Bioremediation of Heavy Metal Contaminated Soil using Biosurfactant

A Dissertation Report
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in
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CERTIFICATE

This is to certify, that the M.Tech project entitled **"Bioremediation of heavy metal contaminated soil using biosurfactant"** is an original piece of work carried out by Ishita Garg, a student of M.Tech (Environmental Science and Technology) for the partial fulfillment of the degree of Master of Technology, Thapar University, Patiala, Punjab under the supervision and guidance of Dr.(Mrs) Asha.A.Juwarkar, Scientist G and Head at Eco-Restoration Division, National Engineering Research Institute (NEERI), Nagpur and Dr. N. Tejo Prakash, Associate Professor, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, Punjab. This project report is upto required standard of its contents and presentation and no part of this project has been submitted for the award of any other degree.

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Declaration

I, the undersigned hereby declare that the research work presented in the M.Tech project entitled " Bioremediation of Heavy Metal Contaminated Soil Using Biosurfactant" has been carried out by me under the supervision and guidance of Dr.(Mrs.) Asha A. Juwarkar, Scientist'G' and Head, Eco Restoration Division, National Environmental Engineering Research Institute (NEERI), Nagpur and Dr. N. Tejo Prakash, Associate Professor, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, Punjab.

Further, I declare that no part of this Dissertation has been submitted for a degree or any other qualification of any other university or examining body in India/elsewhere.

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ABSTRACT

Bioremediation is an efficient tool practiced now-a-days for the removal of contaminants from the contaminated soil or water.

In this report our objective was to remediate arsenic contaminated soil collected from West Bengal using biosurfactant.

We have carried out characterization and fractionation of arsenic contaminated soil, biosurfactant production from *Pseudomonas aeruginosa*, screening of microbial species from soil, lysimeter studies with different treatments for removal of arsenic and collection and characterization of root exudates in presence of arsenic.

From the study conducted, it was found that biosurfactant mediated phytoremediation is effective in removing arsenic from soil. It is also found that in presence of arsenic *Vetiveria* plant produces biosurfactant, with biochemical changes including decrease in carbohydrate content and stable protein content.

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1. INTRODUCTION

There has been a tremendous growth of industries worldwide in the last few decades and the associated anthropogenic activities have often resulted in environmental pollution. Heavy metals such as As, Cr, Pb etc. are prominent components of industrial effluents which are discharged in to the environment and consequently pollute the ecosystem. The presence of these heavy metals in the environment has been a subject of great concern due to their toxicity, non-biodegradable nature and the long biological half-lives for their elimination from biological tissues **(Olatunji *et al*., 2009)**.

Heavy metals are a dangerous group of soil pollutants. Exposure to heavy metals through ingestion, inhalation, or skin contact is usually chronic due to food chain transfer. Chronic problems associated with heavy metal exposure may range from intermediate poisonings to death. Arsenic is present in the environment both naturally and due to certain human activities.

Arsenic is used in industry as a wood preservative and in paints, dyes, metals, soaps, insecticides and semi-conductors. Apart from its natural occurrence it is also released into the environment through burning fossil fuels, paper production, cement manufacturing and mining activities. Arsenic (As) exists in several forms, which vary in toxicity and occurrence of which arsenite and arsenate are the most prevalent toxic forms of inorganic arsenic. Arsenite in reduced state in inorganic is a toxic pollutant in natural environment and is more soluble and mobile than the oxidised state of inorganic arsenic, arsenate **(Elangovan *et al*., 2006)**. Therefore swift removal of metals such as arsenic from contaminated sites is of foremost importance.

Several technologies exist for the remediation of metal-contaminated soils like subsurface barriers, immobilization, pyro-metallurgical, solidification/stabilization, vitrification, extraction, toxicity and/or mobility reduction, electrokinetic treatment, chemical and physical treatments etc. **(Evanko & Dzombak, 1997)**. But all these treatments are very expensive and found to affect the texture of the soil. There is an increased interest in using surfactants to complex metals and remove them from waste streams and in-situ by soil washing and pump and treat remediation technologies **(Herman *et al*., 1995)**.

The term surfactants refers to any compound, which has some influence on interfaces, i.e. **SURF**ace **ACT**ive **A**ge**NTS**, which brings down the interfacial tension between the two liquids and allows easy spreading. The enormous market demand for surfactants is currently met by numerous synthetic, mainly petroleum based, chemical surfactants. These compounds are usually toxic to the environment and most of them non biodegradable, therefore, they may bio-accumulate, whereas their production processes and by-products can be environmentally hazardous. Tightening environmental regulations and increasing awareness for the need to protect ecosystems have effectively resulted in an increase interest for the use of biosurfactants, considered as possible alternatives to chemical surfactants **(Banat *et al.*, 2000).**

Biosurfactants are surfactants that are produced extracellularly or as part of the cell membrane by bacteria, yeasts and fungi. Examples include *Pseudomonas aeruginosa* which produces rhamnolipids, *Candida* (formerly *Torulopsis*) *bombicola* one of the few yeasts to produce biosurfactant, which produces high yields of sophorolipids from vegetable oils and *Bacillus subtilis* which produces a lipopeptide called surfactin **(Mulligan, 2005)**. Biosurfactants have several advantages over the chemical surfactants, such as

- lower toxicity; higher biodegradability
- better environmental compatibility
- higher foaming **(Razafindralambo *et al.*,1996)**
- high selectivity and specific activity at extreme temperatures, pH, and salinity **(Velikonja *et al.*,1993)**
- ability to be synthesized from renewable feedstocks.

Most known biosurfactants are glycolipids. Among the glycolipids, the best known are rhamnolipids, trehalolipids, and sophorolipids **(Desa and Banat, 1997).** Due to the anionic nature of rhamnolipids, they are able to remove metals from soil and ions such as arsenic, cadmium, copper, lanthanum, lead and zinc due to their complexation ability **(Herman *et al.*, 1995).**

An ideal complexing agent to mobilize metals is one that is soluble in water, chemically stable under environmental conditions, not strongly bound to soil particles, and has a high affinity for complexing metals.

The study has been carried out on arsenic contaminated soil collected by NEERI from four districts of West Bengal *viz.,* North 24 Parganas, Nadia, Murshidabad and Burdwan. About 10 % of the total population of the State is exposed to arsenical dermatosis. According to the studies so far made, the cause of arsenic pollution of ground water in West Bengal has been attributed to the hydrolithological phenomena.

In this report, we have made use of rhamnolipid biosurfactant produced by *Pseudomonas aeruginosa* for remediation of arsenic contaminated soil. To evaluate feasibility of rhamnolipid for removal of arsenic from the contaminated soil, lysimeter studies with varying treatments were carried out. The details of treatment are given below:

T1: Arsenic contaminated soil + (200ml water) per day

T2: Arsenic contaminated soil + (150ml water + 50ml of (0.45g/L) biosurfactant per day

T3: Arsenic contaminated soil + (200ml water containing 1g wet wt. of *P.aeruginosa*) per day

T4: Arsenic contaminated soil + *Vetiver* plant + (200ml water) per day

T5: Arsenic contaminated soil + *Vetiver* plant + (150ml water + 50ml of (0.45g/L) per day.

2. OBJECTIVES

The major objectives of the present work are:

- Characterization of arsenic contaminated soil
- Production of biosurfactant using *Pseudomonas aeruginosa* for the remediation of arsenic contaminated soil
- Study of desorption of soil bound arsenic to the plant available form to facilitate metal removal by biosurfactant mediated phytoremediation
- Analysis of root exudates from *Vetiveria* in presence of arsenic

Work Plan

- ✓ Physico-chemical characterization of arsenic contaminated soil
- ✓ Microbial characterization of arsenic contaminated soil
- ✓ Determination of concentration of total arsenic in contaminated soil
- ✓ Fractionation of arsenic in the contaminated soil
- ✓ Production of biosurfactant by *Pseudomonas aeruginosa* using Mineral Salt Medium
- ✓ Quantification of biosurfactant using Orcinol Assay
- ✓ Characterization of biosurfactant
    - Rhamnolipids confirmation using CTAB-methylene blue MSM agar
- ✓ Determination of emulsion stability index of the prepared biosurfactant
- ✓ Use of different treatments for studying removal of soil bound arsenic from the contaminated soil through column lysimeters
- ✓ Collection of root exudates from *Vetiveria* using calcium chloride method and their analysis

3. REVIEW OF LITERATURE

3.1 HEAVY METAL CONTAMINATION IN SOIL

Due to rapid industrialization and urbanization pollution of heavy metals becomes a matter of global concern. Cadmium, copper, arsenic, chromium, lead, mercury, nickel and zinc are considered the most hazardous heavy metals and are included on the EPA's list of priority pollutants. Sources of metals include domestic and industrial effluents, the atmosphere, runoff and lithosphere. Once heavy metals are allowed to pass through the municipal waste treatment facility, they return to the environment where they are persistent, cannot be biodegraded and can thus follow a number of different pathways. The metals can adsorb onto the soil, runoff into rivers or lakes or leach in the groundwater, an important source of drinking water. Exposure to the heavy metals through ingestion or uptake of drinking water particularly where water is reused and foods can lead to accumulation in animals, plants and humans.

