Bioremediation of Arsenic from aqueous solution

A Dissertation Thesis Submitted To Nirma University
In Partial Fulfillment For The Degree Of

Master of Science
In
Biotechnology

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CERTIFICATE

*This is to certify that the thesis entitled "**Bioremediation of Arsenic from aqueous solution**" submitted to the Department of Biochemistry and Biotechnology, Institute of Science, in partial fulfillment of the requirement for the award of the Degree of M.Sc. in Biotecnology, is a faithful record of bonafide research work carried out by **Ms.Rekha Yadav** under the guidance of **Dr. Nasreen Munshi**..*

No part of the thesis has been submitted for any degree or diploma. The candidate possesses minimum 75% attendance in the current academic session.

I further certify that any help or information received during the work on this thesis has been duly acknowledged.

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Rekha yadav

CONTENTS

ABSTRACT

With the rapid industrialization all over the world, pollution is on increase and India is no exception. One of the modes through which the pollutants enter in the atmosphere is industrial effluents. These effluents contain large amount of heavy metals (As, Hg, Pb, Cu, Cr, Se, Zn, etc) which pollutes the soil and water resources. Among all these heavy metals Arsenic (As) is highly toxic to all forms of life. Arsenic toxicity has become a global concern owing to the ever-increasing contamination of water, soil and crops in many regions of the world. This has caused a global epidemic of As poisoning, where many people have developed skin lesions, cancers and other symptoms. The major advantages of bioremediation over conventional treatment methods include: low cost, high efficiency, minimization of chemical or biological sludge, regeneration of biosorbents and possibility of metal recovery. The biosorption capacity of the bacteria isolated from heavy metal contaminated sites was found to be an efficient treatment technique for the removal of arsenic from the aqueous solution. Maximum biosorption of metal was found to be 31 $mg.g^{-1}$ at the pH range of 6 to 7 and at 30ºC.

Introduction

Increase in industrial and chemical based agricultural activities without stringent control on the effluent quality has led to the contamination of environment (Tarique *et al.,* 1996). The presence of pollutants such as the heavy metals in various water bodies has resulted in a number of environmental problems. Hence, it becomes mandatory to control and reduce the levels of these metals ions in wastewaters and bring them to permissible values.

Biosorption using microbial biomass as the adsorbent has emerged as a potential alternative technique to the existing conventional physico-chemical methods for metal removal. The sorbent used generally includes plants, algae, bacteria, fungi and yeast. The physical-chemical processes involved in biosorption are usually identified as ion exchange, complexion, cooordinaton, chelation, micro-precipatation, reverse osmosis, evaporation or adsorption, for the removal of the metal from the solution. For heavy metal removal from waste streams they are not cost effective and hence biological approach i.e. **Bioremediation** has been considered as an alternative remediation for heavy metal contamination (Congeevaram and Dhanaranj, 2007).

Recently, bioadsorbents have emerge as an eco friendly, effective and low cost option. These bioadsorbents include some agricultural wastes, fungi, algae and bacteria. Studies using bioadsorbents have shown that both living and dead microbial cells are able to adsorb metal ions and offer potencial in expensive alternative to conventional adsorbents (Al-Qodah, 2006)

Bioremediation

Bioremediation can be defined as any process that uses microorganisms, fungi, green plants or their enzymes to return the natural environment altered by contaminants to its original condition. Bioremediation may be employed to attack specific soil contaminants, such as degradation of chlorinated hydrocarbons by bacteria.

Microbial activities in the natural environment are the main process which removes, mobilize or detoxify the heavy metals from industrial effluent. The activities can be harnessed to clean up the toxic metal wastes, before they enter into the environment and such biotechnological processes are used to control pollution from diverse sources.

The study of microorganism that are capable of resisting and surviving in polluted environment, provide the basic knowledge for bioremediation. Nowadays natural and genetically engineered species are used for bioprotection and remediation of organic contaminants. Due to non-biodegradability of heavy metals they cannot be treated biologically *in situ* and must instead be extracted from contaminating streams.

The utilization of the microbial biomass for removal of metals from industrial waste waters and polluted waters is a well recognized method for remediation. Metals adversely influence microorganism, affecting their growth, morphology, and biochemical activities, resulting in decreased biomass and diversity.

Despite these toxic stresses, numerous microorganism develop metal resistance and detoxification mechanism, which include volatilization, extracellular precipitation and exclusion, intracellular sequestration, membrane associated metal pumps, entrapment by cellular component, cation exchange and complexation, adsorbtion and degradation of organometals and any of these capabilities of these microorganisms can be used for bioremediation of heavy metals.

Review of literature

Metals in waste water and their toxicity

Toxic metal ions cause physical discomfort and sometimes life threatening illness including irreversible damage to vital body system. Metal ions in the environment bioaccumulate and are biomagnified along with the food chain. Therefore, their toxicity is more pronounced in animals at higher levels. Mine tailing and effluent from non ferrous metals industries are the major sources of heavy metals in the environment. Bio-logical treatment is reasonably effective in removing organic pollutants, heavy metals however tend to accumulate in biological sludge, rendering it unfit as fertilizer and require incineration for its disposal (Tien *et al.*,1991; Kiran *et al.*, 2007).

Heavy metals as pollutants

Heavy metals pollution is a global concern. The levels of metals in all environment including, air, water and soil, are increasing in some cases to the toxic levels, with contributions from a wide variety of industrial sources (Umrania, 2005).

Heavy metals are roughly defined as elements having density over 5g.cm^{-}3. The main cause of water pollution are household wastes (As ,Cu ,Cr ,Ni , Mn), coal fed power station(As, Se, Cu), iron and steel(Cr, Mo, Zn , Sb) and metal smelter (Cd, Ni, Se, Sb). Of these metals, most of the metals are thought to enter rivers and lakes, and their surrounding soils are also heavily polluted by these metals (Markrett,1998).

Among all heavy metals Arsenic is very toxic. Arsenic (As) is ubiquitous in many environments and highly toxic to all forms of life (Abia *et al.*, 2007;). It occurs predominantly in inorganic form as arsenate (As V) and arsenite (As III). Arsenite is an analogue of phosphate and thus interferes with essential cellular processes such as oxidative phosphorylation and ATP synthesis, whereas the toxicity of As (III) is due to its propensity to bind to sulfhadryl groups, with consequent detrimental effects on general protein functioning (Kostal *et al.*, 2004).

Toxic heavy metal ions get introduced to the aquatic streams by means of various industrial activities viz. mining, refining ores, fertilizer industries, tanneries, batteries, paper industries,

pesticides etc. and poses a serious threat to environment (Celik and Demirbas, 2007 Pastircakova, 2004).

The major toxic metal ions hazardous to humans as well as other forms of life are Cr, Fe, Se, V, Cu, Co, Ni, Cd, Hg, As, Pb, Zn etc. These heavy metals are of specific concern due to their toxicity, bio-accumulation tendency and persistency in nature (Friberg and Elinder, 1985; Garget *al.,* 2007; Randall *et al.,* 1974). Several past disasters due to the contamination of heavy metals in aquatic streams are Minamata tragedy in Japan due to methyl mercury contamination and ‘‘Itai-Itai” due to contamination of cadmium in Jintsu river of japan (Friberg and Elinder, 1985; Kjellstrom *et al.,* 1977). Various regulatory bodies have set the maximum prescribed limits for the discharge of toxic heavy metals in the aquatic systems. However the metal ions are being added to the water stream at a much higher concentration than the prescribed limits by industrial activities, thus leading to the health hazards and environmental degradation.

Metal and other pollutants tend to persist indefinitely, circulate and eventually accumulate throughout the food chain (Gadd and White, 1993). Conventional methods for removal of metal ions from aqueous solutions include chemical precipitation, ion exchangers, chemical oxidation/reduction, reverse osmosis, electro dialysis, ultra filtration etc (Zhang *et al.,* 1998).

Heavy metals contaminate the aquatic environment and sources of potable water, because of their known accumulation in the food chain and their persistence in nature, when they are discharged in small quantities by numerous industrial activities. Sources of metal into earth’s environment is depicted in Figure 1.

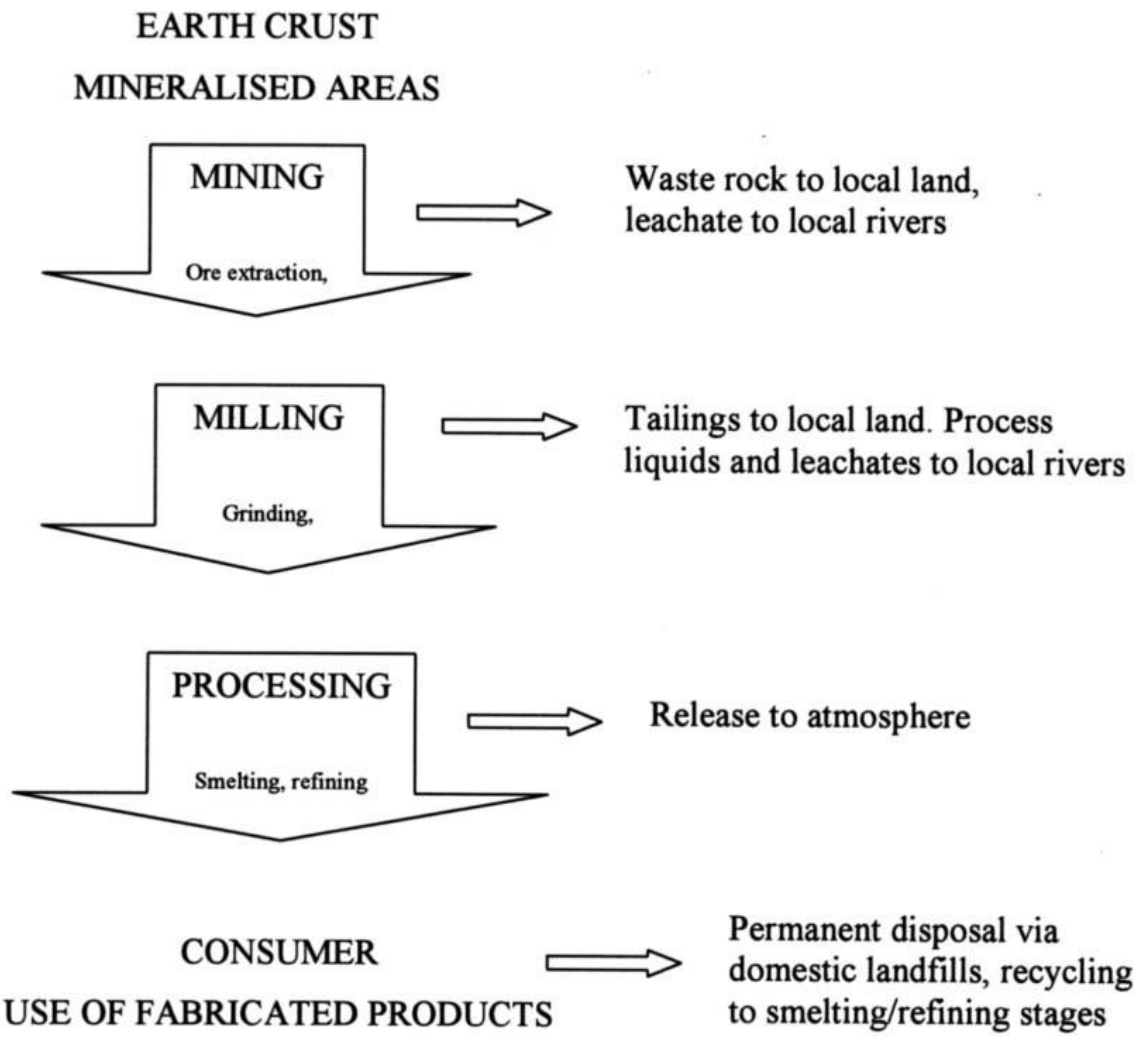


Figure1: Pathways of metals derived from mining activities into earth's surface. Adapted from Salomons (1995) and Hester and Harrison (1994).

