# RESEARCH PLAN PROPOSAL

## DETOXIFICATION OF ARSENITE [As(III)] USING ARSENITE HYPER-TOLERANT BACTERIA

For registration to the degree of

**Doctor of Philosophy**

**IN THE FACULTY OF SCIENCE**

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## INTRODUCTION

Concern for environmental quality has stimulated scientific studies of the chemical behaviour and biological effects of metal contamination in the hydrosphere and lithosphere. A large number of metals are essential for growth, metabolic activities and various other body functions but some are harmful for living cell. From the standpoint of environmental pollution, heavy metals and metalloids are extremely toxic because of their relative accessibility to biological systems (Wood, 1974). This heavy metal and metalloid toxicity is mainly due to the fact that they form complexes with protein molecules which render them inactive, for example, inactivation of enzymes (Kim, 1985). Among the metalloids, arsenic is of major concern due to its hazardous toxicity.

Arsenic (As) is the third element in Group V of the periodic table, thus classified as a metalloid (Wackett *et al.*, 2004). It is having an atomic number of 33 and an atomic weight of 74.92. It was first documented by Albertus Magnus in 1250 (Emsley, 2001). It ranks $20^{th}$ in abundance in the earth crust, $14^{th}$ in sea water and $12^{th}$ in human body (Woolson, 1975). In nature, arsenic exists in three allotropic forms (alpha or yellow; beta or black; gamma or grey) and several ionic forms (Norman, 1998).

Arsenic toxicity is highly dependent on its oxidation state: trivalent arsenicals are at least 100 times more toxic than pentavalent derivatives (Nakamuro and Sayato, 1981). High intake of arsenic is a major risk for human health resulting in irritation of the stomach and intestine, decreased production of white and red blood cells, skin changes, lung irritation, heart disruptions including nerve injury, stomach aches and cancers.

Microorganisms catalyze many transformations of arsenic species including oxidation, reduction and methylation reaction (Cullen and Reimer, 1989; Dowdle *et al.*, 1996). Such metal transformation represents a key component to metal cycling in natural systems (Lloyd & Lovely, 2001). The ability of microorganisms to grow in high metal concentration results from specific mechanism as extracellular precipitations and exclusion of metal ions, binding of metal ions to the outer surface of bacteria and its intracellular sequestration (Satchanska *et al.*, 2005). The oxidation states of a metal ion may determine its solubility, thus many scientists have been trying to use microbes that are able to oxidize or reduce heavy metal like arsenic in order to remediate contaminated sites.

Arsenic exists mainly in two toxic soluble forms, Arsenite [As(III)] and Arsenate [As(V)]. Unlike organic contaminants, which are degraded into harmless chemical species, arsenite cannot

be destroyed, but it can be transformed into less toxic forms (Arsenate). But this chemical oxidation of arsenite to arsenate is very slow process. Moreover, the physico-chemical arsenic treatment processes require strong oxidants, which are expensive and leads to secondary pollution. The bacterial oxidation of As(III) can thus contribute to a natural attenuation of arsenic contamination by decreasing arsenic bioavailability. Bacteria may possess this arsenite oxidation capability either as its detoxification mechanism or to gain energy, where As(III) acts as an electron donor and $CO_2$ as a carbon source. Many researchers have reported some regulatory *ars* operons which confer bacterial resistance for arsenic. Specifically for arsenite oxidation, *aox* genes have been reported which is responsible for the production of an enzyme arsenite oxidase which catalyzes the bio-oxidation of arsenite As(III) to arsenate As(V).

Therefore, it seems important to isolate bacteria from heavy metal contaminated environment which possess mechanism to resist arsenite stress followed by its biotransformation into arsenate, hence, leading to the bioremediation process of native polluted sites.