Among heavy metals Pb, Cd and As are considered potentially important environmental pollutants due to their trends to accumulate on vital organs of humans and animals. The most common metals found at contaminated sites are (U.S. EPA, 1996b), in order: lead (Pb), chromium (Cr), arsenic (As), zinc (Zn), cadmium (Cd), copper (Cu) and mercury (Hg).

The soil-plant system is the fundamental constructive unit of the geo-sphere and biosphere. Therefore, heavy metal pollution of soil has an important influence not only on the yield and quality of crops, but also on the quality of atmospheric and aquatic environment, and even on the health of human beings via food chains. Heavy metals are among the pollutants that need to be removed from such contaminated sites. Several heavy metals such as Cd, As and Cr are considered hazardous waste metals that can accumulate in the human body with a relatively large half-life. It has been stated for example, that Cd has half-life of 10 years once in the human body **(Salt *et al.*, 1995)**. Additionally, some species of Cd, Cr and Cu have been associated with health effects ranging from dermatitis to various types of cancer **(McLaughlin *et al.*, 1999)**.

3.2 GENERAL REMEDIATION APPROACHES

Several technologies exist for the remediation of metals-contaminated soil and water. These technologies are contained within five categories of general approaches to remediation: isolation, immobilization, toxicity, reduction, physical separation and extraction. These are the same general approaches used for many types of contaminants in the subsurface **(LaGrega *et al.*, 1994)**. As is usually the case, combinations of one or more of these approaches are often used for more cost effective treatment of a contaminated site.

3.3 ARSENIC CONTAMINATION

Historically arsenic is known as a poison. It does not often present in its elemental state but is more common in sulfides and sulfosalts such as Arsenopyrite, Orpiment, Realgar, Lollingite and Tennantite. Arsenic is used in industry as a wood preservative and in paints, dyes, metals, soaps, insecticides and semi-conductors. Apart from its natural occurrence it is also released into the environment through burning fossil fuels, paper production, cement manufacturing and mining activities **(Elangovan and Chalakh, 2006)**.

Non-biodegradability of arsenic is responsible for its persistence in the environment; once mixed in soil, it undergoes transformation into various mobile forms before ending into environmental sink. Arsenic (As) exists in several forms, which vary in toxicity and occurrence.

The common valence states of arsenic in nature include –3, 0, +3 and +5. In soils, the most often encountered arsenic forms are inorganic As (III) (arsenite) and As (V) (arsenate). As (V) and As (III) can be volatilized to arsine (AsH_3); MMAA to monomethylarsine (CH_3AsH_2; MMA); DMAA to dimethylarsine [$(CH_3)_2AsH$; DMA]; and TMAO to trimethylarsine [$(CH_3)_3As$), TMA] **(Turpeinen, 2002).** Arsenite As (+3) in reduced state in inorganic is a toxic pollutant in natural environment and is more soluble and mobile than the oxidised state of inorganic arsenic, arsenate As (+5) **(Elangovan and Chalakh, 2006)**. Organic arsenic is 500 times less harmful than inorganic arsenic.

Arsenic, like many other elements in soils, is subject to microbial transformations. For instance, reduction and methylation of arsenic by bacteria can form gaseous arsines leading to volatilization of arsenic or to mineralization of organic arsenic compounds to inorganic arsenic.

These transformations influence arsenic cycling and accumulation in the soil. Accumulation of arsenic in soils is of great concern due to its toxicity and potential to contaminate groundwater. Also, because arsines are the most toxic forms of arsenic, their assessment in contaminated environment is important (**Turpeinen, 2002**).

3.3.1 HAZARDOUS EFFECTS OF ARSENIC

Arsenic and many of its compounds are potent poisons and cause health effects such as:

- Disruption of ATP production through several mechanisms
- Metabolic interferences leading to to death from multi-system organ failure probably from necrotic cell death
- Cardiovascular (heart failure) effects
- Gastrointestinal (burning lips, painful swallowing, thirst, nausea, and severe abdominal colic) effects
- Hematological effects (anaemia and leucopoenia)
- Hepatic effects
- Renal effects
- Neurological effects (headache, lethargy, mental confusion, hallucination, seizures and coma)
- Dermal effects (skin disorder, hyperkeratosis)
- Carcinogenic effects (lung cancer) **(Elangovan and Chalakh, 2006)**.

  In order to minimize the hazards caused due to arsenic in the environment several methodologies for its removal have been suggested by different workers. Bioremediation being one of the promising methods have been used for the removal of arsenic in the present study

3.4 BIOREMEDIATION

Bioremediation is the use of microorganism to remove pollutants from the contaminated environment. Technologies can be generally classified as in-situ or ex-situ. In-situ bioremediation involves treating the contaminated material at the site, while ex-situ involves the removal of the contaminated material to be treated elsewhere. Some examples of bioremediation technologies are bioventing, landfarming, bioreactor,

composting, bioaugmentation, rhizofiltration and biostimulation. The technologies entail the science of understanding natural processes that promote and accelerate destruction, transformation, removal, or stabilization of pollutants **(Dzantor, 1914)**.

Microbe Assisted Bioremediation

Bioremediation can occur on its own (natural attenuation or intrinsic bioremediation) or can be spurred on via the addition of fertilizers to increase the bioavailability within the medium (biostimulation). Recent advancements have also proven successful via the addition of matched microbe strains to the medium to enhance the resident microbe population's ability to break down contaminants. Microorganisms used to perform the function of bioremediation are known as Bioremediators (bioaugmentation).

Metallic pollutants are not degraded during composting but may be converted into organic combinations that have less bioavailability than mineral combinations of the metals **(Bryson and Barker, 2002).**

Many micro-organisms can produce iron-complexing molecules, named siderophores. These molecules are synthesized in case of iron deficiency. Some of these siderophores also have high affinities for heavy metals, and in case of *Pseudomonas aeruginosa* and *Alcallgenes eutrophus* siderophore (pyoverdin and alcaligme E, respectively), synthesis was also induced by heavy metals even in the presence of high iron concentrations. A comparison between negative and constitutive siderophore mutants leads to the conclusion that siderophores or, more generally metallophores, can play a role in metal solubilization.

The metal solubilization and biocrystallization capacity of *A. eutrophus* CH34 was used to treat sandy soils contaminated with heavy metals. The bacterium can solubilize the metals (or increase their bioavailability) via the production of siderophores and adsorb the metals in their biomass, on metal-induced outer membrane proteins, and by bioprecipitation. The difficult point is to find an easy way to separate the biomass, loaded with metals, from the soil matrix. In case of *A. eutrophus* CH34, a special phenomenon was observed. The bacterium was able to improve the settling of the soil by production of some extracellular polymers. In that way, biomass and soil could be separated more easily, e.g., by settling or flotation. The heavy metal resistance,

bioprecipitation capacity, and improved soil flocculation lead to the development of a bioremediation method for heavy metal contaminated soils (**Diels *et al.*, 1999**).

Phytoremediation

In recent years phytoremediation has proved a novel, efficient and economic technique for the recovery of contaminated soils. Phytoremediation is based on the fact that a living plant can be considered as a solar-driven pump, which can extract and concentrate toxic elements from the contaminated soil. This involves principally three processes , phytoextraction involving extraction of heavy metals from the soil; phytofiltration (or rhizofiltration) involving of acquatic and semiaquatic plants in various water purification systems; phytovolatization involving volatization of some toxic metals like Se, As and Hg **(Maiti *et al.*, 2003).**

The technique involves planting contaminated areas with certain plant species which are known to absorb and concentrate toxic substances. These species are referred to as hyperaccumulators. The plants can help to clean various types of contamination including metals, insecticides, explosives and oil; they remove the contaminants from the ground when their roots absorb water and nutrients from contaminated soil and groundwater. Plants are better at cleaning contaminants from the soil when they have deep roots. The plant may store the contaminants in their roots, stems and leaves; converted into less damaging contaminants within the plant; or converted into gases that are released into the air when the plant transpires. Cleaning land by phytoremediation frequently takes several years. The EPA uses phytoremediation because of the advantages it offers. It requires less equipment and work than other methods, as the plants do most of the work. A site can be cleaned without having to remove the topsoil or pump out contaminated groundwater. This prevents workers coming into contact with the contaminants. Phytoremediation has been proven satisfactorily in various places and may be used on contaminated sites **(Esparza, 2006)**.