Conventional methods for heavy metal removal from industrial effluents

- Precipitation
- Ion exchange
- Electro-wining
- Electro-coagulation

- Cementation
- Reverse osmosis

Precipitation

Precipitation is the most common method for removing toxic heavy metals up to parts per million (ppm) levels from water. Since some metal salts are insoluble in water and which get precipitated when correct anion is added. Although the process is cost effective, its efficiency is affected by low pH and the presence of other salts (ions).

Ion Exchange

It is another method used successfully in the industry for the removal of heavy metals from effluents. An ion exchanger is capable of exchanging either cations or anions from the surroundings materials. Moreover ion exchange is nonselective and is highly sensitive to pH of the solution.

Electro-wining

It is widely used in the mining and the metallurgical industrial operations for heap leaching and acid mine drainage. It is also used in the metal transformation and electronics industries for the removal and the recovery of metal.

Cementation

It is a type of precipitation method in which a metal having a higher oxidation potential passes into solution.

Reverse osmosis

It is the process in which semi permeable membrane is used for the recovery and the removal of the heavy metal from diluted waste water.

These conventional techniques suffer from diverse drawback. For example, precipitation process cannot guarantee the metal concentration limit required by regulatory standards and produce wastes that are difficult to treat. On the other hand, ion exchange and adsorption processes are very effective but require expensive adsorbent material for the removal of metal ion. The use of low cost and waste materials of biological origin as adsorbent of

dissolved metal ion has been shown to provide economic solutions to this global problem. In the literature, the capability of either living or nonliving organisms for fixing metal ions is widely described (Cherguia *et al*., 2007).

Biosorption

It is a property of certain type of living, non-living microbial biomass to bind and concentrate heavy metals from even very dilute aqueous solution. Biomass exhibits this property, acting just as chemical substances, as an ion exchanger of biological origin. It is particularly the cell wall structure of certain algae, fungi and bacteria, which was found responsible for this phenomenon. Till now, research in the area of biosorption suggests it to be an ideal alternative for removal of metal containing effluents.

Advantages of biosorption

Biosortion is highly competitive with the presently available technologies like ion exchange, electro dialysis, reverse osmosis etc. Some of the key feature of biosorption as compared to conventional processes includes

- Competitive performance
- Heavy metal selectivity
- Cost effectiveness
- Regenerative
- Process equipment known
- No sludge generation
- Metal recovery possible

Mechanism of biosorption

Various mechanisms of biosorption are presented in Figure 2.

Metal ions could be adsorbed by complexing with negatively charged reaction sites on the cell surface. Microbial cell wall is rich in polysaccharides and glycoprotiens such as glucans, chitin, mannans and phospho-mannans. These polymers form abundant source of metal binding ligands.

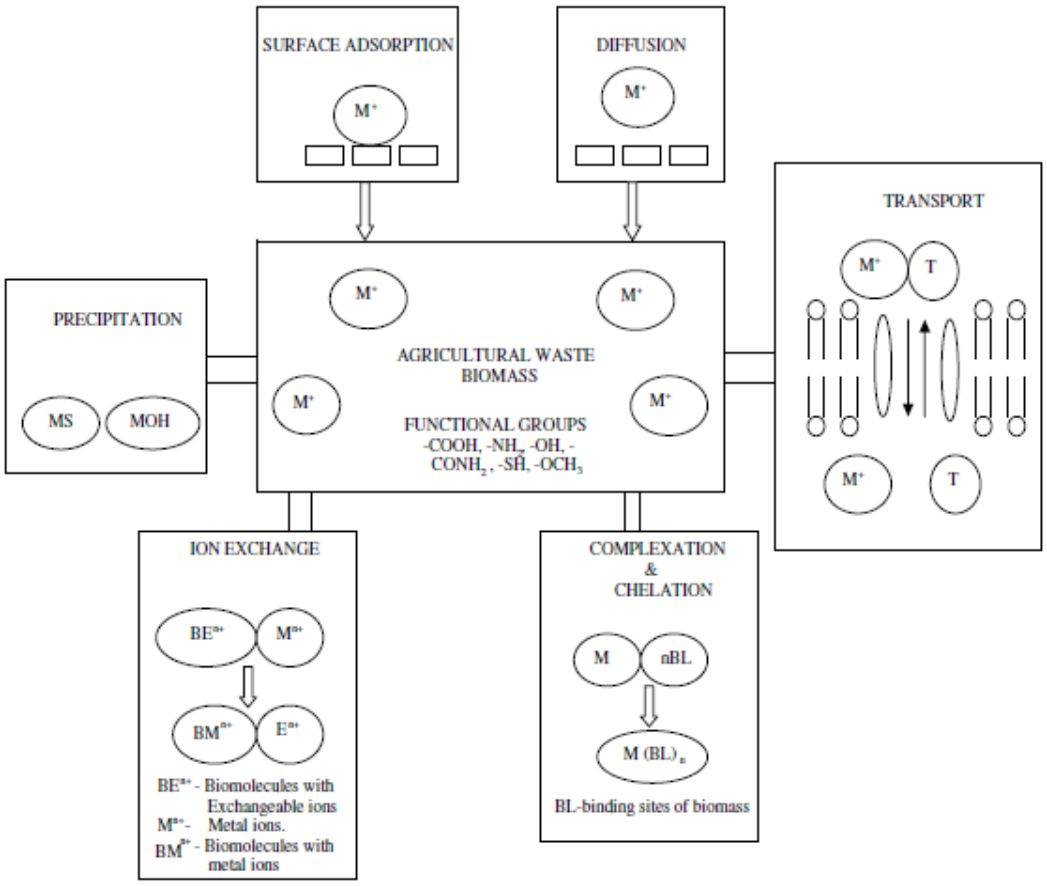


Figure 2: Mechanism of Biosorption

Cell walls of fungi present a multi-laminated architecture where up to 90% of their dry mass consists of amino or non amino polysaccharides. In general the fungal cell wall can be regarded as a two phase system consisting of the chitin skeleton framework embedded in an

amorphous polysaccharides matrix (Farkas, 1980). Up to 30% of *Aspergillus niger* biomass is comprised of an association of chitin and glucan. Chitin and chitisan components of the cell wall are suggested to be important for metal uptake (Fourest and Roux, 1992). Metabolism-independent uptake essentially involves adsorption process such as ionic, chemical, and physical adsorption. A variety of ligands located on the fungal and algal walls are known to be involved in metal chelation. These include carboxyl, amine, hydroxyl, phosphate and sulfhydryl group.

Determination of the exact mechanism is further complicated by complex solution chemistry of the metals and the inability to determine the precise metal complex present in the solution (Tobin *et al.,* 1984), which is not readily amenable to instrumental analysis (kuyucak and Volesky, 1984). Differences in affinities between elements and their ionic species may exist for the various ligand encountered in biological system.

Biosorption can occur by two ways, viz. Active biosorption and Passive biosorption. Passive biosorption is known as biosorption on the surface of cell wall of the organism is rapid, takes short time in completion and is unaffected by metabolic inhibitors, but is affected by physical conditions.

Active biosorption occurs when the metal ions penetrate the cell membrane and enters into the cells. The metals are complexed with specific proteins, such as metallothioneins or it gets contained in the vacuoles. Both passive and active uptake can occur at the same time and the adsorption of metals is relatively non specific in terms of the metal taken up (Scragg, 2007). Passive mode of sorption is independent of energy, mainly through chemical, functional groups of the material, comprising the cell and particularly cell wall whereas active mode of biosorption is metabolism dependent and related to metal transport and desorption (Kostal *et al.,* 2004).

Biogeochemistry of Arsenic

Arsenic is the 20th most abundant element in the Earth's crust and is widely distributed throughout nature as a result of weathering, dissolution, fire, volcanic activity, and anthropogenic input. The last includes the use of arsenic in pesticides, herbicides, wood preservatives, and dye stuffs as well as production of arsenic-containing wastes during smelting and mining operations. In arsenic enriched environment, a major concern is the

potential for mobilization and transport of this toxic element to groundwater and drinking water supplies. In Bangladesh (highly affected area for the arsenic contamination), many people have been exposed to arsenic through contaminated wells. This incident serves as an unfortunate reminder of the toxic consequences of arsenic mobilization and underscores the need to understand the factors controlling the mobility and solubility of arsenic in aquatic systems. In India north eastern area are highly prone to the arsenic toxicity , Ganges delta has highest content of arsenic *(according to WHO).*

Properties of arsenic

Arsenic is a metalloid - a natural element that is not actually a metal but which has some of the properties of a metal. Arsenic can exist in many different chemical forms in combination with other elements. Some forms of arsenic are inorganic – that means they do not contain carbon, while other forms always contain carbon and are classed as organic. Inorganic arsenic exists in four main chemical forms, which are known as valency or oxidation states.

Element symbol: As

Oxidation states: III, IV, V

Atomic number: 33

Atomic mass: 74.9216

Melting point (sublimation): 616 $^{\circ}$C

Colour: Metallic grey, yellow or black

The permissible limit for arsenic in drinking water is 0.01 mg.l^{-1}(10 ppm) as per WHO standards.

Arsenic toxicity

Arsenic occurs in large quantities in the Earth's crust. It is present in a number of minerals and occurs in trace quantities in rocks, soil, water and air. Around a third of atmospheric arsenic is derived from natural sources, e.g. volcanoes and the remaining two-thirds occurs through man-made sources.

High levels of arsenic can be found in drinking water which has been collected from deeply drilled wells or indeed as a natural phenomenon where ground water has been contaminated by minerals dissolving from rocks and soils. This problem is more commonplace in South Asia.

The major industrial processes which causes arsenic contamination of the air, soils and water in the environmental are:

- The mining and smelting of non-ferrous metals
- The burning of fossil fuels (especially coal)
- Semiconducter manufacturing industries as doping agents
- Arsenic based Wood preservatives
- Herbicides and pesticides used in agriculture
- Enameling of glass in glass industries

Effect of arsenic on human health

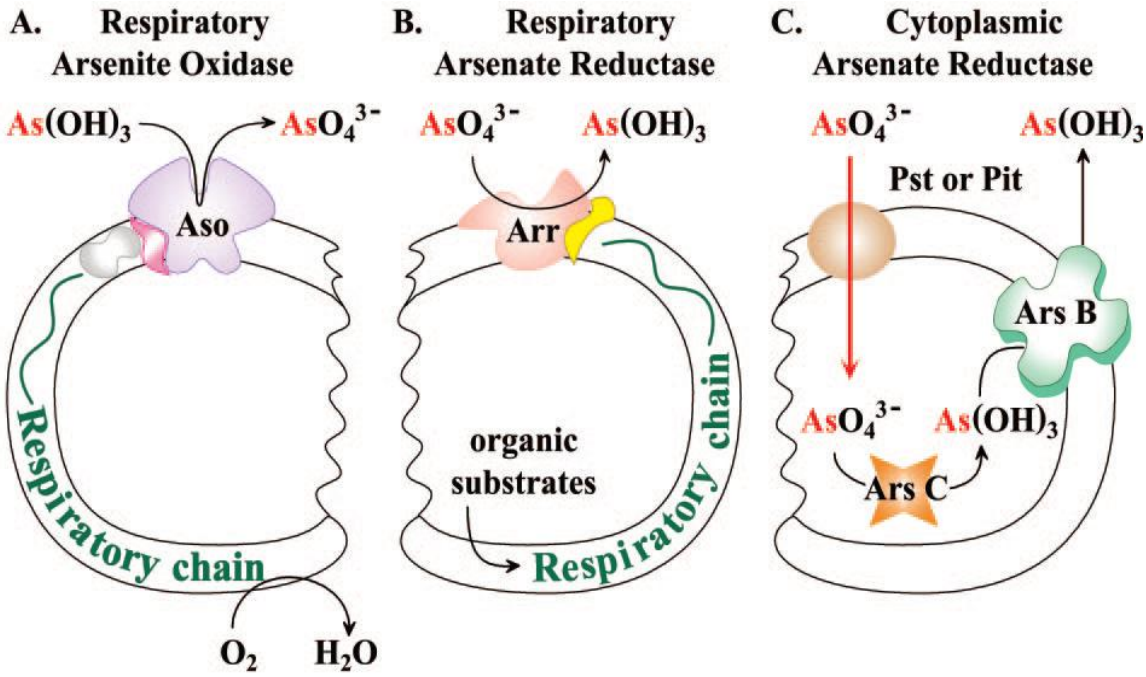
Exposure to high levels of arsenic can cause cancers of the skin, bladder, kidney, and lung, and diseases of the blood vessels of the legs and feet. Diabetes, high blood pressure, and reproductive disorders have also been linked to arsenic exposure. Arsenic in the gaseous form when react with water it produces Hydride gas which is highly haemolytic, i.e. ruptures membranes of the red blood cells and thus causes haemolytic anaemia. A very high exposure to inorganic arsenic can cause infertility and miscarriages with women, and it can cause skin disturbances, decline resistance to infection, brain damage in both men and women. Inorganic arsenic can damage DNA. *(http:www.lentech.com/periodic-chart-elements/As-en.htm)*