# REVIEW OF LITERATURE

## Arsenic and its distribution in the environment

Arsenic is recognized as one of the most toxic oxyanion in the natural environment, which cause severe contamination of soil-water systems and subsequent endemic arsenicosis worldwide. It enters the environment, as the result of both geogenic processes and anthropogenic disturbances. Geogenic sources include natural weathering of rocks, burning vegetation, volatilization and volcanic dust fluxes (Chilvers and Peterson, 1987). It occurs naturally in a wide range of minerals, which together with a widespread use of arsenic in pigments, insecticides, and herbicides, represents the major sources of arsenic in natural waters (Kumaresan and Riyazuddin, 2001). Anthropogenic disturbances as the arsenic sources include use of pesticide, herbicide, wood preservatives and dye stuff as well as production of arsenic containing waste during smelting and mining operations. The primary anthropogenic input derives from combustion of municipal solid wastes, fossil fuels in coal and oil-fired power plants, release from metal smelters and direct use of arsenic containing herbicides by industry and agriculture. Arsenic is also used in the production of glass and semiconductors. In arsenic enriched environment, a major concern is the potential for mobilization and transport of this toxic element to ground water and drinking water supplies (Salam *et al*., 2009).

High concentration of arsenic in ground water has been reported in several countries including Argentina, Bangladesh, Chile, China, India, Japan, Mexico, Mongolia, Nepal, Poland, Taiwan, Vietnam and some parts of United States of America (Anwar *et al*., 2002; Mitra *et al*., 2002; Pandey *et al*., 2002; Smith *et al*., 2001; Chowdhary *et al.,* 2000). The arsenic calamity of Bangladesh can be described as the largest known mass poisoning in the history with an estimated 35-77 million people exposed to arsenic contaminated drinking water (Rabbani *et al*., 2002). This incident serves as an unfortunate reminder of toxic consequences of arsenic mobilization and underscores the need to understand the factors controlling the mobility and solubility of arsenic in aquatic system (Newman *et al*., 1998). According to Union Ministry of Water Resource, the underground water of eight districts of West Bengal and one district of Bihar are arsenic contaminated. Arsenic in drinking water was found to be much above 10µg/litre (WHO recommended provisional guideline value) in the middle Ganga plains of Bihar and U.P. (Chakraborty *et al.,* 2003). Reports on the occurrence of arsenic in ground water resources and the

associated health hazards due to human consumption have been made from various parts of India and world.

## Arsenic Toxicity

Arsenic occurs in several oxidation states (-3, 0, +3, +5). Inorganic forms of arsenic are more toxic than organic forms (Hopenhayn *et al*., 2006). The major arsenic species found in clinical and environmental samples are arsenate, arsenite, arsenous acids ($H_2AsO_3$), arsenic acids ($H_2AsO_4$), monomethylarsonate (MMA), dimethylarsinate(DMA), arsenobetaine(AB), and arsenocholine(AC) (Kumaresan and Riyazuddin, 2001). The trivalent form i.e. Arsenite [As(III)] is 100 times more toxic and mobile than its pentavalent form i.e. Arsenate [As(V)] (Nakamuro and Sayato, 1981, Cullen and Reimer, 1989; Neff, 1997). Both of these forms are toxic and show their toxicity as As(III) reacts with thiol group (-SH), whereas As(V) uncouple oxidative phosphorylation.

Inorganic As(III) inhibits pyruvate dehydrogenase by binding to the sulfhydryl group of dihydrolipoamide. Consequently, the conversion of pyruvate to acetyl coenzyme A (CoA) is decreased; citric acid activity is decreased thereby lowering the production of cellular ATP. The trivalent arsenic inhibits cellular glucose uptake, gluconeogenesis, fatty acid oxidation and further production of acetyl CoA; it also blocks the production of glutathione, which prevents cellular oxidative damage. Due to unionized form at neutral pH, arsenite can passively move across the membrane bilayer or be transported by a carrier protein similar to those that transport unionized organic compound (Marcus, 2009).

