.

### Isolation of arsenite-resistant bacteria

One gram of each soil samples was used to provide series. 0.1 ml of each dilution was added to each of PHG-II agar plates (4 g pepton, 1 g yeast extract and 2 g glucose and 15 g agar per liter) supplemented with 0.5 mM sodium arsenite (pH=7) by spread-plate method. The plates were incubated at 30°C for 3-5 days. This experiment was conducted in three replicates. After isolation of resistant colonies, their enrichment, purification and identification were done by the help of Gram Staining and biochemical tests (catalase- Licetinase- citrate – MR- VP -manitol fermentation and acid production - fermentation of glucose, sucrose and galactose - nitrate reduction test, urease and Esculine Hydrolysis) (Sneat et al., 1989; Cappuccino and Sherman, 1996).

### Minimum inhibitory concentration (MIC) determination

The (MIC) of arsenite at which no colony growth occurred was determined by the agar dilution method. PHG-II agar plates supplemented with different concentration of arsenite the level of resistance (0.5, 1, 2, 4, ... and 192 mM /L) were inoculated aseptically with a culture of bacterial isolates in exponential growth phase. The plates were incubated for 48 h at 35°C. Minimum concentration of arsenite allowing growth of the isolates was an indication of positive tolerance (Hassen et al., 1998).

### Growth curve and arsenite removal by bacterial strains

The growth curve of the most resistant bacteria at sub MIC concentration of arsenite were monitored by measuring the optical density (OD) of the cultures at 600 nm using a spectrophotometer. At each interval a certain volume of medium was removed, after measuring OD at 600 nm. It was centrifuged and filtered. Then, the arsenite and arsenate concentration were measured by the above mentioned method (Chen and Shao, 2008; Revanasiddappa et al., 2007; Pillai et al., 2000). At the end of growth phase arsenite concentration in bacterial cell structure was measured by the method of Takeuchi et al., 2007. Briefly, the culture media were shake at 100 rpm, centrifuged at 5000 xg at 4°C for 20 min. The

**Table 1.** Soil chemical and physical properties.

Depth (cm)	Texture	Clay	Silt (%)	Sand	pH	EC (dS.m <sup>-1</sup> )	P <sub>ava</sub>	K <sub>ava</sub>	As <sub>Total</sub> (mg.kg <sup>-1</sup> )	As <sup>5+</sup>	As <sup>3+</sup>
0-20	SiCL	35.83	48.83	15.34	7.63	2.6	10	203	60.2	40.3	19.7

**Table 2.** The biochemical tests and MIC of the greatest arsenite resistant bacteria.

Bacteria test	Strain 4	Strain 8	Strain 10
Spore	+	-	+
Catalase	+	+	+
Manitol	+	ND	+
VP	-	-	-
MR	+	+	+
Nitrate	+	+	+
Citrate	-	-	+
Glucose	+	+	+
Sucrose	ND	+	+
Urea	ND	+	ND
Probable general and species	<i>B. macerans</i>	<i>C. (vitaromen)</i>	<i>B. (megaterim)</i>
MIC	128	128	128

pelletes were washed twice with distilled water and placed in an oven with 100°C temperature for drying. The dried samples were weighted and digested with nitric acid. Then the arsenite concentration was measured at 617 nm by the spectrophotometric method.

#### Statistical analysis

Statistical analysis was conducted using the SPSS System software. For comparison of means the Duncan test was used at the 5% probability level.

## RESULTS

### Soil physical and chemical properties

The physical and chemical properties of soil is presented in Table 1.

### Resistance to arsenite

The results showed that, 69 and 25% of arsenite resistant isolates were gram positive and negative bacilli, respectively and 6% of them were gram positive cocci.

According to MIC determination results, the greatest resistance to arsenite has been related to gram positive bacilli. Its maximum MIC and MBC were 128 and 192 mM/L respectively, which is related to such bacteria as *B. macerans*, *B. megaterim* and *C. vitarumen* (Table 2).

### Growth curve and arsenite removal by bacterial strains

In Figure 1 growth curves of *B. macerans*, *C. vitaromen*, *B. megaterim* were shown in 128 mM arsenite.