Aerobic arsenite oxidase activity

Bacterial oxidation of arsenite to arsenate has long been recognized, especially with aerobic isolates from arsenic-impacted environments. Similar isolates have also been found in soils and sewage not known to be exposed to elevated levels of arsenic. It is not currently clear

whether arsenite oxidation is limited to a few isolates in each species. For example, although two *Alcaligenes faecalis* isolates have this activity, the activity has not been examined in culture collection isolates of this species. It seems that most environmental isolates lack this potential, although a range of *Bacteria* with arsenite oxidase enzyme activity have been isolated and genes apparently encoding arsenite oxidase are found widely in various groups of *Bacteria* and *Archae* (Silver* and Le T. Phung, 2005)

Transformation of Arsenic by bacterial enzymes



As (III) → As(V) As (V) → As (III) As (V) → As (III)

FIGURE3: Cellular locations and functions of bacterial respiratory arsenite oxidase, respiratory arsenate reductase, and cytoplasmic arsenate reductase (Phung , 2005)

Bioremediation of metals by non-living biomass of plant and microbial origin

Inactive and the dead biomass is an innovative and alternative technology for removing heavy metals, these include nonliving biomass of plants, aquatic ferns algae, seaweeds, waste biomass from plants and mycelial wastes. Their efficiency depends on the capacity, affinity and specificity including physic-chemical nature. Chitin and chitosan are naturally abundant biopolymers which are of interest to research concerning the sorption of metal ions since the amine and hydroxyl groups on their chemical structures act as chelation sites for metal ions.

This study evaluated the removal of copper, chromium, and arsenic elements from chromated copper arsenate (CCA)-treated wood via biosorption by chitin and chitosan. Exposing CCA-treated sawdust to various amounts of chitin and chitosan for 1, 5, and 10 days enhanced removal of CCA components compared to remediation by deionized water only.

Remediation with a solution containing 2.5 g chitin for 10 days removed 74% Cu, 62% Cr, and 63% As from treated sawdust. Remediation of treated sawdust samples using the same amount of chitosan as chitin resulted in 57% Cu, 43% Cr, and 30% As removal. The results suggest that chitin and chitosan have a potential to remove copper element from CCA-treated wood (40 $mg.g^{-1)}$. Thus, these more abundant natural amino polysaccharides could be important in the remediation of waste wood treated with the newest formulations of organometallic copper compounds and other water-borne wood preservatives containing copper (Kartal *et al.*, 2004)

Removal of metals by bacterial biomass

Biomass of bacteria may uptake and accumulate a significant amount of metal ions, resulting in the transfer of metals to a contaminated matrix of biomass (Smith *et al.*, 1994). When suspension of dead biomass of actinomycetes from industrial fermentation was mixed with wastewater, the biosorption of metal cations occurred due to negatively charged sites on bacterial cell wall (Niggemeyer *et al.*, 2001). The selected highly potential isolate (ATh-14) showed maximum adsorption of Ag 73%, followed by Pb 35%, Zn 34%, As 19%, Ni 15% and Cr 9% in chalcopyrite using acidothermophilic autotrophs (Umrania, 2005). *Leptothrix* group such as *Leptothrix ochracea* gave 25 $mg.g^{-1}$ (Zouboulis *et al.*, 2004).

Removal of metals by plant derived biomass

Sorption systems developed from residues from agro-industrial activities are very attractive alternatives for environmental remediation. Lignocellulosic polymers are the most abundant biopolymers in the biosphere. They are structural elements of wood and other plant materials. Coconut *(Cocos nucifera)* husk is attractive due to the high proportion of well-defined polymeric structures in its composition. *Cocus nucifera* shown biosorption capacity of 6.6-165mg.g^{-1} at 25ºC (De Sousa *et al.,* 2009). The polymers viz lignocellulose and hemicellulose show affinity for metal complexation (Marmiroli *et al.,* 2005), and are involved in the metabolic mobilization of metals (Dupont *et al.,* 2004) and in the control of their oxidation states in the environment. Coconut fibers offer cost-effective advantages as a toxic metal sequestering medium for environmental applications (Hasany and Ahmad, 2006; Sousa *et al.,* 2007).

Exposure of treated sawdust to 5 g chitin-containing solution for 10 days removed about 74%, 62%, and 63% of the initial concentrations of copper, chromium, and arsenic, respectively in CCA (chromate copper arsenate) treated sawdust.

Removal of metals by algal biomass

Marine brown algae (*Fucus vesiculosus*), terrestrial moss and birch wood sawdust (*Betula* sp.) have been studied as raw materials for preparation of low cost biosorbents for the removal of metal cations from aqueous solutions.

Biosorption, an inexpensive and reliable method to remove cadmium and lead ions from solution using dry seaweed biomass as adsorbents, was investigated. *Sargassum wightii* (Brown algae) exhibited maximum metal uptake at pH 4–5 and the value ranged from 18% to 29% of dry biomass. The kinetics of metal adsorption was fast with 70–80% taking place within 30 min (Kaladharan *et al.,* 2003). It was also reported, when Cr(VI) concentration was increased from 10 to 50 mg/l approximately in the absence of salts, the adsorption capacity increased from 8.0 to 48.0 mg.g^{-1} of biomass of algae *Lyngbya putealis* (Kaushik *et al.,* 2007)

Removal of metals by fungal biomass

Non-living waste biomass of A*spergillus niger* attached to wheat bran was used as a biosorbent for the removal of heavy metal cation from aqueous solution and metal uptake

was found to be a function of the initial metal concentration, biomass loading and pH. Metal uptake decreased in the presence of co-ions, which was dependent on the concentration of metal ions in two compounds in aqueous solution (Spanelova *et al.,* 2003) The structure and the biosorption properties of fungal biomass of *Aspergillus niger* originated from citric acid fermentation industry was investigated.

In this work, alkaline treatments (1M NaOH/20°C/24h and 10M NaOH/107°C /6 h) were used to evaluate the dependence of sorption properties of biomass on the cell wall composition. The biosorption was studied by the batch method, with the biomass concentration of 1 g.l^{-1}, at pH 6. The adsorption of lead was more effective than that of arsenic.

It was reported that *Penicillium purpurogenum* gives maximum biosorption by using 3.4 mg.g^{-1} of the biomass (Say R. *et al.,* 2003). It was also reported that B.cinenerea gives maximum biosorption by using 12.98 mg.g^{-1} of the biomass (Tunali S. et. al., 2006).

Objectives

1. To isolate different strains of bacteria from Arsenic contaminated environment.

2. Screening of isolates for arsenic biosorption in aqueous solution.

3. Screening of selected bacteria for bioremediation of arsenic containing synthetic waste and process optimization using selected isolate.

4. Morphological and biochemical characterization of efficient strains of bacteria for possible identification.

Materials and methods

Collection of sample and isolation of bacteria

Six soil samples were collected from the nearby area of pesticides manufacturing factories, Nirma University, near sewage outlets and petrol pump located at G.I.D.C., Vatwa. The soil samples were serially diluted (10^{-1} to 10^{-5}) with normal saline.

The 0.1 ml of the inoculum was added to the plates containing nutrient agar and nutrient agar with metal (concentration of arsenic taken was 50 ppm) from each dilution.

After incubation at 30º C for 24 to 48 h, the colonies were counted, and total viable population was calculated by multiplying with the dilution factor. The total viable population was reported as average of triplicate experiments.

The colonies were randomly selected from both N. agar and N. agar with arsenic containing plates on the basis of colony morphology, purified and preserved on N. agar slants.

Preparation of the bacterial biomass

Bacterial isolates which were both metal resistant and nonresistant were grown in N-broth. Cells were harvested by centrifugation at 11000 rpm for 10 min. Harvested biomass were then washed thrice with the sterile deionised water, and the pellets were suspended in the same water. They were preserved in the refrigerator at 5°C temperature. In this way, biomass of 49 isolates were harvested.

One ml of collected biomass was taken on aluminium foil and kept at 55°C overnight in hot air oven and corresponding dry weight of 1 ml was measured. Then amount of liquid sample corresponding to 10 mg was calculated and used for biosorption studies.

Biosorption Studies of the Biomass

For biosorption studies, 10 mg dry weight equivalent of biomass were allowed to treat with 20 $\mu g.ml^{-1}$ arsenic containing waste solution in 250 ml Erlenmeyer flasks for 30 min along with control in which all the chemicals were added except the biomass and the biosorption

obtained by this solution was further substracted from the biosorption obtained by other systems (biomass added), the remaining amount of metal in solution was estimated by spectrophotometric molybdenum blue method (Vogels, 1961). Effect of different environmental parameters were determined for the optimization of process by using the selected cultures.

Estimation of Arsenic

Arsenic was estimated by molybdenum blue method. This method estimates arsenite i.e. Arsenic in trivalent state (Vogels, 1961).

Principle

When Arsenic, as arsenite is treated with ammonium molybdate solution and the resulting heteropoly molybdiarsenate (arseno-molybdate) is reduced with hydrazine sulphate or with stannous chloride, a blue soluble complex "molybdenum blue" is formed. The constitution is uncertain, but it is evident that the molybdenum is present in a lower valency state. The stable blue colour has a maximum absorption at about 830 nm and shows no appreciable change in 24 h.

Standard Arsenic solution

Analytical grade $KAsO_2$ was used to make 100 $\mu g.ml^{-1}$ stock solution. To make the stock solution, 44.24 mg $KAsO_2$ was added and adjusted to 100 ml in volumetric flask.

Reagent

Solution (A)- Dissolve 1.0 g of ammonium molybdate in 10 ml of 6N sulphuric acid. Solution (B)- Dissolve 1.15 g of stannous chloride in 100 ml of water.

Procedure for standard graph

In 25 ml of volumetric flask, arsenic stock was taken (as 0.1 ml, 0.2 ml, 0.3ml, 0.4 ml, 0.5 ml, 0.6 ml); to these 2 ml of solution (A) (ammonium molybdate) and 1 ml of solution (B) (stannous chloride) was added. Then with distilled water it was made to 20 ml. This volumetric flask was heated at 75 to 85 $^{\circ}$c for 15 min. After 15 min it was cooled and adjusted the volume up to 25 ml with distilled water. Since the solution obtained were very dark hence 1:25 dilution was done. The O.D. was taken at 830 nm against reagent blank.

Procedure for sample

In 25 ml of volumetric flask 1ml of sample was taken from the biosorption system and the estimation was done as described earlier. The O.D. obtained was correlated with standard graph to obtain concentration of arsenic remaining in the solution after biosorption by biosorbent.

Determination of the adsorption capacity

Adsorption isotherms show the distribution of solute between the liquid and solid phases equilibrium conditions. Many different isotherm models have been proposed for the adsorption of solutes in a liquid solution onto a solid surface.