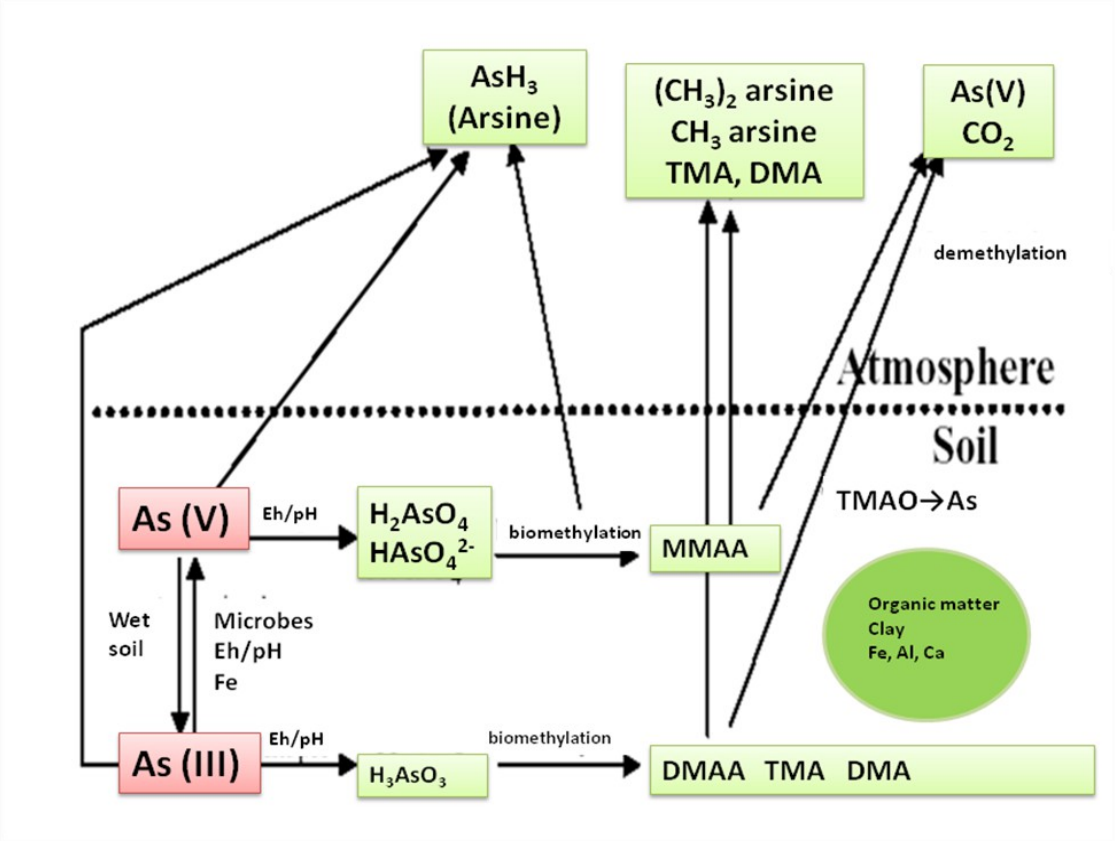
Effects of pentavalent inorganic arsenic As(V), occurs partially because it gets readily transformed into trivalent arsenic and its toxicity proceeds thereafter. More importantly, pentavalent arsenic resembles inorganic phosphate and substitute for phosphate in glycolytic and cellular pathways. High energy phosphate bonds are made and uncoupling of oxidative phosphorylation occurs. For example, in the presence of pentavalent arsenic, adenosine diphosphate (ADP) forms ADP-arsenate instead of ATP. This way the higher energy phosphate bonds of ATP are lost (Marcus, 2009).

In recent years concerns has increased about the release of arsenical compounds in the environment and their toxicity to a wide variety of organisms, including humans. There has been much information on the biological effects of arsenic compounds on mammals, arsenic is able to induce cell transformation (Lee *et al*., 1985), gene amplification in murine cells (Lee *et al*., 1988), gene damage in human alveolar type-II cells (Tezuka *et al*., 1993), and is co-mutagen agent in

exposed hamster cells (Lee *et al.,* 1988). Arsenic compounds elicit a cellular stress response similar to heat shock protein synthesis (Caltabiano *et al.,* 1986; Deaton *et al.,* 1990). Arsenic causes lung and skin cancers in humans (Leonard and Lanwerys, 1980; Pershagen, 1985; Schneidman and Belizaire, 1986). A close relationship appears to exist between ingested inorganic arsenic and bladder, kidney and liver cancers in human (Bates *et al*., 1992). Chronic arsenic poisoning can also cause melanosis (hyperpigmentation or hypopigmentation or white spots), hyperkeratosis (harden skin), restrictive lung disease, peripheral heart disease (black foot disease), gangrene, Diabetes mellitus, hypertension and ischaemic heart disease (Das *et al.,* 2004). In women high concentration of inorganic arsenic can cause infertility and miscarriages and also declining resistance to infections, brain damage and cardiovascular effects (Walton *et al*., 2004). *In vitro* experiments have shown multiple effects at the molecular level following arsenic exposure including differential expression of genes involved in the cell cycle regulation, signal transduction, stress response, apoptosis, cytokine production, growth factor and hormone receptor production (Hossaine *et al.,* 2000; Tabelline *et al*., 2005). In *in vivo* conditions, inhibition of gut flora of rats was observed with a decrease in stool arsenic level and an increase in liver arsenic level. However, the presence of vitamin E reduced the arsenic toxicity (Mittal and Flora, 2007).