Arsenic removing plants

The fern, ***Pteris vittata***, is the first plant found to be a hyperaccumulator of arsenic, that is, it uses arsenic as part of its food. Tests have found levels up to 200 times higher than the concentrations measured in contaminated soils where the fern is growing. A site contaminated by timber treated with a solution of chromium, copper and arsenic, the arsenic concentration in the soil was 38.9 mg/k whilst that in the fern was 7.526 mg/k. In tests using

artificial soil contaminated with arsenic, the concentration of this metalloid in the fern's leaves reached 22,630 mg/k of arsenic, which means that 2.3 percent of the plant consisted of arsenic **(Esparza, 2006)**.

Research indicates that **edenfern** accumulates an arsenic concentration, in the above ground plant tissue, more than 200-fold higher than any other plant species tested. Edenfern, accumulates consistent high arsenic concentrations in its shoots from successive harvesting. Based on the arsenic concentration in the soil solution of 34 mg/L, edenfern was able to concentrate arsenic in its shoots more than 16,000 fold higher than the arsenic concentration in the soil solution (**Komar *et al.*, 2001**).

Vetiveria zizanioides and *Vetiveria nemoralis* were chosen for arsenic removal experiments. They were grown in the soil containing sodium arsenate ($Na_2HAsO_4.7H_2O$) at different concentrations (control, 50, 75,100, 125, and 150 mgAs/kg soil).

Accumulation of arsenic in the roots of both species was higher than in the leaf. The amount of arsenic accumulation in *V. Zizanioides* was more than in *V. nemoralis*. In addition, arsenic removal efficiency of both species increased with increasing exposure time **(Srisatit *et al.*, 1999)**.

Mechanism of hyperaccumulators to heavy metal tolerance

The mechanisms of plants to heavy metal tolerance may range from exclusion, inclusion and accumulation of heavy metals. Plant roots produce and secrete various compounds into rhizosphere. Root exudation includes the secretion of ions free oxygen and water, enzymes, mucilage, low molecular weight compounds like sugar, amino acid, organic acid, nucleotide etc and high molecular weight compound like polysaccharides, fatty acids, sterols, flavanones, secretory compounds **(Shimp and Hawkes, 1993)**.

Plant release exudates in the soil environment that may help to stimulate the degradation of organic chemicals by inducing enzyme systems of existing bacterial populations and stimulating growth of new species those are able to degrade the wastes. Stimulation of soil microbes by plant root exudates can also result in alteration of the geochemical conditions in the soil, such as pH, which may result in the transport of inorganic contaminants.

Vetiver is a perennial grass with strong ecological adaptability, large biomass and is easy to manage and grow in different soil conditions. It has great potential for various applications including hillside soil and water conservation, sustainable agriculture, fixing sandy riverbank and pollution control. *Vetiver* is an extremely hardy grass species with many characteristics that makes it ideal for environmental protection. *V. zizanioides* can tolerate and grow in high metal contaminated soil. The total dry weight of *V. zizanioides* grown in 250 mgAs/Kg soil significantly decreased with the arsenic accumulating more in the roots than in the leaves **(Srisatit *et al.*, 1999)**.

On the basis of literature, it was revealed that many of the scientific studies has been under taken to evaluate *Vetiveria zizanioides* for its root exudates potential in phytoremediation and its antioxidant activity under the stress of different compounds such as metals, benzene, toluene etc.

3.5 BIOSURFACTANT

Biosurfactants are surface-active substances synthesized by living cells. They have the properties of reducing surface tension, stabilizing emulsions, promoting foaming and are generally non-toxic and biodegradable. Interest in microbial surfactants has been steadily increasing in recent years due to their diversity, environmentally friendly nature, possibility of large-scale production, selectivity, performance under extreme conditions and potential applications in environmental protection.

Surfactants are amphipathic molecules with both hydrophilic and hydrophobic moieties present within the same molecule. The hydrophobic moiety of a biosurfactant is either a long-chain fatty acid, hydroxy fatty acid or α-alkyl β-hydroxy fatty acid and the hydrophilic moiety can be a carbohydrate, amino acid, cyclic peptide, phosphate, carboxylic acid, or an alcohol.

Biosurfactants are produced extracellularly or as part of the cell membrane by bacteria, yeasts and fungi. Examples include *Pseudomonas aeruginosa* which produces rhamnolipids, *Candida* (formerly *Torulopsis*) *bombicola* one of the few yeasts to produce biosurfactant, which produces high yields of sophorolipids from vegetable oils and *Bacillus subtilis* which produces a lipopeptide called surfactin **(Mulligan, 2005)**.

3.5.1 Classification

Microbial surfactants are categorized by their chemical composition and microbial origin. **Rosenberg and Ron (1999)** suggested that biosurfactant can be divided into low-molecular-mass molecules, which efficiently lower surface and interfacial tension, and high-molecular-mass polymers, which are more effective as emulsion stabilizing agents. The major classes of low-mass surfactants include glycolipids, lipopeptides and phospholipids, whereas high mass includes polymeric and particulate surfactants. Examples of low molecular mass molecules are rhamnolipids, sophorolipids whilst food emulsifiers and biodisperan are examples of high molecular-mass polymers.

3.5.2 Biosurfactant Facilitated Remediation of Metal Contaminated Soil

Bioremediation of metal-contaminated soils is more complex because microbial cells or large exopolymers do not move freely through the soil. The use of microbially produced surfactants (biosurfactants) is an alternative with potential for remediation of metal-contaminated soils. The distinct advantage of biosurfactants over whole cells or exopolymers is their small size, generally biosurfactant molecular weights are less than 1500. A second advantage is that biosurfactants have a wide variety of chemical structures that may show different metal selectivities and thus, metal removal efficiencies.

A review literature shows that complexation capacities of several bacterial exopolymers were similar to the complexation capacity of a rhamnolipid biosurfactant produced by *Pseudomonas aeruginosa* ATCC 9027.

Rhamnolipid are most common biosurfactants in which one or two molecules of rhamnose are linked to one or two molecules of β-hydroxydecanoic acid are the best-studied glycolipids. Production of rhamnose-containing glycolipids was first described in *Pseudomonas aeruginosa* by Jarvis and Johnson.

There are four types of rhamnolipids **(Tsuji, 1998).** Type 1 (R1) is rhamnosyl-β hydroxydecanoyl-β-hydroxydecanoate (mono-rhamnolipid) of molecular mass 504 Da, type II (R2) is L-rhamnosyl-β-L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate (di-rhamnolipid) of molecular mass 650 Da. The other two types of rhamnolipids contain either two rhamnose attached to β-hydroxydecanoic acid or one rhamnose connected to the identical fatty acid. Rhamnolipid type I and type II are suitable for soil washing and heavy metal removal **(Jeneil, 2001).**

3.5.3 Mechanism of Interaction of Metal with Biosurfactant

Biosurfactants have the potential to impact the major factors that cause the removal of heavy metals from soils to be so difficult, namely, sorption, rate-limited mass transfer, and resistance to aqueous-phase transport. The addition of a biosurfactant may promote desorption of heavy metals from solid phases in two ways. The first is through complexation of the free form of the metal residing in solution. This decreases the solution phase activity of the metal and, therefore, promotes desorption according to Le-Chatelier's principle. The second is that under conditions of reduced interfacial tension, biosurfactants will accumulate at the solid-solution interface. This may allow direct contact between the biosurfactant and the sorbed metal**. (Miller, 1995).**