Langmuir isotherms:

Langmuir model is probably the most popular isotherm models due to its simplicity and its good agreement with experimental data. The Langmuir model, the saturated monolayer isotherm, can be described by the linear form:

Ce/qe=(1/KLqmax)+Ce/qmax

where Ce is the equilibrium metals concentration in aqueous phase (mmol L^{-1}); qm is the qe for a complete monolayer (mmol g^{-1}), a constant related to sorption capacity (the maximum amount of metal ion per unit weight of adsorbent); and KL is a constant related to the affinity of the binding sites and energy of adsorption(l mmol-1). By plotting Ce versus Ce/qe, qmax and KL can be determined (Ruiz, 2009).

Freundlich isotherm:

The equilibrium established between the adsorbed metal ions (qe) and that remained free in the solution (Ce) was also represented by the Freunlich adsorption isotherm, the linear equation as following:

ln qe= ln KF+ (1/n)lnqe

where KF and n are the Freundlich constants related to the adsorption capacity and adsorption intensity of the sorbent, respectively.

Equilibrium experiments were carried out by contacting 10mg of biomass with 40 mL of mercury chloride solution of different initial concentration, 10 ppm -75 ppm. Series of such conical flasks was then shaken at a constant speed of 300 rpm on a orbital shaker with temperatures 30ºC. The samples were then centrifuged and analysis was performed as said before (Ruiz, 2009)

Effects of different parameters

Static and shaking condition

20 ml reaction system (4ml arsenic stock solution + 16 ml double distilled water) was taken in which 10 mg equivalent biomass was added, the systems were put in the static condition and on the shaker for 60 min. followed by centrifugation at 10,000 rpm for 10 min. and arsenic estimation was done as mentioned previously.

Influence of bivalent cations

The effect of bivalent cations on arsenic bioremediation by isolate RA07 was studied using 0.05 mM final concentration of Ca, Mg, Zn, Ni and Cu. For each case, 10 mg equivalent dry weight of biomass was added to 20 ml arsenic system in which 1ml of arsenic solution was added, followed by centrifugation and remaining Arsenic estimation

Effect of different biomass concentration

Different amount of biomass ranging from 1, 5, 10, 20, 30, 40, 50 and 60 mg equivalent in 20 ml systems were taken and effect of this on arsenic biosorption was studied with isolate RA07 as mentioned previously.

Effect of reaction pH

To determine the influence of reaction pH by isolate RA07, 0.1 N HCl and 0.1 N NaOH was used to set pH 2.5 to 9. Four ml of arsenic stock solution (100 $\mu g.ml^{-1}$) and 10 mg dry weight equivalent biomass in 1 ml amount was added in 250 ml Erlenmeyer flask followed by 16 ml of double distilled water, thus giving final arsenic concentration of 20$\mu g.ml^{-1}$. All the systems were incubated on shaker for 60 minutes and remaining lead in solution was estimated after centrifugation at 10,000 rpm for 10 minutes.

Effect of temperature

The effect of temperature on bioremediation by isolate RA07 was done at 35°C, 40°C and 50°C temperature. After addition of 10 mg dry weight equivalent biomass to the systems, they were incubated at respective temperatures in shaker incubator for 60 minutes followed by centrifugation at 10,000 rpm for 10 min and remaining arsenic was estimated.

Effect of metal concentration

Reaction system of different concentration for arsenic (2.5, 5, 10, 20, 30, 40, 50, 60 $\mu g.ml^{-1}$) were taken adding 10 mg equivalent dry weight biomass of isolate RA07 followed by 60 minutes shaking and remaining arsenic in solution was estimated after centrifugation at 10,000 rpm for 10 minutes.

Effect of drying

To study the drying effect on biomass on isolate RA07, 10 mg dry weight equivalent of biomass was added to the 20 ml system followed by the heat treatment for 60°C, 100°C and 150°C temperature. The remaining systems were again made up to 20 ml, put on shaker for 60 minutes then used for the biosorption study as mentioned previously.

Effect of sonication on biomass

10 mg dry weight equivalent biomass of isolate RA07 was sonicated for different time intervals (1,5 and 10 min). For this, biomass in 1 ml volume was first sonicated and after that added in 250 ml Erlenmeyer flask thus giving the final arsenic concentration of 20 $\mu g.ml^{-1}$. All the systems were incubated on shaker for 60 minutes and remaining arsenic in solution was estimated after centrifugation at 10,000 rpm for 10 minutes.

Effect of contact time

Cell suspension equivalent to 10 mg dry weight of biomass (Isolates RA07,RA06, RA43) was taken in 250 ml Erlenmeyer flask and 4 ml of arsenic stock solution (100 $\mu g.ml^{-1}$) was added and final volume was made up to 20 ml with distilled water, thus giving final arsenic concentration of 20 $\mu g.ml^{-1}$ in the system. The system was incubated on shaker (300 rpm) and the samples were centrifuged at 10,000 rpm for 10 minutes at desired intervals (30min,

60min, 120min), after that supernatants were collected. The supernatants were examined for remaining arsenic by the method mentioned previously.

Effect of one and two stage of contact time or influence of cycle

The experiment was conducted in two ways. First in which after first cycle, the biomass was removed and fresh biomass added for second cycle whereas in second case after first cycle additional biomass was added with removing the previous biomass. In first case 5 mg of biomass was added and removed by centrifugation after 30 min, again in this fresh 5 mg biomass was added, put on shaker, centrifuged followed by the centrifugation. In the second case 5mg biomass was added and fresh biomass was added without removing the previous one.

Identification of screened isolates by biochemical tests

Cultures were identified by morphological characteristics, microscopy and biochemical tests. Initially Gram staining reactions were confirmed 2 to 3 times for young cultures. Morphology of cells were recorded and their arrangement were noted. Gram staining reactions were also confirmed by performing KOH test. Then catalase test was performed and following biochemical tests were performed.

Carbohydrate Fermentation Test

24 hour old culture was inoculated in nutrient sugar broth with andrade's indicator for dextrose, lactose, maltose, xylose, sucrose, mannitol, adonitol and galactose. The cultures were incubated overnight at 37°C to check for acid production. The colour change is from peach to pink after 24-48 hours of incubation which is a positive result.

Oxidation-fermentation (Hugh and Leifson) test

Hugh and leifson medium has low peptone and high carbohydrate constituents. Formation of acid shifts the pH and a yellow colour is formed as a positive reaction due to indicator bromothymol blue. For fermentative metabolism, the medium is covered with sterile paraffin oil.

Methyl red Test

The pH indicator methyl red detects a pH change to the acid range as a result of acidic end products such as lactic, acetic, and formic acids. This test is of value in distinguishing between a mixed acid fermenter and a butanediol fermenter. At pH 4, the methyl red indicator turns red indicates a positive methyl red test whereas at pH 6, the indicator turns yellow indicates a negative methyl red test.

Voges proskauer Test

The Voges-Proskauer test identifies bacteria that ferment glucose, leading to 2, 3-butanediol accumulation in the medium. The addition of alpha-napthol and KOH will detect the presence of acetoin, a precursor in the synthesis of 2,3-butanediol. In the presence of the reagents and acetoin, a cherry-red color develops. Development of a red color in the culture medium 15 minutes following the addition of reagents represents a positive VP test; absence of a red color is a negative VP test.

Citrate utilization test

The citrate utilization test is done to see whether bacteria are able to utilize citrate as sole source of carbon. The Simon citrate agar slants were streaked and incubated at 35°C for 24-48 hours. The slants will turn blue from green if the test is positive.

Indole Production Test

Bacteria contain tryptophanase enzyme that metabolize tryptophan to indole, pyruvic acid and ammonia. The presence of indole can be detected by reacting with kovac's reagent giving red colour. Sulphide indole motility broth tubes were inoculated or stabbed with the culture and kept at 37°C for 24 hours. Next day 10 drops of kovac's reagent were added.

Hydrogen Sulphide Production Test

By lead acetate paper strip test

This test is for detection of H_2S production from sulphur containing amino acids. Organisms possessing the enzyme amino acid desulphurase can remove the sulphur found in the amino acid (e.g. cystiene) as H_2S.

Blackening of paper strip is due to formation of lead sulphide which indicates H_2S production by organism.

Lysine decarboxylase test

Bacteria those are able to produce the enzymes lysine decarboxylase can decarboxylate lysine and use the amines as precursors for the synthesis of other needed nitrogenous molecules. Decarboxylation of lysine can be detected by culturing bacteria in a medium containing the desired amino acid, glucose, and a pH indicator (bromocresol purple). Before incubation, sterile mineral oil is layered onto the broth to prevent oxygen from reaching the bacteria and inhibiting the reaction. The acids produced by the bacteria from the fermentation of glucose will initially lower the pH of the medium and cause the pH indicator to change from purple to yellow. The acid pH activates the enzyme that causes decarboxylation of lysine or ornithine to amines and the subsequent neutralization of the medium. This results in another color change from yellow back to purple.

Phenylalanine deamination Test

This test helps in differentiating the enteric bacteria. The enzyme phenyl alanine deaminase removes amine group from phenyl alanine in phenyl alanine agar slant and hence produces phenyl pyruvic acid which reacts with ferric chloride solution giving green coloured compound.

Urea hydrolysis test

A strongly buffered medium (Stuart's broth, pH 6.8) in which urea is the only nitrogen source is used. Urease is an enzyme possessed by many species of microorganisms that can hydrolyse urea.Ammonia produced reacts in the solution to form ammonium carbonate, resulting in alkalization which results in change in colour of indicator phenol red to pink.

Nitrate reduction test

Organisms possessing nitrate reductase when grown in a medium containing nitrate as the sole nitrogen source will reduce nitrate to nitrite. The formation of which is detected by adding sulphanilic acid which forms a diazonium salt which in turn reacts with alpha-napthylamine thereby leading to formation of soluble red azo dye.

Ammonia production test

When nitrates or nitrites are reduced by microorganisms, ammonia is liberated which while trying to escape from the tube converts litmus paper hanging from the neck of the tube from red to blue.

Starch Hydrolysis test

The cultures were streaked on the starch agar plate and then zone of clearance for hydrolysis of starch was visualised by adding Gram's iodine after incubation.

Gelatin hydrolysis test

Gelatin is a protein which is hydrolyzed by some of bacteria that produce gelatinase enzyme. When the plate is flooded with acidic mercuric chloride solution after incubation, the unhydrolysed gelatine is precipitated as cloudy white precipitate. Formation of clear zone around the colony is due to gelatinase production indicating positive test.

Casein hydrolysis test

Caseolytic organisms produce casease which hydrolyse casein to soluble form paracasein. Hence the clear zone is observed in the casein agar medium surrounding the growth.

Catalase activity

Many bacteria possess enzymes that can protect themselves against reactive super oxides which was identified by adding 30% hydrogen peroxide to the culture of nutrient agar. Positive test is indicated by appearance of brisk effervences on adding 30% hydrogen peroxide.

Triple sugar iron agar test

The Triple sugar iron agar slants were inoculated with 24-48 hours old culture grown in n-agar and checked for acid production and H_2S production. The colour change of slants to yellow indicates acid production aerobically indicates positive test. Yellow colored butt indicates acid production fermentatively, blacking of butt indicates H_2S production and bubble in butt indicates gas production, where as pink butt indicates alkali production. All reaction are used for identification.

Growth at different environmental conditions

Growth at different environmental conditions was also checked.

- N-broth with pH 4.
- N-broth with pH 9.
- Growth at 44^{o}C in N-broth.
- Growth at 4^{o}C in N-broth.
- Growth in presence of 2% NaCl in N-broth.
- Growth in presence of 5% NaCl in N-broth.

Results and Discussions

Total viable population

A good amount of microorganisms were obtained on the both type of plates from all the soil samples. Soil samples from different contaminated sites were collected, serially diluted and then cultures were grown on the plate containing N-agar n the plates containing N-agar with metal (As), then incubated for 36 hrs and the data is presented in Table 1.