## Resistance and Biotransformation of Arsenic in Bacteria

The ubiquity of arsenic in the environment has led to the evolution of arsenic defence mechanism in microbes. The biogeochemical cycle of this element strongly depends on microbial transformation that affects the mobility and distribution of arsenic species in environment (Tamaki and Frankenberger, 1992). Despite of its toxicity, a number of micro-organisms are capable of using either the oxidized form of inorganic As(V) or the reduced form As(III) in their metabolism (Silver and Phung, 2005). These redox reactions are generally carried out by micro-organisms either for detoxification or for energy generation to support cellular growth. Micro-organisms have evolved a variety of mechanisms for triumphing the arsenic toxicity, including minimizing the amount of arsenic that enters the cell (e.g. through increased specificity of phosphate uptake), catalyze transformations of arsenic including oxidation, reduction, and methylation (Cullen and Reimer, 1989; Dowdle *et al*., 1996; Newman *et al*., 1998).




**TMA- Trimethylarsenate** **DMA- Dimethylarsenate** **MMA- Monomethylarsenate**

**DMAA- Dimethylarsonic acid** **MMAA- Monomethylarsonic acid**

**Biogeochemical cycle of Arsenic (O´Neill 1990; Tonner-Navarro *et al.*, 1998; Jain and Ali, 2000)**

Microbial resistance to arsenite have been frequently observed from sources like monitoring wells (Zelibor *et al.,* 1987), soil (Jaroenmit *et al.,* 1987), agricultural drainage water and evaporation tanks (Huysman and Frankenberger, 1990), aquifers in West Bengal (Suresh *et al.,* 2004 ab), bio-oxidation tanks (Rawling, 2008), arsenic contaminated sites (Cai *et al.,* 2009a), Gold mine and bioreactor (Dave *et al.*, 2010) all of which have been reported to be contaminated with arsenic.

Some microbial strains possess genetic determinants that confer resistance to arsenic. Genetic determinants for arsenic resistance in bacteria can be either chromosomal or plasmid (Silver and Misra, 1988; Silver and Walderhang, 1992; Cervantes *et al.*, 1994). Bacteria such as *Escherichia coli* (Touw *et al.,* 2007), *Acidithiobacillus caldus*, *Leptospirillum ferriphilum* (Rawlings, 2008) and *Cornyebacterium glutamicum* (Mateos *et al.,* 2006), *Microbacterium oxydans, Achromobacter sp.* and *Ochrobactrum anthropi* (Aksornchu *et al.,* 2008) have evolved arsenic resistance using *ars* operon that is regulated by *ArsR,* a repressor protein that dissociates from DNA when As(III) bind

(Touw *et al.,* 2007). Chang *et al.,* 2007 studied the DNA sequence homology analysis of *ars* genes in arsenic resistant bacteria isolated from soils and sediments of Gold mines highly contaminated with arsenic. This study showed that arsenite oxidation is mediated by *ars*AB genes encoding the efflux pump as well as *ars*R and *ars*D regulatory genes. The *ars*R and *ars*D leader gene are required for an arsenic resistance system when the high homology genes are controlled by the *ars* inducer-independent regulatory amino acid sequence. In addition, the strains with the ability of As(V)-reduction involved the *ars*C gene homologues. Plasmid governed bacterial resistance to arsenic was first discovered by Novick and Roth (1968), in a group of *Staphylococcus aureus* β-lactamase plasmid.

Due to this resistance mechanism, bacterial strains have been observed to tolerate varying levels of arsenite as up to 1.0 gm/L (Jaroenmit *et al.,* 1987; Huysmans and Frankenberger, 1990), 2-80 mM (Chen and Shao, 2009), 10mM (Joshi *et al.,* 2009), 100ppm (Salam *et al*., 2009), 40 mM (Chitpirom *et al.,* 2009), 120 mM (Dave *et al.,* 2010).