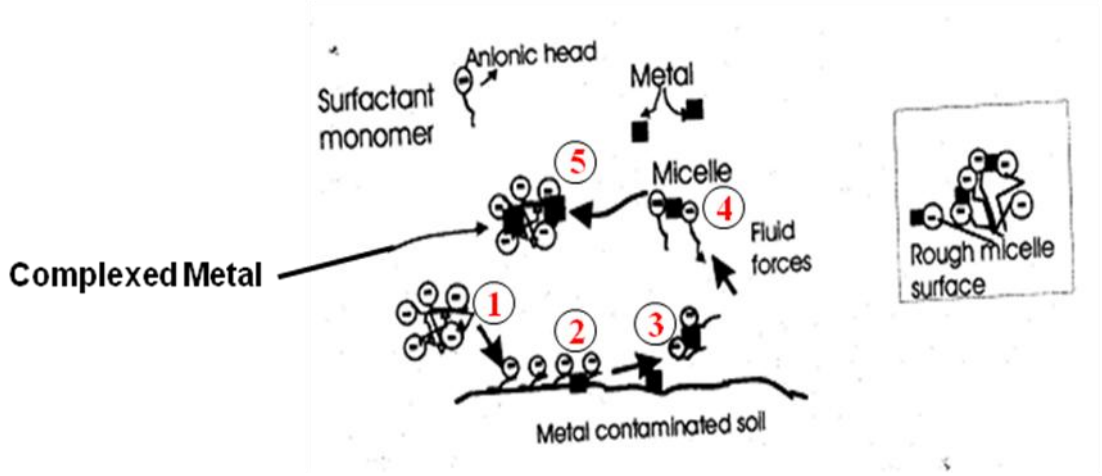


Fig.(1) Potential mechanism for the metal removal by biosurfactant. Insert depicts rough surface of micelle with hydrophobic portions of the surfactant protruding into the outer surface.

1. **Adsorption of micelle on soil surface**
2. **Interaction with sorbed metal**
3. **Desorption of surfactant and metal**
4. **Transportation of metal and surfactant away from the soil**
5. **Incorporation of the metal into micelle.**

3.5.4 Application of Biosurfactant

There are many areas of industrial application where chemical surfactants could be substituted by biosurfactants in fields as diverse as agriculture, food and beverage industries, industrial cleaning, cosmetics, pharmaceutical industry, petroleum and petrochemical industries, waste treatment, pipeline transportation, additives for rolling oil, as therapeutic agents, in pulp and paper industry, textiles and ceramics.

Potential applications of biosurfactants are in field of soil and marine bioremediation, including polyaromatic hydrocarbon (PAHs) or polychlorinated biphenyls (PCBs) or pesticide bioremediation, bioreclamation of soil, metal contaminated soils bioremediation, and also in oil storage tank cleaning, microbial enhanced oil recovery.

The review of literature indicated limited work on bioremediation of heavy metal contaminated soil using biosurfactant at field level.

4. MATERIALS AND METHODS

4.1 PHYSICO-CHEMICAL CHARACTERIZATION OF ARSENIC CONTAMINATED SOIL

The soil used for the studies was analyzed for physico-chemical properties as per the standard methods **(Black *et al.*, 1965).**

4.1.1 Chemical Characterization of Arsenic Contaminated Soil

4.1.1.1 Determination of Soil pH

Soil reaction is one of the important soil characteristics that indicate the availability of plant nutrients .It is measured as soil pH, which is a measure of activity of ionized hydrogen (H+) in the soil solution. It is one of the most indicative measurements of the chemical properties of the soil. It is generally both a symptom of the soils condition and a cause of many of the reactions that occur. For the measurement of soil pH, soil is brought to saturation stage with distilled water in the ratio of 1:2:5 and pH is measured with help of glass electrode pH meter.

Reagents:

- 4.0 pH Buffer**:** Prepared by dissolving 10.12 g Potassium hydrogen phthalate in 1 liter distilled water
- 7.0 pH Buffer**:** Prepared by dissolving 3.387 g Potassium dihydrogen phosphate and 3.533 g Disodium hydrogen phosphate in 1 liter distilled water
- 10.0 pH Buffer**:** Prepared by dissolving 2.092 g Sodium bicarbonate and 2.64 g Sodium carbonate in 1 liter distilled water.

Procedure:

- 10g dried soil was taken in a beaker and 20 ml distilled water was added.
- Contents were stirred for 30 minutes and then allowed it to settle down.
- Calibrate the pH meter with standard buffers & note the pH reading with the help of pH meter.

4.1.1.2 Determination of Electrical Conductivity in Soil

The conductivity of a soil is the specific conductivity of a soil water suspension at a defining ratio.

Reagents:

- Standard potassium chloride solution (0.01 N): Prepared by dissolving 745.6 mg potassium chloride (anhydrous) in 1000 ml distilled water (specific conductivity of this standard solution was 1.413 μS/cm at 25^{o}C)

Procedure:

- 10g dried soil was taken in a beaker and 20 ml of distilled water was added.
- The above solution was stirred for 30 minutes in order to allow bulk of the soil to settle.
- Conductivity meter was calibrated with standard potassium chloride solution and the conductivity was measured with the help of conductivity meter.

4.1.1.3 Cation Exchange Capacity

Reagents:

- Sodium acetate (NaOAc) 1N: 136.08g sodium acetate was dissolved in 900 ml of distilled water and the pH was adjusted to 8.2 using NaOH/acetic acid. The reagent was made up to 1 litre using double distilled water.
- Isopropyl alcohol 99%
- Ammonium acetate (1N)

Procedure:

- 4-6 g of air dried soil was shaked in 50ml centrifuge tube containing 33ml of NaOAc of pH 8.2 for 5 minutes. The contents were centrifuged and the supernatant was discarded. The sample was treated in the same manner with 3 additional 33 ml NaOAc solution and the supernatant was discarded.
- Then 33ml of 99% isopropyl alcohol was added and soil was shaked for 5 minutes on shaker. The contents were centrifuged and the supernatant was discarded. The

procedure was repeated twice with 33ml of isopropyl alcohol and supernmatant was discarded.

- The soil treatment was continued with 33ml of 1N NH_4OAc 3 times as above and the supernatant of each extraction was collected in 100 ml of volumetric flask and the volume was made up with NH_4OAc. Sodium was estimated in the extract using flame photometer and CEC was calculated.

Calculation:

Sodium, meq/100 g soil = CEC, meq/100g soil

CEC, meq/100 g soil = (10/100)*R*(V/1000)*(100/S)*(1/23)

R = Galvanometer reading for Na

S = Weight of soil sample

V = volume of extracting solution

10/100 = ten mg/l Na standard adjusted to 100 readings.

4.1.1.4 Determination of organic carbon in soil

Soil organic matter is defined as the organic as the organic fraction of soil including plant, animal and microbial residues, fresh and at all stages of decomposition, and the relatively resistant soil humus. Organic carbon in soil samples is oxidized by dichromate sulphuric acid and the amount of dichromate remaining determined by titration with a standard solution.

$$K_2Cr_2O_7 + H_2SO_4 \longrightarrow K_2SO_4 + Cr_2(SO_4)_3 + 4H_2O + 3(O)$$

$$K_2Cr_2O_7 + 6FeSO_4 \longrightarrow K_2SO_4 + Cr_2(SO_4)_3 + Fe_2(SO_4)_3 + 4H_2O$$

Reagents:

- Potassium dichromate (1N) - 49.04gms of Potassium dichromate was dried overnight in oven. Prepared by dissolving 49.04 gm of Potassium dichromate in water and dilute to 1000 ml.
- Ferrous ammonium sulfate (0.5 N) - Prepared by dissolving 200 gm of Ferrous ammonium sulfate in about 800 ml of water, add 20 to 40 ml conc. Sulphuric acid and cool and dilute to 1 litter.

- Diphenylamine indicator - Prepared by dissolving 500 gm of Diphenylamine indicator in a mixture of 100 ml conc. Sulphuric acid and 20 ml distilled water.
- Concentrated Orthophosphoric acid (H_3PO_4) - (85%)

Procedure:

- 1 gm of soil sample was taken in a 500 ml conical flask.
- 10 ml potassium dichromate solution and 20 ml concentrated sulphuric acid was added and mix gently by swirling the flask.
- The content in the flask was allowed to stand for 30 minutes. After 30 minutes 200 ml distilled water, 10 ml Ortho-phosphoric acid and 1 ml ferroin indicator were added.
- The above solution was titrated with 1N ferrous ammonium sulphate.
- At the end point the colour of the solution changes from dull green through turbid blue to brilliant green to brick red.
- Similarly blank was prepared without soil and the same procedure was followed.