Isolation of cultures for arsenic biosorption

Forty-nine cultures were isolated based on the visual dissimilarity and colony characteristics (Table2). On subculturing, five cultures were lost, hence screening for arsenic biosorption is reported for 44 isolates.

Screening of the bacteria for the biosorption

On the basis of percentage biosorption, bacterial isolates were screened. The results are presented in Table 3 which shows arsenic bioremediation by 49 isolates. Amongst which, the isolates showing more than 60% As sorption were selected for the further study. Thus, first three isolates (RA07, RA06 and RA43) were taken.

Initial characterisation

Initial characterisation was done for the all isolates by cell morphology, Gram's staining (Table 4) and 3% KOH test (for the confirmation of gram staining).

Table 1: Total viable count for soil samples obtained from N-agar and N-agar containing 50 µg.ml^{-1} potassium arsenite.

Sr. No.	Source	Viable count on N-agar (CFU.g^{-1})	Viable count on N-agar+Arsenic
1.	Soil from Chemet chemicals	(7.94 ± 2.3) X 10^5	(7.12±2.1)X 10^5
2.	Soil from Nirma campus	(4.70 ± 2.5)X 10^5	(3.48±3.7) 10^4
3.	Soil near industrial sewage	(1.76 ± 1.5) X 10^6	(1.16 ±3.23)X10^6
4.	Soil near petrol pump	(4.16 ± 2.5)X 10^6	(7.75±2.75)X10^3
5.	Soil from Sriram chemicals	(6.59 ± 1.3)X 10^4	ND
6.	Soil from Metal casting industry	(7.12 ± 2.38) 10^4	ND

ND: Not done

A high population was obtained from the soil sample collected near the Chemet chemicals and soil from Sriram chemicals. Low population was obtained from the soil sample collected from soil near industrial sewage and from soil of nirma campus. Arsenic resistant colony were taken from the soil sample collected from Chemet chemicals.

Table2: Following number of isolates selected randomly From Different Soil Samples for screening for Arsenic bioremediation

S No.	Source	No. of isolates
1	Soil from Chemet chemicals industry	18
2	Soil from Nirma campus	6
3	Soil near industrial effluent sewage	5
4	Soil near petrol pump	6
5	Soil near Sriram chemicals	5
6	Soil near Metal casting industry	9
	Total number of isolates	49

Maximum number of isolates (18) were taken from the soil sample collected near the Chemet chemicals. They were Arsenic resistant and non resistant.

Plate 1: Isolates collected from Chemet chemicals (RA01, RA02, RA04, RA05, RA06 and RA07)

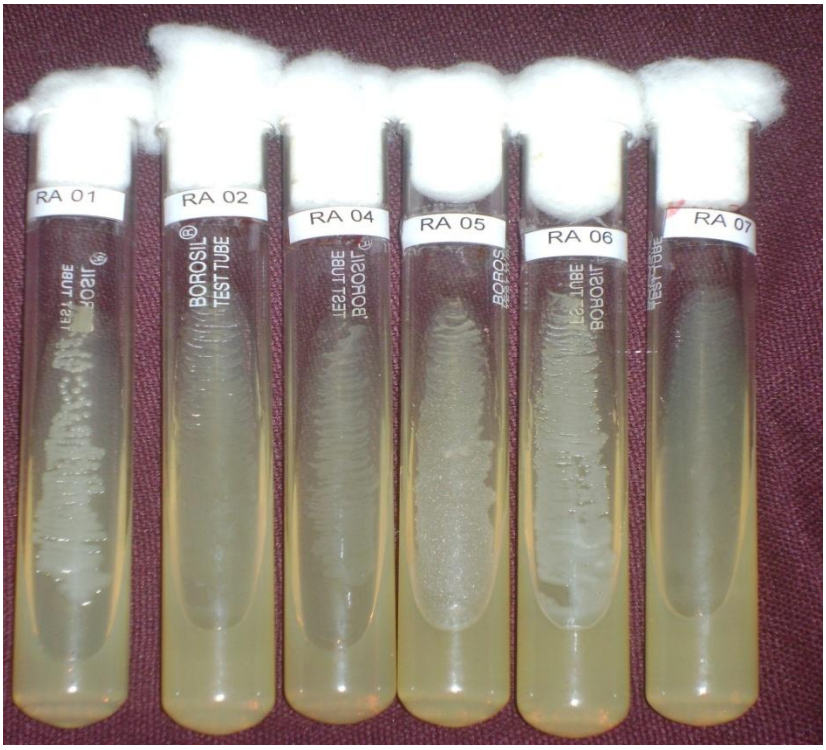


Plate 2: Arsenic resistant isolates selected from soil sample from Chemet Chemicals (RA03, RA08, RA09, RA10, RA11, RA12, RA13, RA14, RA15, RA16, RA17 and RA18)

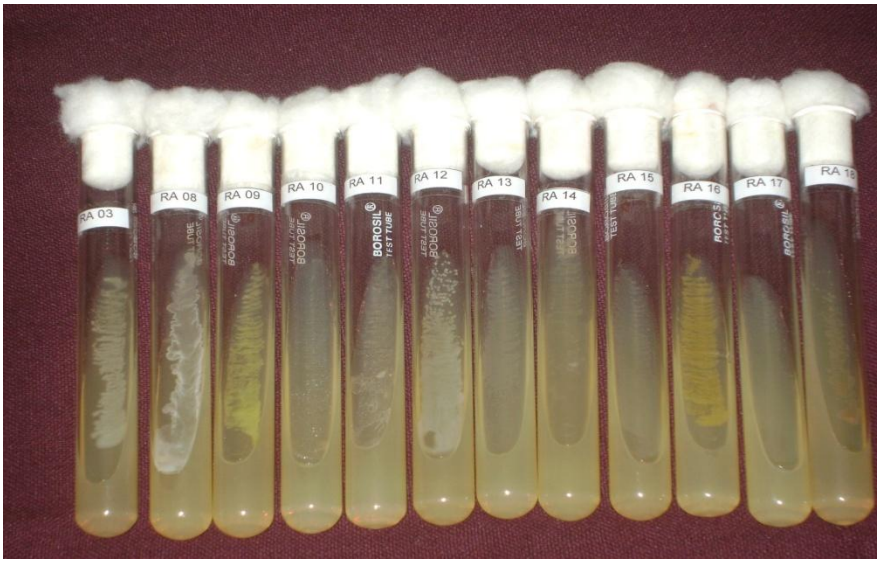


Plate3: Isolates collected from Sriram chemicals – Five isolates had taken from this soil sample

Plate4: Isolates collectected from Nirma soil, Metal casting industry soil and soil near industrial sewage

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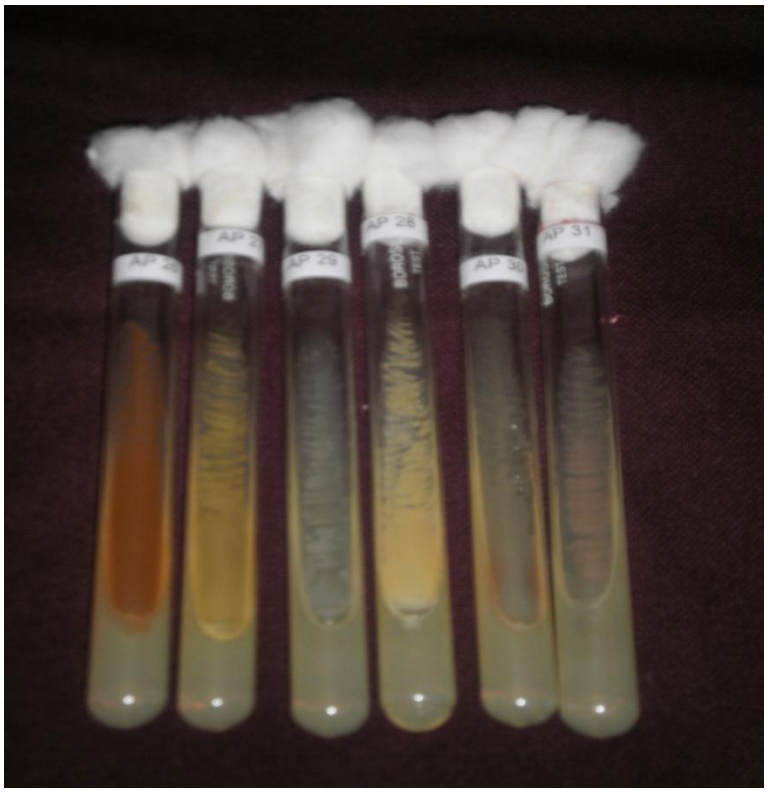


Table3: Screening of 44 isolates for the Arsenic biosorption in aqueous solution

S.no	Isolate number	Remaining arsenic in the system µg.ml^{-1}	Average Biosorption (%)	Biosorption (mg.g^{-1})
1	RA7	4.5	77.5±1.5	31±0.35
2	RA6	6.25	68.75±1.7	27.5±0.53
3	RA43	7.25	63.75±1.5	25.5±0.35
4	RA48	9.5	52.5±1.5	21±0.53
5	RA49	9.25	51.25±1.4	21.5±0.86
6	RA41	11.5	42.5±0.82	17±0.70
7	RA28	12.5	37.5±1.2	15±0.70
8	RA29	12.5	37.5±1.7	15±0.70
9	RA27	12.75	36.25±0.53	14.5±0.17
10	RA30	13.25	33.75±0.88	13.5±0.21
11	RA25	13.5	32.5±1.06	13±0.35
12	RA5	13.75	31.25±0.88	12.5±0.35
13	RA35	13.25	31.25±1.94	13.5±0.35
14	RA04	14	30.0±1.41	12±0.53
15	RA11*	14.25	28.75±1.23	11.5±0.35

……Table continue

S.no	Culture name /culture no.	Remaining arsenic in the system µg.ml^{-1}	Average Biosorption (%)	Biosorption (mg.g^{-1})
16	RA11*	14.25	28.75±0.88	10.5±0.53
17	RA8*	14.75	26.25±1.76	9±0.53
18	RA40	15.5	22.5±1.76	8.5±0.35
19	RA1	15.75	21.5±1.06	8.5±0.35
20	RA2	15.75	21.5±0.707	8±0.53
21	RA46	16	20±0.88	7.5±0.17
22	RA23	16.25	18.75±0.35	7±0.53
23	RA20	16.5	17.5±0.35	7±0.35
24	RA22	16.5	17.5±0.25	6±0.17
25	RA39	17	15±1.2	5.5±1.06
6	RA26	17.25	13.75±1.06	5±0.35
27	RA21	17.5	12.5±1.5	4.5±0.35
28	RA44	17.75	11.25±0.70	4±0.17
29	RA47	16.25	10±0.35	3.5±0.35
30	RA33	18.25	8.75±1.23	3.5±0.35

……Table continue

S.no	Culture name /culture no.	Remaining arsenic in the system µg.ml^{-1}	Average Biosorption (%)	Biosorption (mg.g^{-1})
31	RA36	18.25	8.75±1.23	3.5±0.53
32	RA45	18.25	8.75±1.23	3±0.53
33	RA24	19.25	7.5±1.41	2.5±0.35
34	RA32	18.75	6.25±0.35	2±0.17
35	RA12*	19	5±0.176	2±0.17
36	RA19	19	5±0.35	2±0.17
37	RA31	19	5±0.35	2±0.35
38	RA37	19	5±0.35	2±0.35
39	RA38	19	5±0.53	2±0.17
40	RA48	19	5±0.17	1±0.17
41	RA4	19.5	2.5±0.35	1±0.17
42	RA3	19.5	2.5±0.17	0
43	RA9*	20	0	0
44	RA10*	20	0	0