## Bioremediation using arsenite oxidizing bacteria

Unlike organic contaminants, which are degraded into harmless chemical species, Arsenite cannot be destroyed, but it can be transformed into less toxic forms (Arsenate). Conventional methods for removing Arsenic from industrial effluents include oxidation, co-precipitation and adsorption onto coagulated flocs, lime treatment, adsorption onto sorptive media, ion exchange resin and membrane techniques. These processes are ineffective and extremely expensive. Furthermore, the oxidation of arsenite to arsenate is a pre-requisite for all of the treatment processes. The oxidation of arsenite by chemical process requires strong oxidants as chlorine, hydrogen peroxide, ozone etc (Kim and Nriagu, 1999), which is slow (Tamaki and Frankenberger, 1992), expensive and also creates secondary pollution.

Bacteria capable of oxidizing arsenite to arsenate could be one of the promising strategies for bioremediation; as an alternative to supplement existing physico-chemical method of arsenic oxidation. Various heterotrophic bacteria have been reported which generally oxidize As(III) for detoxification (Anderson *et al.,* 1992; Martin and Pederson, 2004). Similarly, various reports have been available on arsenite oxidation by Chemoautrophic or Chemolithotrophic bacteria, which use As(III) as an electron donor and carbon dioxide as a sole carbon source (Santini *et al*., 2000; Bernhardt and Santini, 2006).

*Bacillus arsenoxydans* from cattle dipping fluids in South Africa was the first ever reported arsenite oxidizing bacteria by Green, 1918. Thereafter, many bacterial strain capable of oxidizing arsenite into arsenate like *Alcaligenes faecalis* (Philips and Taylor, 1976; Santini *et al.,* 2002), *Agrobacterium* from gold mines, Australia (Santini *et al.,* 2000), from aquatic macrophytes (Salamassi *et al.,* 2002), *Acinetobacter, Flavobacterium, Pseudomonas, Sinorhizobium, Sphingomonas* from arsenic contaminated soil, Thailand (Kinegam *et al.,* 2008), *Pseudomonas strutzeri* strain GIST-BDan2 from natural and constructed wetlands (Chang *et al.,* 2010) and *Pseudomonas lubricans* from heavy metal laden industrial waste water (Rehman *et al.,* 2010), have been isolated. Various extremophilic environments also harbour arsenite oxidizing bacteria like *Hydrogenobacter* strain, *Sulfurihydrogenibium* strain from geothermal arsenic contaminated environment, Russia (Hamamura *et al.,* 2010), *Alkalilimnicola ehrlichii* from Mono Lake, an alkaline hyper-saline salt lake, California (Hoeft *et al.,* 2007), *Thermus aquaticus* and *Thermus thermophilus* from hot springs, thermally pollutes domestic and industrial waters (Gihrang *et al.,* 2001).

## Genes and enzymes responsible for arsenite oxidation

Chromosomally determined arsenite resistance generally results from oxidation of As(III) which represents a potential detoxification process in microorganisms. The *aoxB* genes as a functional marker of aerobic As(III) oxidisers are responsible for this oxidation which results in the formation of As(III) oxidase facilitating biotransformation of As(III) to As(V) species (Quemeneur *et al*. 2008). The genes *arsR, arsD, arsAB, arsA, arsB, arsC, arsH, arrA, arrB, aoxA, aoxB, aoxC, aoxD, aroA* and *aroB* may be useful for arsenite oxidizing bacteria in abandoned arsenic contaminated mines. *Ochrobactrum tritici* SCII24, a heterotrophic organism contains two different *ars* operons and is able to oxidize arsenite to arsenate. The presence of arsenite oxidase genes in this organism was evaluated, and sequence analysis revealed structural genes for an As(III) oxidase (*aoxAB*), a *c*-type cytochrome (*cytC*), and molybdopterin biosynthesis (*moeA*) (Branco *et al.* 2009). Of the arsenite-oxidizing bacteria, *Alcaligenes faecalis* (Anderson *et al*., 1992), NT-26 (Santini and vanden Hoven, 2004), and NT-14 (vanden Hoven and Santini, 2004) have been studied in detail and their arsenite oxidases purified and characterized.