Calculation:

$$\text{Organic Carbon (\%)} = \frac{10\,(B - T) \times 0.003 \times 100}{B \times \text{wt. of soil sample}}$$

Where B = Blank (ml of FAS solution)

T = Sample (ml of FAS solution)

4.1.1.5 Determination of free calcium carbonate of soil

1g of soil was treated in 250ml conical flask with 50ml of standardized sodium potassium phthalate solution. The contents were vigorously shaked for one hour and were allowed to settle and filtered. 10ml filtrate was taken in another flask, 1ml of phenolphthalein indicator and was titrated with standard NaOH solution (0.25N), (prepared by dissolving 10gm NaOH in distilled water up to 1 litre volume) till the colour change was observed. Simultaneously blank was run with 10ml of HCL.

Calculations:

Wt of soil (g)	: 1gms
Volume 0.5N HCL added (ml)	: 50ml
Volume of filtrate taken for titration (ml)	: 10ml
Normality of NaOH (calculated on the basis of standardization)	: N
Volume of standard NaOH required to neutralize 10ml of HCL	: B
Volume of standard NaOH required to neutralize excess of 0.5N HCL in flask	: T

Percentage of free calcium carbonate = (B-A) (N of NaOH) × 50/10 × (0.05)*100/wt of soil (g)

Soil characterization on the basis of $CaCO_3$ content:

Free $CaCO_3$ %	***Category***
Less than 3	base enriched soil
4-7	slightly calcareous soil
8-20	calcareous soil
21-60	strongly calcareous soil
Above 60	lime stone

4.1.2 Physical characterization of arsenic contaminated soil

Soil physical conditions play a vital role that is governed by various mechanical fractions of soils. Because of different sizes, shapes and arrangements of soil particles, the pore space of soil varies greatly both in size and proportion of the total soil volume .These features are of great importance because the pore volume controls the amount of water and air contained in a soil. The size distribution of the pores affects both the permeability of the soil to air and water. This, in addition, controls the movement and retention of water in the soil available to the plant.

4.1.2.1 Keen-Raczkowski Box (Kr Box) Experiment

Bulk Density: Bulk density of the soil is an important parameter, which is an indicator of soil health. It varies according to the distribution of soil separates (sand, silt and clay) and the organic matter content of the soil.

Porosity: It is the part of the soil, which is not occupied by soil solid that is space occupied by soil, water and air.

Water Holding Capacity: The water content of soil at which all the soil pores are filled with water is referred to as saturation or maximum water holding capacity.

Apparatus:

- Analytical balance
- Oven
- Sieve (0.5 mm)
- Keen- Box (Keen-Raczkowski Box)
- Knife spatula
- Filter paper (Whattman 1 cm to 5 cm diameter)
- Aluminium dish

Procedure:

- Filter paper was fitted into the box and the brass ring was inserted to hold it in position. The box was placed in an aluminum container and was weighed (a). For brevity, the Box + Filter Paper Container will be termed as Box etc.
- The box was filled with soil (< 0.5 mm) by adding a few grams at a time and after each such addition the box was tapped several times to ensure uniform packing. When the box was nearly full, sufficient soil was added to allow the surface to be struck off flat with the spatula. The edge of the box was tapped with the edge of the spatula. More soil was added and striked off flat once more. The process was repeated several times, until there was no appreciable settling, and then weighed the Box etc. + Soil was weighed (b)
- The box was placed in a flat-bottomed dish containing about 1 cm depth of distilled water and was left overnight until the soil becomes saturated. If an appreciable amount of water was taken up by the soil, more water was added to maintain the level in the

dish. The box from the dish was removed, the outside was dried with a duster, and Box etc + Saturated soil was weighed (c).

- The portion of the soil that has expanded above the top of the box was cut off by placing a straight edged knife on its rim at a slight angle to the horizontal, and drawing the across the top. Surplus soil was transferred to a dish and was weighed (g). Box etc. + Residual Soil was also weighed (d). Both the dish and the Box etc. were dried in an oven. Cooled in a desiccator and reweighed them (h) and (e) respectively.

Calculation:

- Volume of the ring (v) $= \frac{\pi D^2 H}{4}$
- Bulk density $= \frac{b-a}{v}$
- Water holding capacity $= \frac{(c-b)*100}{b-a}$
- Porosity % $= \frac{(c-d)*100}{V}$

Weight of the box + filter paper	=	a
Weight of the box +air dry soil	=	b
Weight of the box +saturated soil	=	c
Weight of the box +oven dry soil	=	d
Weight of the ring	=	w
Height of the box	=	h
Diameter of the box	=	D
Volume of the ring	=	V

4.2 MICROBIOLOGICAL ANALYSIS OF ARSENIC CONTAMINATED SOIL

The soil used for batch studies was analyzed separately for different types of microbial groups viz. bacteria, fungi, actinomycetes, and nitrogen fixing strains of *Rhizobium* and *Azotobacter* by following standard procedures for soil microbial populations with specific media (purchased form Hi-Media Laboratories Pvt. Ltd., Mumbai, India) detailed below and were expressed in terms of colony forming units (CFU/g). The population of bacteria, fungi and actinomycetes were determined by using nutrient agar, Rose Bengal Chloramphenicol agar medium, and Kenknight and Munaier's medium respectively. *Rhizobium* species was selectively enumerated by using the Congo red yeast extract mannitol agar. *Azotobacter* population was counted using a nitrogen-free sucrose mineral salt Jensen's medium. The compositions of different media are described below: -

4.2.1 Enumeration of Bacteria

Nutrient agar medium was used for the total bacterial count present in the soil.

Media Composition (Nutrient Agar):

It was prepared by dissolving 3 gm beef extract and 5 g peptone in 1000 ml of distilled water. The pH of the medium was adjusted to 6.8 – 7.0 using 0.1 N NaOH. Then 15.0 g agar-agar was added, and sterilized at 121^{o} C for 30 minutes.

Procedure:

- 1g of rhizospheric soil sample was aseptically added to 9ml sterilized water blank and shaken thoroughly for 20 –30 minutes. Serial dilutions from 10^{1} to 10^{7} were prepared by pipetting appropriate amount of soil suspension.
- Then, 0.1ml of aliquot from 10^{4} to 10^{7} dilutions was pipetted into sterilized petri dishes containing nutrient agar and spread it by alcohol dip spreader. The plating was done in duplicate for each dilution.
- After spreading, the plates were incubated at 28 – 30^{o}C in an inverted position for 3 days. The plates were removed after completion of incubation period and only those plates, which show the count between 30 and 300 colonies, were selected for enumeration.

Calculation:

$$\text{Bacteria (CFU/g)} = \frac{\text{No. of colonies x dilution}}{\text{Wt of soil}}$$

4.2.2 Enumeration of Fungi

Rose Bengal Chloromphenicol agar was used for total fungal count in the soil.

Media Composition (Rose Bengal Chloromphenicol Agar, Rbca)

RBCA was prepared by dissolving 10 g Dextrose, 5 g Peptone, 1.0 g Dipotassium sulphate, 0.05 g Rose Bengal, 0.10 g Chloromphenicol, 0.5 g Magnesium sulphate, and 15.0 g Agar in 1000 ml Distilled water. The pH of the medium was adjusted to 7.2 ± 0.2 using 0.1 N HCl and then the medium was sterilized at 121 ^{o} C for 30 minutes.

Procedure:

- 1 g of rhizospheric soil samples was aseptically added to 9 ml sterilized water blank and shaken thoroughly for 20 –30 minutes. Serial dilutions from 10^1 to 10^4 were prepared by pipetting appropriate amount of soil suspension.
- Then, 0.1 ml of aliquot from 10^2 to 10^4 dilutions was pipetted into sterilized petri dishes containing nutrient agar and spread it by alcohol deep spreader. The plating was done in duplicate for each dilution.
- After spreading the plates were incubated at 28 – 30 ^{o}C in an inverted position for 5 days. The plates were removed after completion of incubation period and only those plates, which show the count between 30 and 300 colonies, were selected for enumeration.

***Calculation*:**

$$\text{Fungi (CFU/g)} = \frac{\text{No. of colonies x dilution}}{\text{Wt of soil}}$$

4.2.3 Enumeration of Actinomycetes

Kenknight and Munaier's Medium was used for the total actinomycetes count present in the soil.

Media Composition (Kenknight and Munaier's Medium, K&M)

K&M was prepared by dissolving 1.0 g dextrose, 0.1 g dipotassium dihydrogen phosphate, 0.1 g sodium nitrate, 0.1 g KCl, 0.1 g magnesium sulphate and 15.0 g agar- agar in 1000 ml distilled water and the pH was adjusted to 7.2 using 0.1 HCl / 0.1 N NaOH. Then, the medium was sterilized at 121 ^{o} C for 30 minutes.