***denotes the arsenic resistant isolates**

Table 4 : Initial characterisation of all the isolates

source	Media	Microorganism	Gram staining	KOH test	Sporulating
Soil from Chemet chemical industry	N agar	RA1	Purple short rods	Gram positive	Central spore
		RA2	Pink rods	Gram negative	-
		RA4	Purple cocci	Gram positive	-
		RA5	Pink short rod	Gram negative	-
		RA6	purple rod	Gram positive	-
		RA7	Purple rod	Gram positive	-
	Nagar + arsenic (50ppm)	RA3	Purple rod	Gram positive	Central spores
		RA8	Purple short rods	Gram positive	Central spores
		RA9	Purple cocci	Gram positive	-
		RA10	Purple rod	Gram positive	Central spore
		RA11	Purple rod	Gram positive	-
		RA12	Purple long rods	Gram positive	Terminal spore
		RA13	Purple short rods	Gram positive	-
		RA14	Purple short rod	Gram positive	Central spores
		RA15	Pink rods	Gram negative	-
		RA16	Purple short rods	Gram positive	Central spore
		RA17	Purple very short rods	Gram positive	-
		RA18	Purple thin rods	Gram positive	-

.....Table continue

Source	Microorganism	Gram staining	KOH test	Sporulating
soil from Sriram chemicals	RA36	Purple rods	Gram positive	Central spores
	RA37	Purple rods	Gram positive	Central spores
	RA38	Purple short rods	Gram positive	-
	RA39	Pink rods	Gram negative	-
	RA40	Purple rods	Gram positive	Central spores
soil from Nirma campus	RA19	Purple rods	Gram positive	Central spore
	RA20	Purple short rods	Gram positive	-
	RA21	Pink rods	Gram negative	-
	RA23	Pink rod	Gram negative	-
	RA24	Pink rods	Gram negative	-
	RA25	Purple rods	Gram positive	Central spore
	RA26	Purple rods	Gram positive	Central spore
	RA27	Pink rods	Gram negative	-
	RA28	Purple rods	Gram positive	Central spore
soil near Petrol pump	RA30	Purple rods	Gram positive	-
	RA31	Purple rods	Gram positive	-
	RA32	Purple rods	Gram positive	-
	RA33	Purple rods	Gram positive	Central spores
	RA34	Purple rods	Gram positive	Central spores
	RA35	Purple rods	Gram positive	Central spores

…..Table continue

Source	Microorganism	Gram staining	KOH test	Sporulating
Soil near Metal casting industry	**RA41**	**Purple rods**	**Gram positive**	**-**
	RA42	**Purple rods**	**Gram positive**	**Central spore**
	RA43	**Purple long rods**	**Gram positive**	**Central spore**
	RA44	**Purple rods**	**Gram positive**	**-**
	RA45	**Purple rods**	**Gram positive**	**Central spore**
	RA46	**Pink rods**	**Gram positive**	**-**
	RA47	**Purple rods**	**Gram positive**	**Central spore**
	RA48	**Purple rods**	**Gram positive**	**-**
	RA49	**purple rods**	**Gram positive**	**-**

Out of the 49 isolates, 38 were Gram positive and 11 were Gram negative bacteria. Amongst 38 Gram positive isolates, 20 were sporulating and 18 were non sporulating.

Screened cultures showing more than 60 percent (RA07, RA06, RA43) Arsenic bioremediation were selected for the further studies.

Effects of different parameters on Arsenic bioremediation

Bioremediation of heavy metals is influenced by various parameters like pH, temperature, different bivalent cations, dry conditions etc. (kortal *et al.,* 2003)

Effect of static and shaking condition on Arsenic biosorption

Three cultures viz. RA07, RA06 and RA43 were giving more than 60 percent of arsenic bioremediation which were further characterize for biosorption in comparative manner in static and shaking conditions (shown in Figure 4) as well as for their efficiency to remove As(III) in the presence of the bivalent cations.

In comparision to the shaking condition the percent biosorption for the arsenic in the static system decreased in all the cultures. The results are supported by the work of kiran *et al.,* (2006) Cu^{+2} biosorption by algal biomass, when they increase the agitation efficiency of their systems, biosorption was also found increase.

The reason behind the decreased biosorption may be the less supply of aeration and agitation i.e. in the static conditions the system was not properly mixed so as to provide better bioactive sites for efficient metal biosorption.

Effect of presence of different bivalent cation on biosorption of arsenic by isolate RA 07 and RA06

RA43 was left for the further parameters studies because it was slow growing and was not giving reproducible results for the arsenic bioremediation.

Influence of different bivalent metal ion on biosorption of arsenic was done on RA07 and RA06. Biosorption of the arsenic was significantly decreased in both the isolates (Figure5). The similar results has been reported by Kaushik *et al.,* 2006 when they studied the biosorption of heavy metals (As, Cd, and Cu) by algal biomass *(Lyngbya putealis)* in the presence of metal salts.

This may be due the reason that the different bivalent cations compete with As(III) to bind with the active binding sites on the bacterial cell wall, as a result of which As was unable to

get adsorbed and remained in the system, hence the biosorption decreased (Ziagova *et al.*2007). Maximum negative influence was shown by Zn. Overall capacity of Arsenic biosorption of this biomass is reduced to less than 50 percent.

Figure 4: Effect of shaking and static conditions on As bioremediation by isolates RA43, RA07 and RA06.

System: 20ml
Isolates name: RA07, RA06, DH03
Biomass taken: Equivalent to 10mg dry weight
Metal conc.: 20 µg.ml^{-1}
Contact time: 60 min.
Shaking condition: 300 rpm

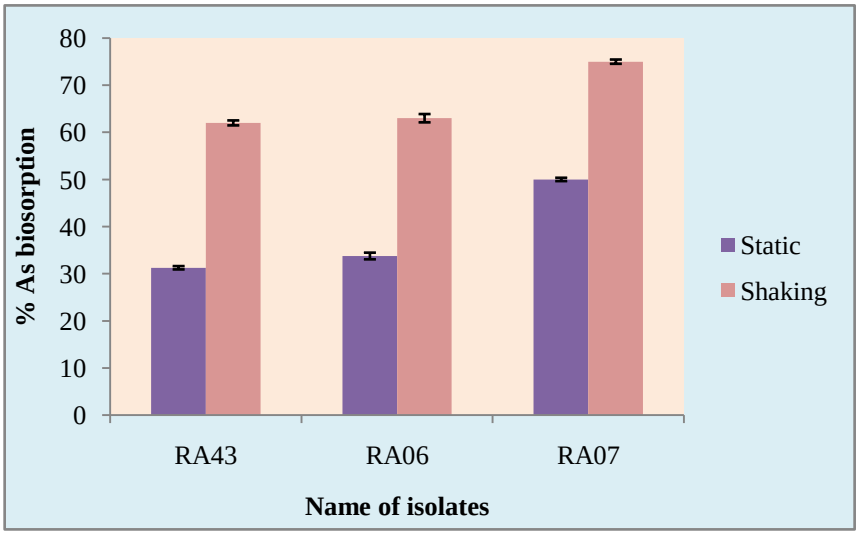
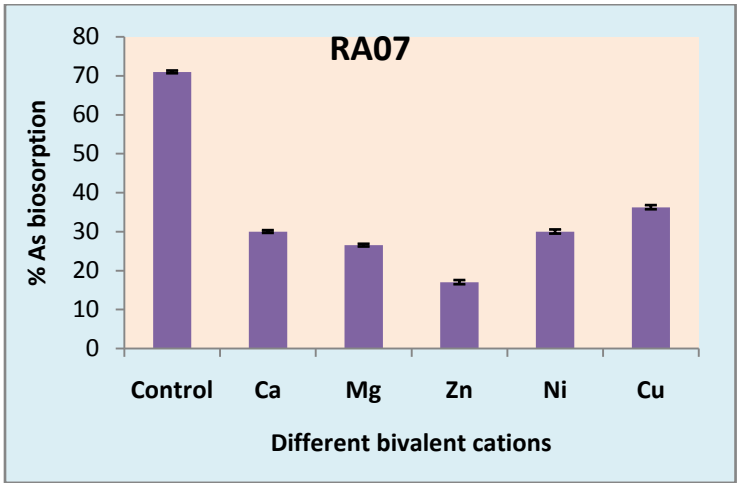
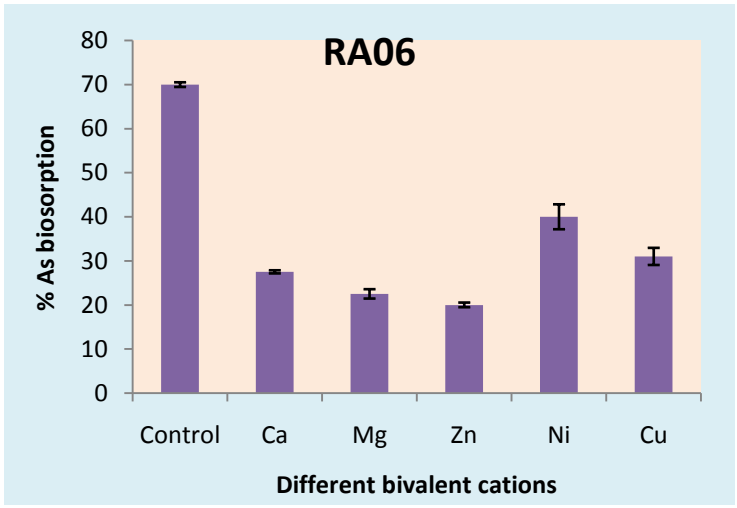


Figure5: Effect of presence of different bivalent cations on biosorption of arsenic.

Isolate taken: RA07,RA06 Contact time: 60 min.
System volume: 20ml Conc. of bivalent ions: 0.05mM
Biomass: Equivalent to 10mg dry weight Volume taken of bivalent metal solution: 1ml





Rest of the parameters were investigated for isolate RA07. Though there were no major difference in the biosorption ability of both the cultures, RA07 was preferred because it was found to be easily growing on the N-agar plate as compare to RA06, hence it was taken for further investigations.

Effect of different amount of biomass taken

Effect of different amount of biomass of isolate RA07 on biosorption of As(III) was investigated. When we increased the amount of biomass the biosorption first increased and after that started decreasing (Figure.6).

High biosorbent conc. are known to cause cell agglomeration and consequent reduction in the inter-cellular distance. This is reported to produce "screen effect" among the dense layer of cells, leading to protection of the binding sites from the metal ions. In other words, metal uptake is higher when the intercellular distance is more (at low biosorbent concentration), as this condition ensure optimal electrostatic interaction between cells, a significant factor for biosorption (Ruiz *et al.,* 1997). That is why in our case the amount of biomass higher than 10-30 mg had resulted lower As biosorption, hence 10mg optimized biomass was taken for all the studies.

Effect of pH on the Arsenic biosorption

The influence of pH on As(III) biosorption by bacterial isolate RA07 was investigated. The effect of pH was tested in the range of 2 to 9. The optimum range of pH obtained was 6 to 7 on which the maximum biosorption was studied, biosorption on the lower pH and pH above 8 was less (Figure7).

Generally low pH causes protonation of cell wall components which decreases the metal uptake of the biomass, whereas on increasing pH, the negative charge density increases, due to the deprotonation of the metal binding sites. At low pH the cell surface sites are closely linked to the H^+ ions, thereby making these unavailable for the cations. However, with an increase in pH, there is an increase in ligands with negative charges which results in increased binding of cations (Rafael *et al.,* 2003). Hence for all the studies, pH was taken as 7.