Moreover, a crystal structure of the *Alcaligenes faecalis* arsenite oxidase has been elucidated (Ellis *et al*., 2001). Enzymes from different species appear to vary considerably. For example, the enzyme of *Alcaligenes faecalis* is a $\alpha_1\beta_1$ dimer (Ellis *et al.,* 2001) while that from the

chemolithoautotroph arsenite oxidizer NT-26 (Santini and vandenHoven, 2004) is a $\alpha_2\beta_2$ tetramer, and the enzyme from *Hydrogenophaga* is a $\alpha_3\beta_3$ hexamer (vandenHoven and Santini, 2004). Genes encoding As(III) oxidases (*aox*) have also been identified and sequenced in several organisms, showing a common genetic organization, *aoxA-aoxB*, that encodes the small and large subunits, respectively. These *aox* operons usually contain additional genes, e.g., *cytC*, which encodes a cytochrome *c*, and *moeA*, which encodes an enzyme involved in molybdenum cofactor biosynthesis (Silver and Phung, 2005).

## Development of a bioprocess for arsenic bioremediation

These bacterial properties, like high tolerance to arsenite, its oxidizing ability with the help of *aox* genes and the enzyme arsenite oxidase, have been used to develop a whole cell immobilized bioprocess for removing arsenic from a mining effluent by using the activity of arsenic metabolizing bacteria indigenous to the contaminated site (Battaglia-Brunet *et al.,* 2006). Cell immobilization has some advantages when compared with free cell culture (Abd-El-Haleem *et al*., 2003). The reaction speed can be accelerated, it is less susceptible to the effect of inhibitory compounds and nutrient depletion, protect the cells against damage and (Marques *et al.,* 2006) reduced susceptibility to contamination. Also, cell immobilization increases productivity and stability, ease of separation, repeated use etc. (Prabakaran and Hoti, 2008).

Mokashi and Paknikar, 2002 isolated *Microbacterium lacticum* from mining sewage which was able to oxidize upto 50 mmole $lit^{-1}$ As(III). The culture was immobilized on brick pieces and these pieces were packed into a glass column to check the complete oxidation of As(III). The oxidised As(V) was then removed from ground water using 3 different methods: zero-valent iron, activated charcoal and ferric chloride.

Simeonova *et al.,* 2005 described As(III) oxidation by immobilized cells in the batch reactors with an alginate immobilized heterotrophic ULPAs1 strain As(III) oxidizers.

Lugtu *et al.,* 2009 isolated arsenite oxidizing bacteria from mine tailing, *Ensifer adharens* LMG20216 and *Sinorhizobium* sp. CAF63, which grew chemolithotrophically and used As(III) and $CO_2$ as electron donor and carbon source respectively. One of these oxidizing bacteria was immobilized in calcium-alginate beads and thereafter oxidation rate was determined as 10.1~33.7 mM As(III) oxidized $g^{-1}$ dry cell $h^{-1}$. Although, heterotrophic conditions when provided to the bacterium (yeast extract supplement), enhanced the growth and oxidation rates as compared when the chemolithotrophic conditions were given.

We hypothesize that arsenic contaminated soil harbours arsenite oxidizing bacteria, possessing *aox* gene for the production of arsenite oxidase. These bacteria can be successfully immobilized in a column and used to oxidize arsenite to arsenate from contaminated water.

# OBJECTIVES OF RESEARCH

- **Characterization of Arsenite Hyper-tolerant bacteria**
- **Kinetics of arsenic oxidation in bacteria**
- **Development of a whole cell immobilized system as a bioreactor for the treatment of arsenic contaminated water**

# METHODOLOGY

1. **Soil sample collection:** Soil samples will be collected at different distances (100-400meters) and at various depths (10-60 cm) from the source of industrial effluent, in a sterile polypropylene zip lock bags. The soil samples will be analyzed for the presence of microbes as soon as possible after sample collection and stored at 4$^{o}$C for physico-chemical characterization.

2. **Physicochemical Characterization of soil:** The soil will be physico-chemically characterized for pH, electrical conductivity, water holding capacity, organic carbon, organic matter, exchangeable calcium and metal content (APHA, 2005; Maiti, 2003).