Procedure:

- 1 g of rhizospheric soil sample was aseptically added to 9 ml sterilized water blank and shaken thoroughly for 20 –30 minutes. Serial dilutions from 10^{1} to 10^{4} were prepared by pipetting appropriate amount of soil suspension.
- Then, 0.1 ml of aliquot from 10^{2} to 10^{4} dilutions were pipetted into sterilized petri dishes containing nutrient agar and spread it by alcohol deep spreader. The plating was done in duplicate for each dilution.
- After solidification of agar, the plates were incubated at 28 – 30 ^{o}C in inverted position for 5 days. The plates were removed after completion of incubation period and only those plates was selected for enumeration, which shows the count between 30 and 300 colonies.

Calculation:

$$\text{Actinomycetes (CFU/g)} = \frac{\text{No. of colonies x dilution}}{\text{Wt of soil}}$$

4.2.4 Enumetation of Azotobacter

Jensen's medium was used for the total Azotobacter count present in the soil.

Media Composition (Jensen's Medium, Jam)

Jensen's medium was prepared by dissolving 200 g sucrose, 1.0 g K_2HPO_4, 0.05 g $MgSO_4$ $7H_2O$, 0.5 g NaCl, 0.01g $FeSO_4$, 0.005 g Na_2MoO_4 and 2.0 g $CaCO_3$ in 1000 ml distilled water and the pH was adjusted to 7.0 – 7.2 by 0.1 HCl/0.1N NaOH. Then 15 g agar-agar was added and the medium was sterilized at 121 ^{o} C for 30 minutes.

Procedure:

1 g of rhizospheric soil samples was aseptically added to 9 ml sterilized water blank was taken and shaken thoroughly for 20 –30 minutes. A serial dilution from 10^1 to 10^4 was prepared by pipetting appropriate amount of soil suspension.

- Then 0.1 ml of aliquot from 10^2 to 10^4 dilutions were pipetted into sterilized petri dishes containing nutrient agar and spread it by alcohol deep spreader. The plating was done in duplicate for each dilution.
- After solidification of agar, the plates were incubated at 28 – 30 ^{o}C in inverted position for 5 days. The plates were removed after completion of incubation period and only those plates, which shows the count between 30 and 300 colonies was selected for enumeration.

Calculation:

$$\text{Azotobacter (CFU/g)} = \frac{\text{No. of colonies x dilution}}{\text{Wt of soil}}$$

4.2.5 Enumeration of Rhizobium

Yeast extract mannitol agar medium was used for total *Rhizobium* count present in the soil.

Media Composition (Yeast Extract Mannitol Agar Medium, YEMA)

YEMA was prepared by dissolve 10 g mannitol, 0.5 g K_2HPO_4 0.2 g $MgSO_4$ $7H_2O$, g NaCl and 0.1 g yeast extract in 1000 ml distilled water. The pH of the medium was adjusted at 6.8 – 7.0 and 15.0 g agar- agar was added. The medium was sterilized at 121 ^{o} C for just 30 minutes. After sterilization the medium was cooled to 50 ^{o} C and 10 ml of sterilized 0.25 % congo red was added just before pouring into the plates.

Procedure:

- 1 g of rhizospheric soil samples was aseptically added to 9 ml sterilized water blank and shaken thoroughly for 20 –30 minutes. Serial dilutions from 10^1 to 10^4 were prepared by pipetting appropriate amount of soil suspension.
- Then 0.1 ml of aliquot from 10^2 to 10^4 dilutions was pipetted into sterilized petri dishes containing nutrient agar and spread it by alcohol deep spreader. The plating was done in duplicate for each dilution.
- After solidification of agar, the plates were incubated at 28 – 30 ^{o}C in an inverted position for 5 days. The plates were removed after completion of incubation period and only those plates, which show the count between 30 and 300 colonies, were selected for enumeration.

Calculation:

$$\text{Rhizobium (CFU/g)} = \frac{\text{No. of colonies x dilution}}{\text{Wt of soil}}$$

4.2.6 Screening of Biosurfactant producing isolates by Cetyltrimethylammonium Bromide – Methylene Blue

The isolates for screening for rhamnolipid production were streaked on the plates composed of the mineral salts medium with the addition of 200µg/ml cetyl-tri-methyl-ammonium bromide (CTAB; Himedia), 5 µg/ml methylene blue, and 1.5% agar (**Siegmund and Wagner, 1991**). The rhamnolipid producing cultures shows dark blue colonies

4.3 BIOSURFACTANT PRODUCTION AND QUANTIFICATION

4.3.1 Rhamnolipid Production Using *Pseudomonas aeruginosa*

For biosurfactant production, strains of *Pseudomonas aeruginosa* (reported to produce rhamnolipid biosurfactant) was inoculated into sterile mineral salts medium (**Dubey and Juwarkar, 2001**). The composition of MSM used is

COMPOSITION	CONCENTRATION (g/l)
$NaNO_3$	2.5
K_2HPO_4	1.0
KH_2PO_4	0.5
$MgSO_4$	0.5
KCl	0.1
$FeSO_4$	0.01
$CaCl_2$	0.01
Na_2HPO_4	5.67
NH_3NO_3	0.39
5*minimal salt	3.0
Glucose	30

The pH of the medium was adjusted to 7.0 prior to sterilization (15 lbs, 121º C, 20 min). Glucose was autoclaved separately and added at the time of inoculation and inoculated medium was incubated for 96 hrs at 37^{o}C on a rotary shaker at 120 rpm.

4.3.2. Quantification of Biosurfactant by Orcinol Assay

Orcinol assay is a chemical method of indirect quantification of rhamnolipid, the estimated amount of rhamnose in the biomass separated MSM, is directly proportional to the rhamnolipid present. Using this method rhamnolipid concentration was estimated, the OD was read at 421 nm. The rhamnolipid concentration was calculated from a standard curve of L-rhamnose and expressed as rhamnose equivalents. The concentration of rhamnolipid produced was also estimated using orcinol assay method **(Chandrashekharan and Bemiller, 1980).**

Procedure:

- Biosurfactant was centrifuged at 10,000 rpm for 20 minutes and the rhamnolipid thus obtained was then filtered through 0.22µm membrane filter.
- Biosurfactant (0.1 ml) and 0.9 ml of orcinol (0.19 % in 53% sulphuric acid) were mixed and heated in a water bath for 30 min.
- Optical density was read at 421nm.
- The rhamnolipid concentration was calculated from a standard curve of L-rhamnose and expressed as rhamnose equivalents.

4.3.3 Emulsification Stability:

This test has been carried out for measuring stability of biosurfactant. The emulsification index was measured by adding 2 ml of rhamnolipid to 3 ml of kerosene in a test tube and it was then vortexed at high speed for 2 minutes. After 24 hours, the height of the stable emulsion layer is measured. The emulsion stability index (ES%) can be calculated as follows:

$$EV\% = \frac{Emulsion\ height(mm) \times cross\ section\ area(mm^2)}{Total\ liquid\ volume\ (mm^3)} \times 100$$

And,

$$ES\% = \frac{EV\%\ at\ time\ 't', hr.}{EV\%\ at\ '0'\ hr.} \times 100$$

Where,

EV% = Emulsion volume (%)

ES% = Emulsion stability (%)

4.4. ANALYSIS OF TOTAL ARSENIC USING MICROWAVE DIGESTION (USEPA METHOD)

For determination of total metal concentration in sample, microwave digestor is used so that all the organic content of the sample is decomposed in the presence of concentrated