Figure6: Effect of different amount of biomass taken on isolate RA07

Isolate taken: RA07 Contact time: 60 min.
System volume: 20ml Biomass: 1, 5 ,10, 20, 30 ,40, 50 and 60 mg equivalent dry weight
pH:7±0.2

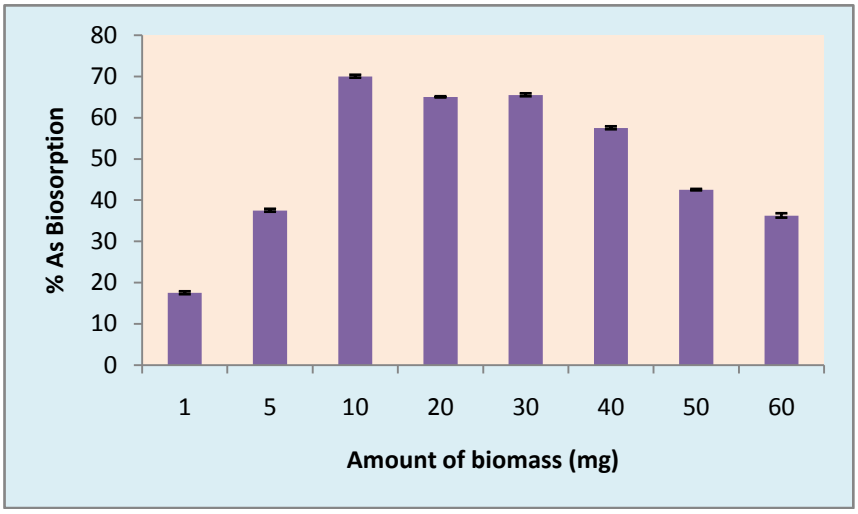
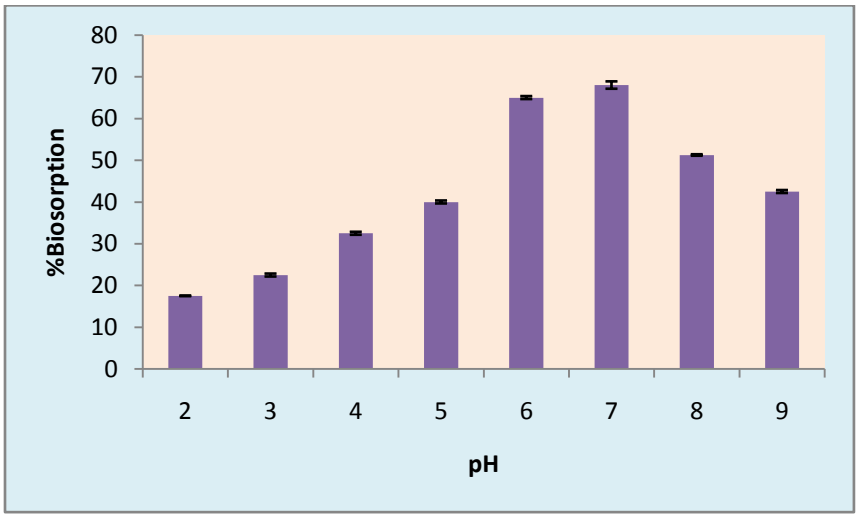


Figure7: Effect of pH on the biosorption of As(III)

Isolate taken: RA07 Contact time: 60 min.
System volume: 20ml Biomass added: 1ml (0.191ml biomass+0.809ml d/w)



Effect of different temperature

When the investigation were carried out for the biosorption of Arsenic(III) it was found that on increasing the temperature, biosorption was decreased (Figure8) and the optimum As bioremediation was obtained at 30º C.

The results of obtained were contradictory to the results obtained by (Umrania *et al.*, 2006). They had reported that on increasing temperature upto 55 to 60°C the amount of biosorption increased for Ag, Pb, Zn ,As, Ni (73%, 35.5%, 36%, 54%, 20%) respectively. This may be because the organism is sensitive to higher temperature and cannot function effectively for As biosorption as some cell activity may be involved in As biosorption, showing that multiple mechanisms are involved in As remediation by isolate RA07.

Effect of different conc. of Arsenic

Effect of As concentration on biosorption was investigated for 2.5, 5, 10, 20, 30, 40, 50, 60 µg.ml^{-1}. At the lower concentration (viz. 2.5, 5 and 10µg.ml^{-1}) the percent biosorption obtained was 96%, 90%, respectively which were very high values. On the other hand when the concentration of the arsenic was raised i.e. for 20, 30, 40, 50, 60 µg.ml^{-1} percent biosoprtion was found to be decreased (Figure9), the value obtained for 20µg.ml^{-1} was 70%.

The high biosorption capacity at low metal concentrations due to dilution of the metal in the system to allow efficient bioremediation whereas As concentration higher than 20µg.ml^{-1} showed low As biosorption potential. For As concentration 20-60 µg.ml^{-1} the As bioremediation range between 65 to 70 percent.

This may indicate that at the lower concentration of the metal bacterial metal binding sites wcan be easily available for the biosorption but as the concentration increases the metal occupies all the binding sites and the system reached at equilibrium state i.e. no more metal can bind to the binding sites of the bacteria (Ziagova *et al.*, 2007).

Fig8: Effect of temperature on biosorption of As(III)

Isolate taken: RA07 Contact time: 60 min.
System Volume: 20ml Biomass: Equivalent to 10mg dry weight
pH:7±0.2 Temperature: 30, 40 and 50°C

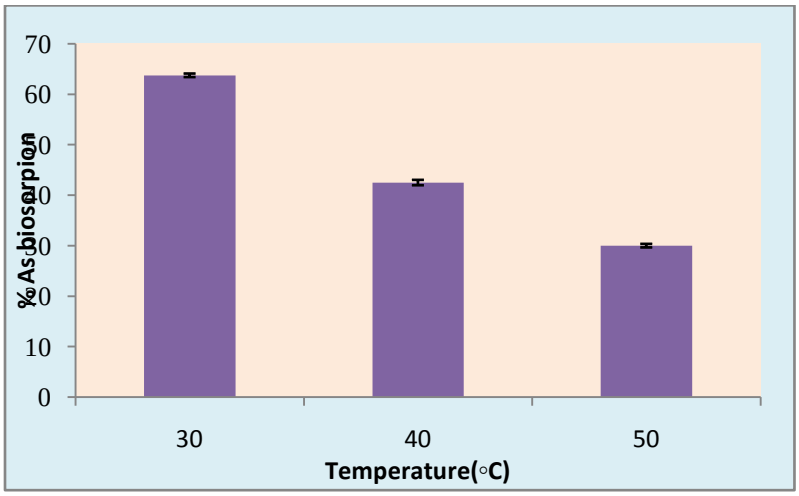
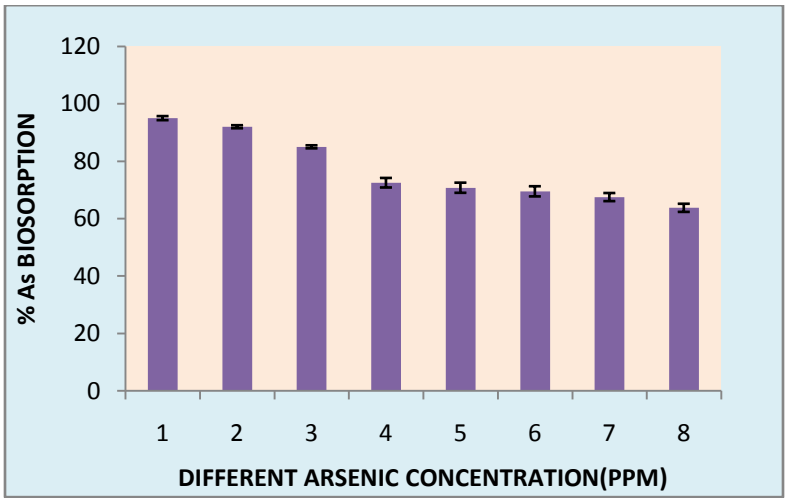


Fig9: Effect of different concentration of arsenic

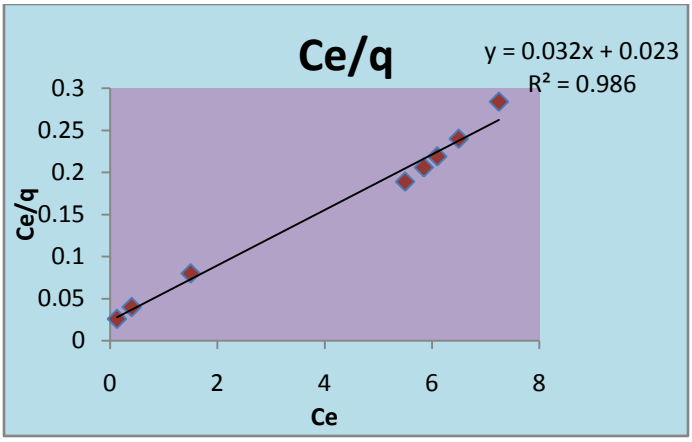
Isolate taken: RA07 Contact time: 60 min.
System volume: 20ml Biomass:10mg equivalent dry weight
pH;7±0.2



Isotherms study

Done on the parameter different arsenic concentration as discussed above and the following results were obtained when Langmuir and Freunlich isotherms were studied.

Langmuir isotherm



Freundlich isotherm

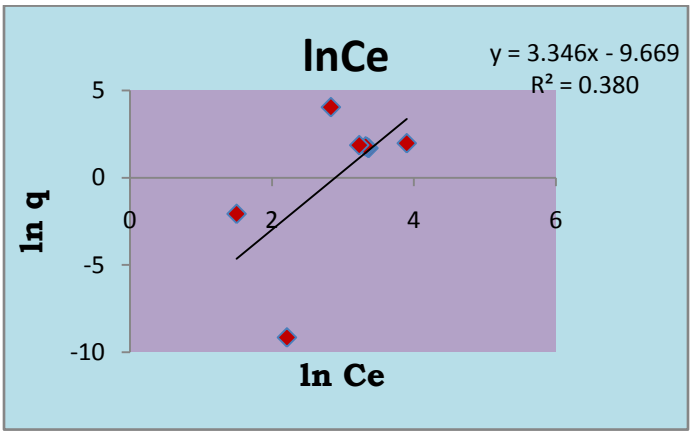


Table5: Freundlich and Langmuir constants for arsenic biosorption by isolate RA07 at 30°C, for 60 min and at pH 7

Isotherm type	qmax($mg.g^{-1}$)	Kd(mgL^{-1})	1/n	Kf($mg.g^{-1}$)	r^2
Freundlich	-	-	3.346	-9.10	0.380
Langmuir	31.25	1250	-	-	0.986

Discussion for isotherms

The analysis of the data for isothermic studies is depicting that biosorption mechanism is more supported by Langmuir isotherm since the value of R^2 is high in case of this isotherm. Langmuir model is valid for modeling monolayer adsorption onto a homogeneous surface with constant adsorption energy, the results obtained are more comparable with Langmuir isotherm (Gazso, 2001).

Freundlich equation posits a heterogeneous surface and considers that molecules attached to a surface site surface site have an affect on the neighbouring sites (Volesky,1990b). The results obtained from the studies of isotherms were not supported by Freunlich model and further investigations are required for the data analysis.

Effect of different cycles on arsenic bioremediation

The experiment was conducted in two ways. First in which after first cycle the biomass was removed and fresh biomass added for second cycle whereas second case after first cycle additional biomass was added without removing the previous biomass (Figure10).

In other case once the maximum active sites were bound by As(III), fresh biomass added in second cycle gave more active sites for free As(III) as well as providing chance for remaining free active sites of earlier biomass to fully saturate. Also in both the cycles, metal concentration varies which is encountered by the two sets of biomass being responsible for the observed results (Tuzen *et al.*,2009)

Effect of sonication

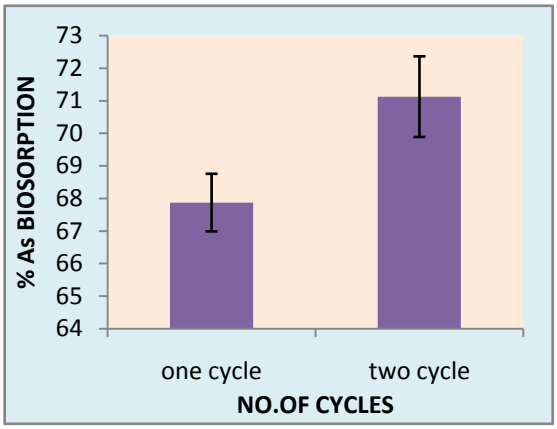
Data shown in the Figure11 suggest that sonication has negative effect even for one minute for arsenic biosorption. This was done to check whether the mode of biosorption by isolate is physical or metabolism dependent phenomenon. The result shows that increase in the time of sonication treatment leads to decrease in arsenic removal. This may be because of lysis of some cells upon sonication.