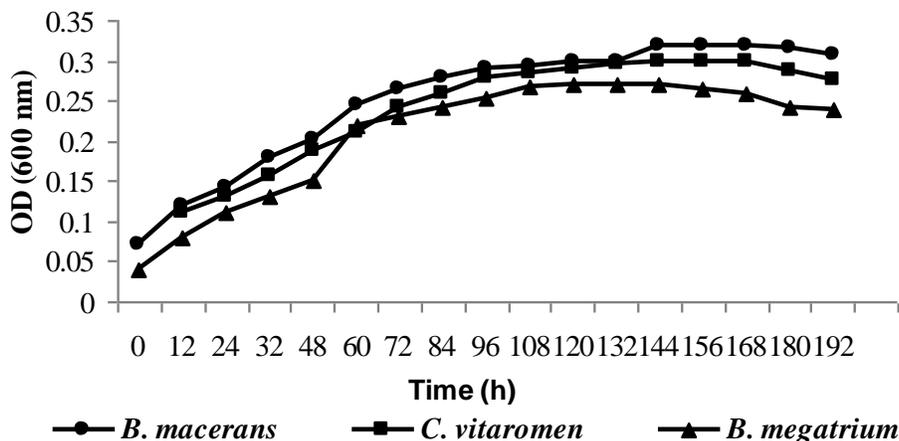
The removal efficiency of arsenite for *Bacillus macerans*, *Corynebacterium (vitaromen)* and *Bacillus megaterim* were 60, 43 and 38% after 48 h of growth, respectively. While after 144 h of *Bacillus macerans*, *Corynebacterium (vitaromen)* growth and 120 h of *Bacillus megaterim* growth, the removal efficiency of arsenite were 92, 80 and 73% respectively (Figures 2, 3 and 4).

Finally, the percentage of arsenite in bacterial mass (bioaccumulation), arsenate from oxidation and remained arsenite were determined. The results were shown in Figure 5. The highest percentage of arsenite in biomass (36%), arsenate from oxidation (27%) were related to *B. macerans*, *B. megaterim* and *B. megaterim*.

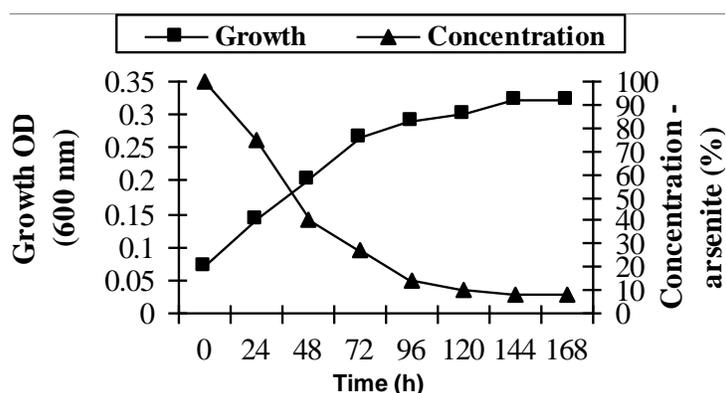
There is a significant difference ( $P < 0.01$ ) between three isolates in arsenite removal potential and arsenite bioaccumulation (Figure 6). The maximum percentage of arsenite removal potential (92%) and arsenite bioaccumulation (36%) were related to *B. macerans*.

## DISCUSSION

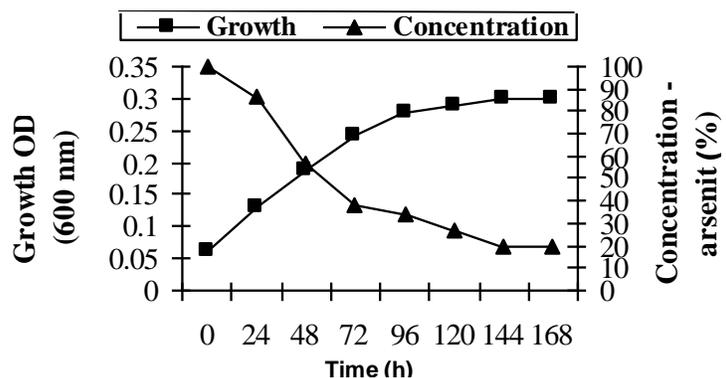
The first step in the identification of bacteria with the ability of bioremediation is isolation of resistant bacteria



**Figure 1.** Bacterial growth curves of *Bacillus macerans*, *Corynebacterium vitaromen* and *Bacillus megaterium* in 128 mM of arsenite.