3. **Isolation of arsenite hyper-tolerant bacteria:** Dilution plating technique will be used to isolate arsenite resistant bacteria by culturing them in defined medium supplemented with 1000ppm (7.69mM or 1g/L) of sodium arsenite and incubated at 37°C for 24-48 h at 120 rpm. The Pure colonies will be obtained with repeated spreading and streaking. The strains will be preserved in 15 % v/v of glycerol.

4. **Minimum Inhibitory Concentration (MIC) determination:** The pure bacterial colonies will be inoculated in defined medium supplemented with increasing doses of sodium arsenite (1000-10000ppm) at different temperature and durations of time. The MIC of each bacterial strain will be determined on the basis of negligible growth in terms of optical density at 600nm (UV-Vis Spectrophotometer). The lowest concentration at which the growth ceases will be considered its MIC (Courvalin *et al.*, 1985; Muller *et al.,* 2003).

5. **Optimization of growth conditions:** The arsenite oxidizing bacterial growth conditions will be standardized for

    - Media composition
    - pH

- Temperature
- Metal sensitivity (Novick and Roth, 1968; Zelibor *et al*., 1987 and Huysmans and Frankenberger, 1990).

6. **Antibiotic Sensitivity Tests:** The method of Novick and Roth (1968), Zelibor *et al*. (1987) and Huysmans and Frankenberger (1990) will be used to screen isolates for resistance to a variety of antibiotics.

7. **Chemical analysis of oxidizing ability:** The isolated bacteria showing highest MIC will be checked for their oxidizing ability by following techniques-

   - Qualitative Test- Qualitative analysis for arsenite oxidation will be done by Silver Nitrate Screening in agar plates (Lett *et al.*, 2001).

   - Quantitative Test-
     - Molybdene Blue Spectrophotometric Method (Zhou, 1990; Lenoble *et al.,* 2003; Cai *et al.,* 2009b)
     - Ion Exchange Chromatography followed by Hydride Generation- Atomic Absorption Spectroscopy (Le *et al.,* 2000; Samanta *et al.,* 1999).

   Oxidation rate of Arsenite [As(III)] biotransformation to Arsenate [As(V)] will be determined.

8. **16S rDNA sequencing and biochemical characterization of the isolated strains:** The oxidizing strains will be identified by 16S rDNA sequencing. The sequencing results will be supported by biochemical characterization (Bergey's Manual of Determinative Bacteriology, 1994).