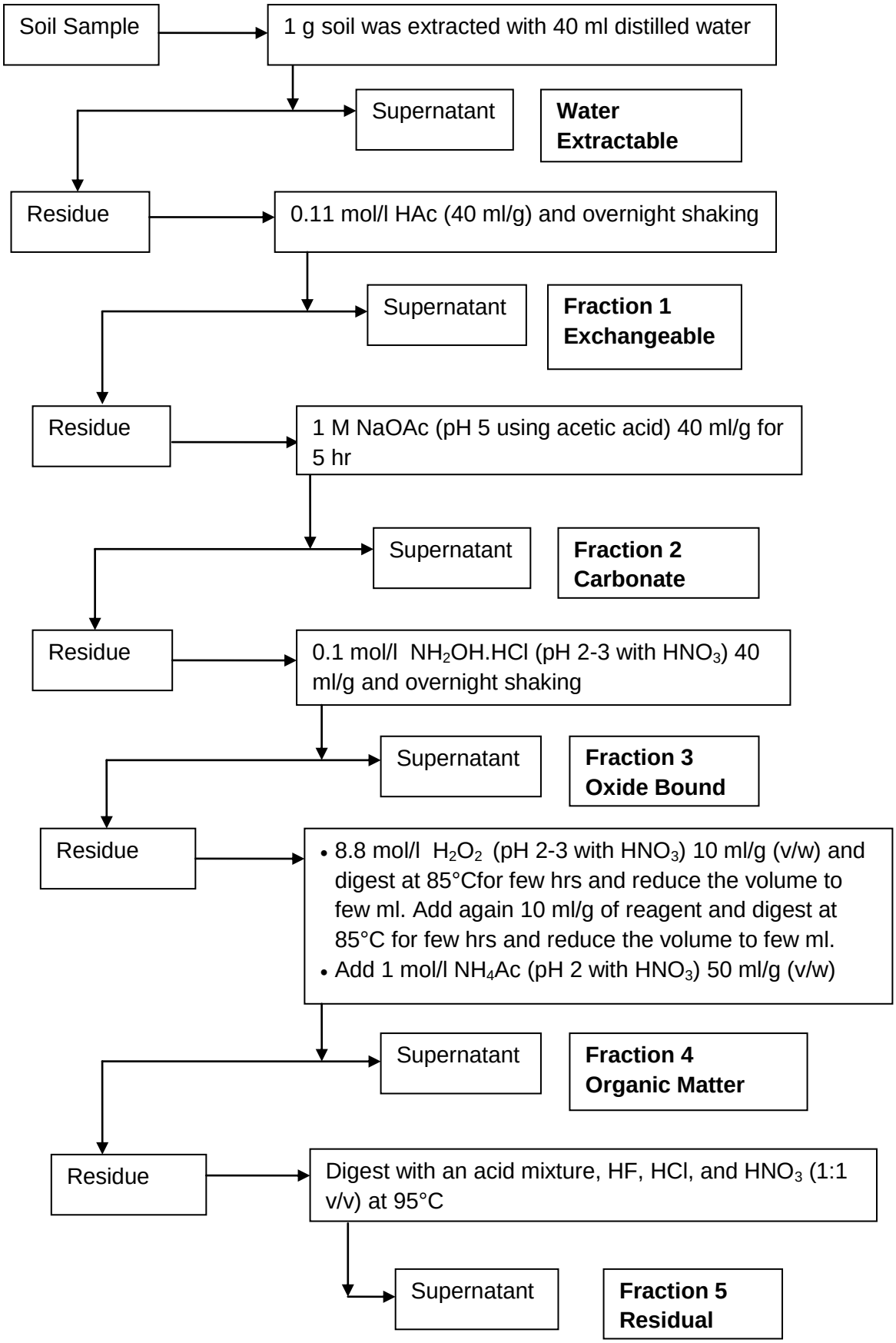
acids, thus leaving only the metal component. This metal concentration can be analyzed using ICP-AES.

Procedure

- Firstly, 1g of As contaminated soil was taken in the microwave vessels and 9 ± 0.1 ml HNO_3 and 3 ± 0.1 HF (hydrofluoric acid)(variable up to 2ml) was added.
- The programme of the microwave was set such that the digestion was carried out at a temperature of 180 ± 5°C for 55 minutes.
- The containers were removed after cooling for 1 day. The digest was filtered with whatmann filter paper #1 in 100 ml volumetric flask and the volume was made up to 100ml with distilled water.
- Finally, the analysis was carried out in ICP-AES to determine the metal concentration in the given sample.

4.5 FRACTIONATION OF ARSENIC

Fractionation of As in the contaminated soil was carried out as per the method described by **Tessier *et al.*, (1979)**. Air dried soil (2g) was treated with respective solutions as mentioned in the schematic diagram. The experiment was performed in duplicate and the average values are presented.


Soil Sample
1 g soil was extracted with 40 ml distilled water
Supernatant
Water Extractable
Residue
0.11 mol/l HAc (40 ml/g) and overnight shaking
Supernatant
Fraction 1 Exchangeable
Residue
1 M NaOAc (pH 5 using acetic acid) 40 ml/g for 5 hr
Supernatant
Fraction 2 Carbonate
Residue
0.1 mol/l NH2OH.HCl (pH 2-3 with HNO3) 40 ml/g and overnight shaking
Supernatant
Fraction 3 Oxide Bound
Residue
8.8 mol/l H2O2 (pH 2-3 with HNO3) 10 ml/g (v/w) and digest at 85°Cfor few hrs and reduce the volume to few ml. Add again 10 ml/g of reagent and digest at 85°C for few hrs and reduce the volume to few ml.
Add 1 mol/l NH4Ac (pH 2 with HNO3) 50 ml/g (v/w)
Supernatant
Fraction 4 Organic Matter
Residue
Digest with an acid mixture, HF, HCl, and HNO3 (1:1 v/v) at 95°C
Supernatant
Fraction 5 Residual

4.6 LYSIMETER STUDY TO ASSESS THE REMOVAL OF ARSENIC FROM CONTAMINATED SOIL

Different treatments were screened to evaluate the effective metal removal from arsenic contaminated soil. The treatments undertaken to study arsenic removal in plant – biosurfactant system and effect of biosurfactant application on arsenic contamination in groundwater are given below:

T1: Arsenic contaminated soil + (200ml water) per day

T2: Arsenic contaminated soil + (150ml water + 50ml of biosurfactant (0.45g/L) per day

T3: Arsenic contaminated soil + (200ml water containing 1g wet wt. of *P. aeruginosa*) per day

T4: Arsenic contaminated soil + Vetiver plant + (200ml water) per day

T5: Arsenic contaminated soil + Vetiver plant + (150ml water + 50ml of biosurfactant (0.45g/L) per day)

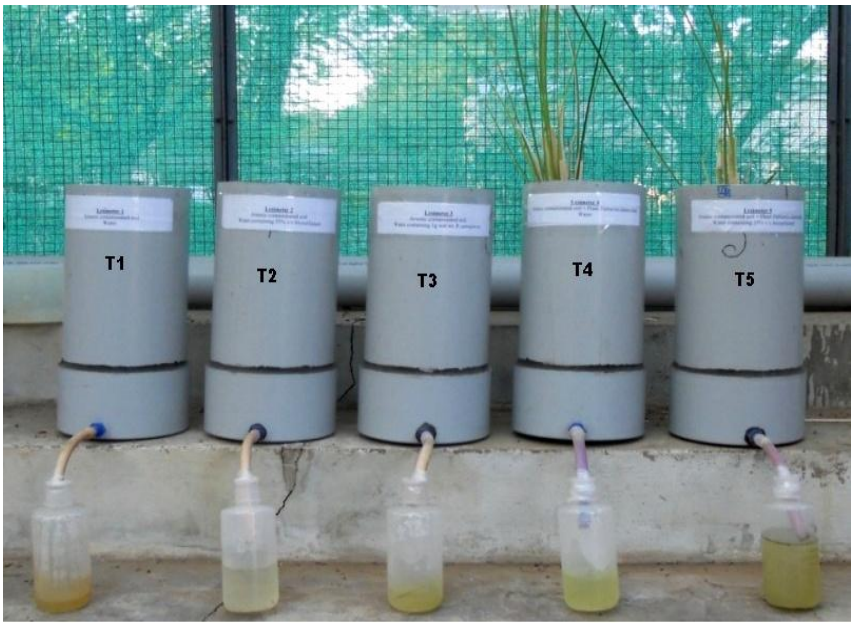


Fig 2: Lysimeter studies for arsenic removal

The soil sample, leachate and plant biomass (root and shoot) were collected from each lysimeter after application of biosurfactant. The collected samples were acid digested (HNO_3 + $HClO_4$) on hot plate at 80°C to remove the organic matter leaving back the metal in the solution. Acid mixture is added 20 ml in the ratio 1:4 for soil and 4:9 for plant biomass. The acid solution is then filtered through Whatmann 42 filter paper and the filtrate is measured for metal concentration using ICP-AES. Calculate the % metal removal from the above data using the formula given below:

$$\%\ metal\ removal = \frac{Final\ Arsenic\ Concentration\ (ppm)}{Initial\ Arsenic\ Concentration\ (ppm)} \times 100$$

4.7 COLLECTION AND CHARACTERIZATION OF ROOT EXUDATES FROM VETIVER PLANT IN PRESENCE OF ARSENIC

Plant release exudates in the soil environment that may help to stimulate the degradation of organic chemicals by inducing enzyme systems of existing bacterial populations and stimulating growth of new species those are able to degrade the wastes. Stimulation of soil microbes by plant root exudates can also result in alteration of the geochemical conditions in the soil, such as pH, which may result in the transport of inorganic contaminants.