**Figure 2.** Growth and arsenite removal curves of *Bacillus macerans*.



**Figure 3.** Growth and arsenite removal curves of *Corynebacterium vitaromen*.

which tolerate high concentrations of heavy metals (Trevors et al., 1985). Most arsenic resistant bacteria are separated from arsenic-rich environments. In natural environments, the number of arsenite resistant bacteria is

less than arsenate resistant bacteria. Arsenate is more toxic than arsenite (Jackson et al., 2005). Among the isolated resistant strains from contaminated soil, three strains demonstrated dramatic resistance to arsenite 128

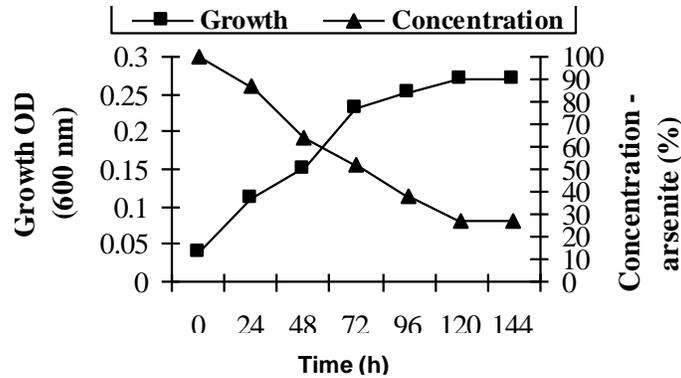


Figure 4. Growth and arsenite removal curves of *Bacillus megaterium*.

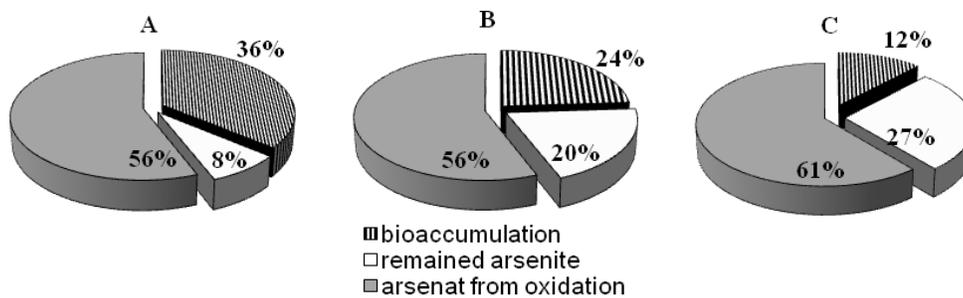


Figure 5. Arsenite in biomass (bioaccumulation), arsenate from oxidation and remained arsenite percentage of A: *Bacillus macerans*, B: *Corynebacterium vitaromen* and C: *Bacillus megaterium*.

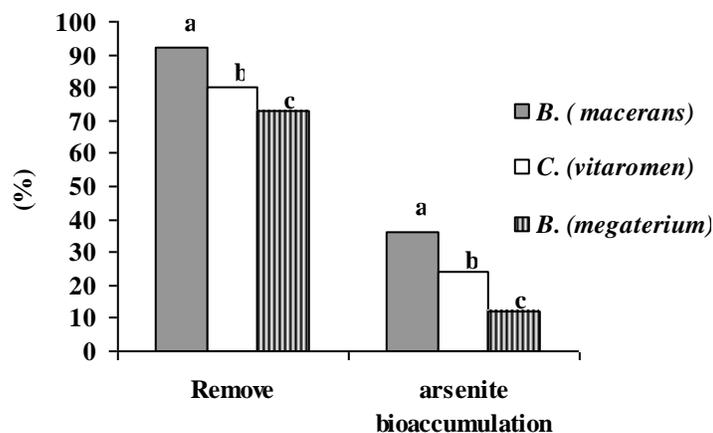


Figure 6. The comparison of arsenite removal potential in 3 isolates.

Mm. These arsenite-resistant strains were probably *B. macerans*, *C. vitaromen* and *B. megaterium*. Concentrations of metals used in this study are also used in the similar studies for bacteria that their medium contains extracted yeast. Abu-shnab et al. (2003) showed that in a contaminated soil the 11.1% of isolated bacteria

were resistant to As with the MIC of 20 mM/L. High levels of soil metal concentration can lead to achieving such a high MIC in resistant strains. Also Chitpirom et al. (2009), in Thailand, isolated arsenic-resistant bacteria from tannery effluent and agricultural soils that were belonged to *Klebsiella*, *Pseudomonas*, *Comamonas* and