Figure10: Effect of cycles on the Arsenic bioremediation

Isolate taken: RA07 Contact time: 60 min.

System volume: 20ml Biomass: 10 mg, 5mg equivalent dry weight

pH:7±0.2



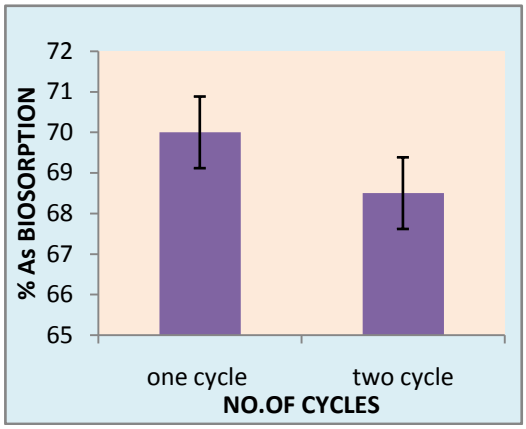
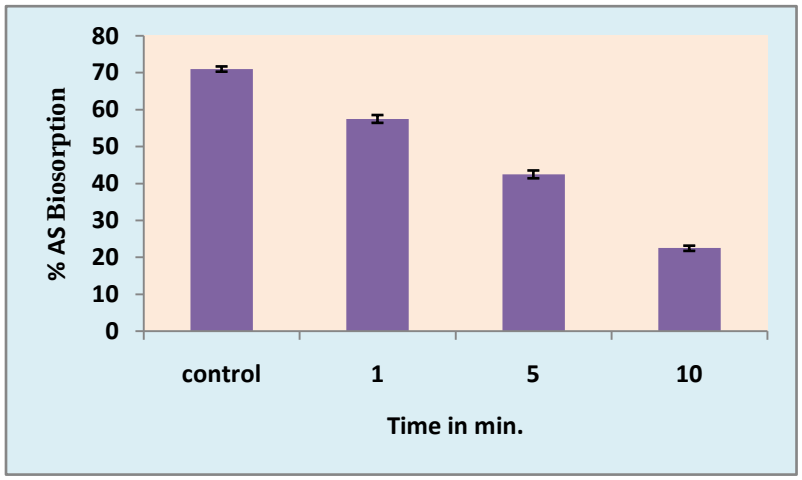


Figure11: Effect of sonication on Arsenic bioremediation

Isolate taken: RA07 Contact time: 60 min.

System volume: 20ml Biomass:10mg equivalent dry weight

pH:7±0.2



Effect of Drying on the As biosorption by isolate RA07

Pretreatment and killing of biomass either by physical or chemical treatments are known to improve biosorption capacity of biomass. Goksungur *et al*. has reported that heat treated yeast cells gave higher metal uptake than untreated biomass.

Effect of pre-treatment of biomass by temperature was investigated at 60°C, 100°C, 150°C for 60 minutes on the biomass of isolate RA07 and obtained data are shown in the (Figure12). The reduction in arsenic removal was found to be directly related with the increase in biomass treatment temperature. That is, with increased temperature used for pretreatment of biomass the effect was more drastic on arsenic biosorption. So the data obtained here are contradictory to the results reported earlier. This may be because the cells may be sensitive to high temperature stress and affecting viability of cells & viability of cells is required for effective arsenic biosorption.

Effect of contact time on Arsenic Biosorption by isolate RA07

Contact time is one of the important parameters for successful use of a biomass for practical application and rapid sorption is among desirable parameter. The experiment was conducted for 1, 2, 5, 10, 30, 60, 120, 300 minutes. Figure13 shows the effect of contact time on the biosorptive removal of arsenic by isolate RA07. It can be observed from the fig that equilibrium reached within 60 to 120 min. However there is increase in biosorption before contact time of 60 min. Gupta VK *et al*, (2008) indicated that maximum sorption took place within first 90 and 70 min for *Oedogonium sp.* and *Nostoc sp.*, respectively.

Figure12:Effect of Drying on the arsenic biosorption

Isolate taken: RA07 Contact time: 60 min

System volume: 20 ml Biomass:10 mg equivalent dry weight

pH: 7±0.2 Pretreatment by drying at 60 ºC,100 ºC and 150ºC

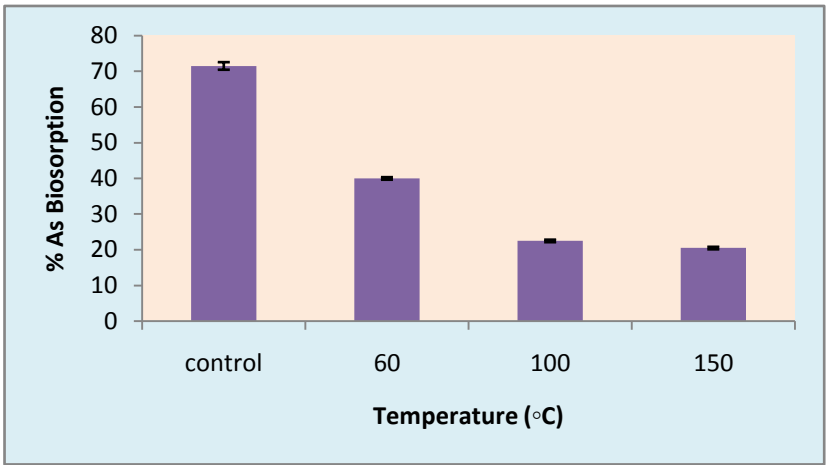


Figure13:Effect of contact time on the Arsenic biosorption

Isolate taken: RA07 Contact time: 1, 2, 5, 10, 30, 60, 120 and 300 min

System volume: 20ml Biomass: 10 mg equivalent dry weight

pH: 7±0.2

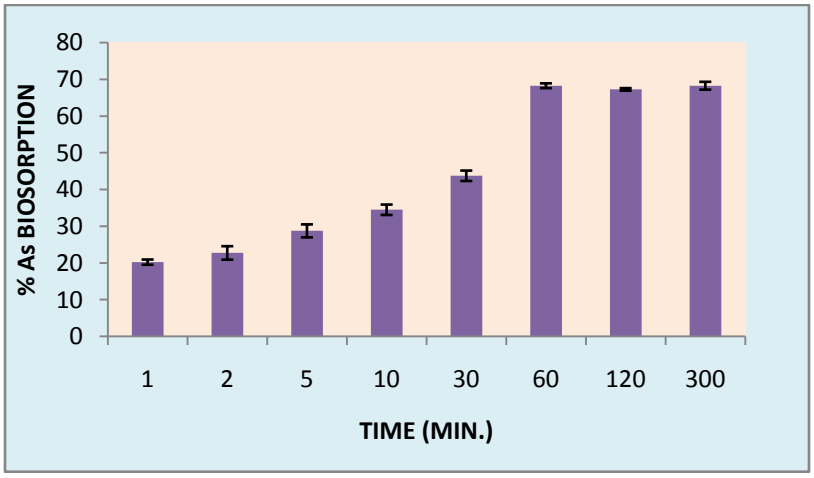


Table5: showing the biosorption of arsenic by different biomass

S.no.	Name of the biomass used	Biosorption ($mg.g^{-1}$)	Refrences
1	*Leptothrix ocracea*	25	Zouboulis *et. al.,* 2004
2	*Cocus nucifera*	6.6-165	De souse et.al., 2009
3	*Lyngbya putealis*	8-48	Kaushik *et. al.,* 2007
4	*Penicillum purpurogenum*	3.4	Say *et. al.,* 2007
5	*B. cinerea*	12.98	Tanali *et. al.,* 2006

The above is showing biosorption capacity by different types of biomass and we did experiment on bacterial isolate RA07 which was giving maximum biosorption capacity of 31 $mg.g^{-1}$ of biomass at 20 $\mu g.ml^{-1}$ concentration of arsenic in 20 ml sytem volume.

Table6: Results of different biochemical tests of selected isolates

TEST NAMES	RA07	RA06	RA43	RA48	RA49
Carbohydrate fermentation test:					
• Glucose	+	+	+	+	+
• Sucrose	-	+	+	+	+
• Maltose	+	+	+	+	+
• Mannitol	+	+	+	+	-
• Lactose	+	+	-	+	-
• Xylose	-	+	+	+	-
Hugh and leifson test:					
• Oxidative metabolism	-	-	+	-	-
• Fermentative Metabolism	+	+	–	+	+
• Nonsacchrolytic					
M R Test	-	-	-	-	-
V P Test	+	+	+	+	+
Citrate utilization test	+	+	+	-	+
Indole production test	+	+	-	+	+
H_2S production test	-	-	-	-	-
Lead acetate test	-	+	-	+	_
Phenylalanine Deamination test	+	+	+	+	_
Urea hydrolysis test	+	-	+	+	-
Decarboxylase test					
• **Lysine**	-	-	-	-	-
• **Ornithine**	-	-	-	-	-
Nitrate reduction test	+	+	-	+	-
Ammonia production test	-	+	-	+	-
Caesin hydrolysis test	-	-	-	-	-
Gelatin hydrolysis test	-	-	-	-	-

Starch hydrolysis test	+	+	+	-	+
TSI test: • Non-fermentor • Lactose non-fermentor • Lactose fermentor • H_2S production	–	-	+	+	-
	-	+	-	-	-
4°C	-	-	-	-	-
44°C	-	+	-	+	-
Macconky test	-	-	-	-	-
EMB test	-	-	-	+	-
2% NaCl	+	+	+	+	+
5% NaCl	-	-	-	+	+
pH 4	-	-	-	-	-
pH 9	+	+	+	+	+
Dehydrogenase test	-	-	-	+	-

Table7: Identification of isolates on the basis of morphology and biochemical test

All the identification was done by Bergey's manual and The Prokaryotes

Isolate name	**Tentative identification**
RA07	*Corynebacterium* spp.
RA06	*Clavibacterium mishiganensis*
RA43	*Bacillus coagulans*
RA48	Unidentified
RA49	*Corynebacterium amycolatum*

Conclusion

From the series of experiments carried out on the bioremoval of arsenic, following conclusion can be drawn.

- Work was done on forty nine isolates out of which RA07, RA06, RA43, RA48, RA49 were taken for optimization, characterization and identification.
- Initial characterization was done by Gram's staining and 3% KOH test for forty-nine isolates. Primary screening for all the forty-nine cultures were done for arsenic biosorption.
- Static conditions negatively influences the biosorption of arsenic by the isolate RA07 in comparision to shaking condition.
- In the presence of different bivalent cations the biosorption of arsenic significantly decreased.
- Maximum biosorption was obtained by 10 mg equivalent dry weight of biomass.
- The bioremoval process was found to be pH dependent and the range obtained was 6 to 7.
- Temperature change (30ºC, 40ºC and 50ºC) during the process detrimentally decreased the biosorption capacity.
- Biosorption of arsenic was found to be maximum at range of 20 to 30 $\mu g.ml^{-1}$.
- Sonication treatment detrimentally decreased the biosorption of arsenic.
- Dry conditions adversely affected biosorption of arsenic.
- Maximum removal of the arsenic was obtained upto 120 min of contact time.
- Different cycles also affected positively and negatively the arsenic biosorption.

Finally, further studies can be done for the desorption and recovery of the metals from bacterial isolates, moreover isolates can be used for scale up phenomenon.

Studies on molecular mechanisms involved in the biosorption phenomenon can also be done on the isolate. Various statistical and other analysis can be done on the data reported.

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