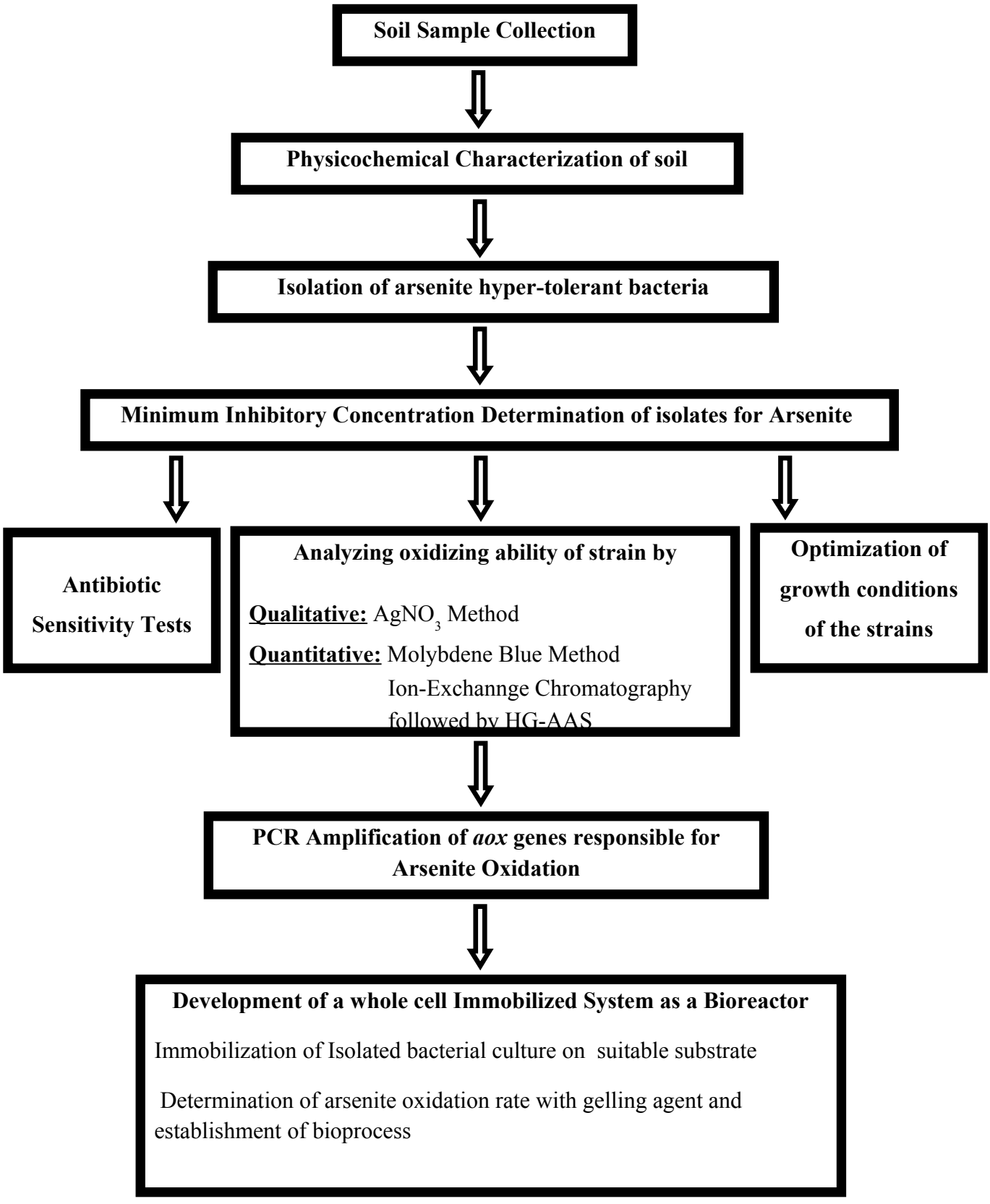
9. **Molecular analysis of oxidizing ability:** The genome of the arsenite oxidizing bacteria will be analysed or the presence of *aox* gene which is responsible for the production of arsenite oxidase (enzyme responsible for the oxidation process of arsenite to arsenate). This will be done by PCR amplification of *aox* genes using specific primers (Quemeneur *et al.*, 2008).

10. **Immobilization of isolated bacterial culture:** Various gelling agent such as agarose, alginate or phytogel will be used for immobilization of bacterial isolates (Roger *et al.,* 1996). For this purpose, a pure bacterial culture will be inoculated in suitable broth and grown in their optimized condition. After reaching a particular optical density, bacterial cell culture will be centrifuged and entrapped in bead by adding the culture to the gelling agent.

11. **Determination of arsenite oxidation rate with gelling agent and establishment of bioprocess:** The immobilized culture will be packed in a column and ground water supplemented with increasing doses of arsenite will be passed through this column at specific flow rate. After treatment, the eluent will be tested for arsenate and arsenite content and percent removal of arsenite will be calculated by Silver Nitrate preliminary test and Ion Exchange Chromatography followed by Hydride Generation- Atomic Absorption Spectroscopy (HG-AAS)**.** The most suitable substrate for immobilization will be determined on the basis of maximum oxidation rate (Mokashi & Paknikar, 2002).

12. **Statistical Analysis:** Observations and all the experiments will be done in triplicates. Mean and standard error of the mean will be calculated.

# PLAN OF WORK


Soil Sample Collection
Physicochemical Characterization of soil
Isolation of arsenite hyper-tolerant bacteria
Minimum Inhibitory Concentration Determination of isolates for Arsenite
Antibiotic Sensitivity Tests
Analyzing oxidizing ability of strain by
Qualitative: $AgNO_3$ Method
Quantitative: Molybdene Blue Method
Ion-Exchannge Chromatography followed by HG-AAS
Optimization of growth conditions of the strains
PCR Amplification of aox genes responsible for Arsenite Oxidation
Development of a whole cell Immobilized System as a Bioreactor
Immobilization of Isolated bacterial culture on suitable substrate
Determination of arsenite oxidation rate with gelling agent and establishment of bioprocess

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