*Enterobacter* genera with the MIC of 40 mM (arsenite) and 400 mM (arsenate). Pepi et al. (2007) isolated 3 arsenic resistant genera (*Aeromonas*, *Bacillus* and *Pseudomonas*) from contaminated sediments with the MIC of 16.66 mM (arsenite) and 133.47 mM (arsenate). They also concluded that these bacteria are suitable for arsenic bioremediation in contaminated sediments. In a study by Luis et al. (2006) in Spain with the aim of biological removing of arsenic, *Corynebacterium glutamicum* with over 60 mM arsenite resistance identified as one of the most tolerant species to arsenic. This results are in agreement with our findings but our isolates could tolerate the higher concentration of arsenite that was related to high level of arsenite in soil. In the study after 144 h of *B. macerans*, *C. vitaromen* growth and 120 h of *Bacillus (megaterium)* growth, the removal efficiency of arsenite were 92, 80 and 73% respectively. The highest percentage of arsenite in biomass (36%), arsenate from oxidation (27%) were related to *B. macerans*, *B. megaterium* and *B. megaterium*. Among resistant isolates, *B. macerans* was able to remove 92% of arsenite in the medium and also store 36% of it in the cell mass which is introduced as superior strain in this regard. Studies by Mondal et al. (2008) on three strains of *Ralstonia eutropha*, *Pseudomonas putida* and *Bacillus indicus* showed that these strains were able to remove (67, 60 and 61% respectively) arsenic from wastewater.

Takeuchi et al. (2007) could isolate a non- genetically engineered potent arsenic accumulating bacterium, *Marinomonas communis*, from marine and non marine environment in Japan which accumulated 2290 µg Asg dw<sup>-1</sup> of arsenic in presence of 5 mg As/ l of arsenat (45.8%). Our results are in agree with Takeuchi et al. (2007) and although details of such mechanisms are not yet clear, accumulation of arsenic into the cell would be a result of higher uptake and lower efflux. The high effective concentration of As in this study and previous study (Takeuchi et al., 2007) could be related to the presence of arsenic resistance systems such as regulatory protein of the *ars* operon that has a specific binding site available for arsenite. However, presence of *ars* operon in bacteria is known to extrude arsenate from the cell by an efflux system. Consequently, arsenic is not accumulated in bacteria. Furthermore, the other known arsenic-resistant system, the phosphate-specific transport (Pst) system, would also lead to lower uptake of arsenat by the cell. Therefore, the present results obtained in our isolates and previously isolated *M. communis* with its higher resistance and higher accumulation of arsenic contradict the known arsenic-resistant systems, suggesting existence of an as yet unknown arsenic resistance system for these strains. Cai et al. (1998) also could isolate *Pseudomonas* strains without the *ars* operon with a yet unknown arsenic resistance system.

These results express the probability of finding more

arsenic accumulation bacteria from the contaminated soil environment. It can be concluded that arsenic resistant and/or accumulating bacteria are widespread in the polluted soil environment, and that arsenic-accumulating bacteria such as *Bacillus* sp. are valuable candidates for arsenic contaminated ecosystems bioremediation.

## REFERENCES

- Abu-Shanab RAI, Delorme TA, Angle JS, Chaney RL, Ghanem K, Moawad H, Ghazlan HA (2003). Phenotypic characterization of microbes in rhizosphere of *Alyssum murale*. *Int J Phyto* 5: 367-380.
- Almond H (1953). Field method for determination of trace of arsenic in soils. *J. Anal. Chem.*, 25: 1766-1767.
- Andrews JM (2001). Determination of minimum inhibitory Concentration. *J. Antimicrob. Chemother.*, 48:5-16.
- Banaa AN, Hoodaji M, Afyuni M (2010). Use of EDTA and EDDS for enhanced zeamays' phytoextraction of heavy metals from a contaminated soil. *J. Residual. Sci. Tech.*, 7(3): 139- 145.
- Brookes PC (1995). The use of microbial parameters in monitoring soil pollution by heavy metals. *J. Biol. Fertile Soils* 19:269-279.
- Cai J, Salmon K, Du Bow MS (1998). A chromosomal *ars* opran homologue of *Pseudomonas aeruginosa* confers increased resistance to arsenic ana antimony in *Escherichia coli*. *J. Microbiol.*, 144:2705-2713.
- Cappuccino J, Sherman N (1996). *Microbiology (a laboratory manual)*. Benjamin/ Cumming publishing company INC.
- Chen Sh, Shao Z (2009). Isolation and diversity analysis of arsenite-resistant bacteria in communities enriched from deep- sea sediments of the South west Indian Ocean Ridge. *J. Extremophiles* 13:39-48.
- Chitpirom K, Akaracharanya A, Tanasupawat S, Leepipatpibooim N, Woong Kim K (2009). Isolation and characterization of arsenic resistant bacteria from tannery wastes and agricultural soils in Thailand. *J. Ecol. Environ. Microbiol.*, 59(4): 649-656.
- Duquesne K, Lieutaud A, Ratouchniak J, Muller D, Lett M-C, Bonnefy V (2008). Arsenite oxidation by a chemoautotrophic moderately acidophilic *Thiomonas* sp.: from the strain isolation to the gene study. *Environ. Microbiol.*, 10: 228-237.
- Evangelou MWH, Ebel M, Schaeffer A (2007). Chelate assisted phytoextraction of heavy metals from soil. Effect, mechanism, toxicity and fate of chelating agents. *Chemosphere* 68: 989-1004.
- Garbisu C, Alkorta I (2001). Phytoextraction: a cost- effective plant-based technology for the removal of metals from the environment. *J. Biores Technol.*, 77: 229-236.
- Hassen A, Saidi N, Cherif M and Boudabous A (1998). Resistance of environmental bacteria to heavy metals. *Biores Technol.*, 64: 7-15.
- Inskeep WP, Maser RE, Hamamura N, Warelow TP, Ward SA, Santini JM (2007). Detection, diversity and expression of aerobic bacterial arsenite oxidase genes. *Environ. Microbiol.*, 9:934-943.
- Jackson CR, Dugas SL, Harrison KG (2005). Enumeration and characterization of arsenat-resistant bacteria in arsenic free soils. *J. Soil Biol. Biochem.*, 37(12): 2319-2322.
- Kelly JJ, Tate RL. (1998). Effects of heavy metals contamination and remediation on soil microbial communities in the vicinity of a zinc smelter. *J. Environ. Qual.*, 27. pp: 1458-1467.
- Luis M, Ordóñez E, Letek M, Gil J (2006). *Corynebacterium glutamicum* as a model bacterium for bioremediation of arsenic. *Int. J. Microbiol.*, 9: 207-215.
- Mondal P, Majumder CB, Mohanty B (2008). Growth of three bacteria in arsenic solution and their application for arsenic removal from wastewater. *J. Basic Microbiol.*, 48(6): 521-525.
- Nwuche CO, Ugoji EO (2008). Effects of heavy metal pollution on the soil microbial activity. *J. Environ. Sci. Technol.*, 5:409-4140.
- Pais IJ, Benton Jons JR (1997). *The hand book of trace elements*. Publishing by: St. Luice press Boca Rrton Florida.
- Pepi M, Volterrani M, Renzi M, Marvasi M, Gasperini S, Franchi E, Focardi SE (2007). Arsenic-resistant bacteria isolated from contaminated sediments of the Orbetello Lagoon, Italy, and their characterization. *J. Appl. Microbiol.*, 103(6):2299-308.

- Pillai A, Sunitha G, Gupta VK (2000). A new system for the spectrophotometric determination of arsenic in environmental and biological samples. *J. Anal. Chem. Acta* 408: 111-115.
- Revanasiddappa HD, Dayananda BP, Kumar TNK (2007). A sensitive spectrophotometric method for the determination of arsenic in environmental samples. *J. Environ. Chem. Lett.*, 5: 151-155.
- Santini JM, Sly LI, Schnagl RD, Macy JM (2000). A new chemolithotrophic arsenite-oxidizing bacterium isolated from a gold mine: phylogenetic, physiological and preliminary biochemical studies. *Appl. Environ. Microbiol.*, 66:92-97.
- Sneath PH, Mair ANS, Sharp ME, Holt JG (1989). *Bergey's manual of systematic bacteriology*. Vol (2). Williams and Wilkins.
- Takeuchi M, Kawahata H, Prasad Gupta L, Kita N, Morishita Y, Ono Y, Komai T (2007). Arsenic resistance and removal by marine and non-marine bacteria. *J Biotechnol* 127: 434-442.
- Trevors JT, Oddie KM, Belliveau BH (1985). Metal resistance in bacteria. *FEMS Microbiol. Lett.*, 32:39-54.