4.7.1 Collection of root exudates by calcium chloride*:*

Procedure:

- Plants were uprooted very carefully to minimize root injuries.
- Roots were transferred into a washing solution (0.05 mM $CaCl_2$) for 1 hr.
- The $CaCl_2$ solution was renewed and roots could exudates into fresh solution for 2 hrs as cold water soluble exudates (CSE).
- Then roots were transferred into a new $CaCl_2$ solution at 60°C for 5 min to gain warm- water soluble extract (WSE).
- One batch of plant was incubated overnight with $CaCl_2$ solution.
- Extracts were freez-dried to concentrate using lyophilization.
- Different treatment flasks were kept to collect root exudates in presence of As (+3) and As (+5) at 5ppm, 15ppm, 25ppm concentration in both shock treatment and overnight method.

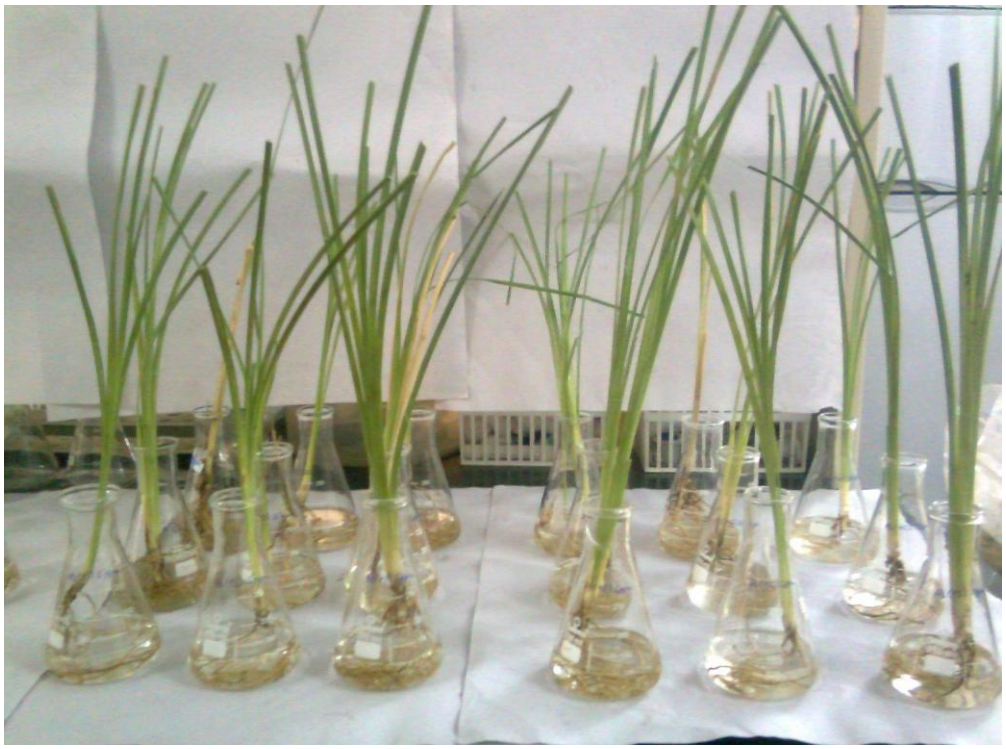


Fig 3: Root Exudates collection by $CaCl_2$

1. *Vetiveria* plant + $CaCl_2$ solution
2. *Vetiveria* plant + $CaCl_2$ solution + As (+3) (5 ppm)
3. *Vetiveria* plant + $CaCl_2$ solution + As (+3) (15 ppm)
4. *Vetiveria* plant + $CaCl_2$ solution + As (+3) (25 ppm)
5. *Vetiveria* plant + $CaCl_2$ solution + As (+5) (5 ppm)
6. *Vetiveria* plant + $CaCl_2$ solution + As (+5) (15 ppm)
7. *Vetiveria* plant + $CaCl_2$ solution + As (+5) (25 ppm)

4.7.2 Characterization of root exudates

Root exudates were analyzed for the presence of sugars, proteins and biosurfactant

Reagents:

- BSA standards
- Sugars standards
- Anthrone reagent
- Conc. H_2SO_4
- 95% ethanol
- 85% o- phosphoric acid
- Bradford reagent
- Phosphate buffer saline

❖ **Total carbohydrate- Anthrone's test**

The total carbohydrate content of root exudates collected was determined using Anthrone's reagent (**Dreywood, 1946)**. Anthrone reagent was prepared fresh before use by dissolving 200 mg of anthrone in 100 ml of ice cold 95% H_2SO_4.

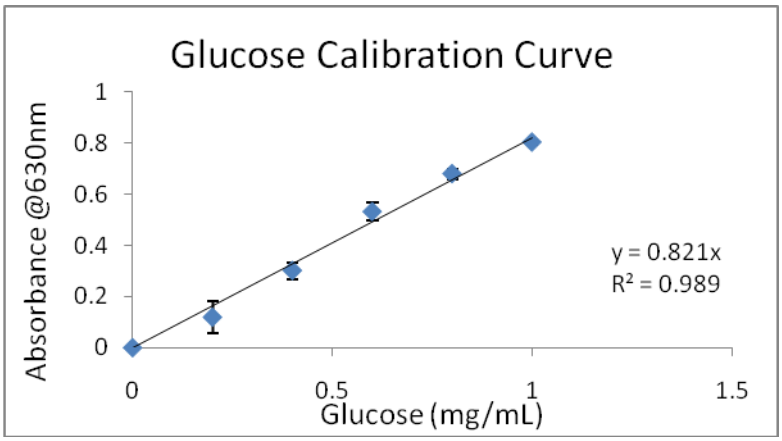


Fig 4: Standard graph of glucose

❖ **Total proteins- Bradford's test**

Root exudates were analyzed for protein content by Bradford's assay using bovine serum albumin (BSA) as standard **(Bradford, 1976)**. Bradford reagent was prepared by dissolving 100 mg of coomassie blue BG in 50 ml of 95% ethanol and 100 ml of o-phosphoric acid and make up the volume to 200 ml. For use 1 volume of Bradford reagent and 4 volume of distilled water was mixed.

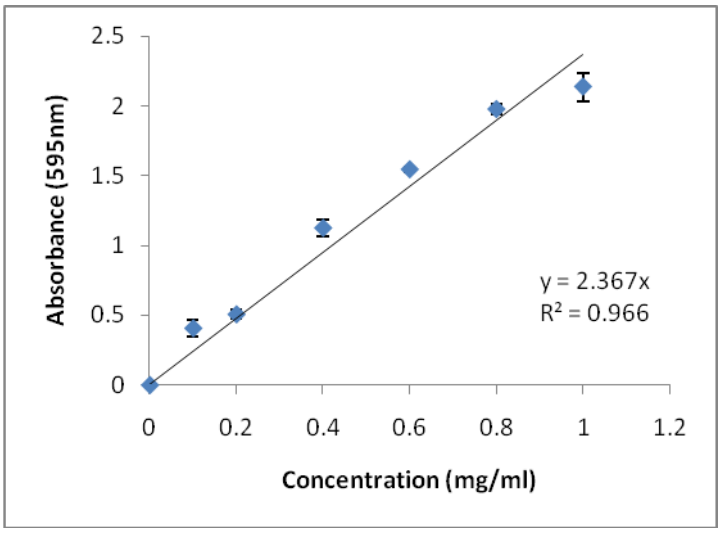


Fig 5: Standard graph of BSA (Protein)

❖ **Emulsification property:**

Emulsification property of concentrated root exudates was observed with reference to diesel oil. 1 ml diesel oil was added to 4 ml of extract and was vigorously vortexed for 2 min. The mixture was allowed to stand for 10 min and then absorbance was taken at 540nm. A comparison with microbial surfactant (by *P. aeruginosa*) and chemical surfactant (Triton X100) was also made **(Cheng *et al.*, 2008).**

4.8 OPERATION OF INSTRUMENT

➢ *Flame Photometer*

Principle

Flame photometry is based on the fact that compounds of alkali and alkaline earth metals can be thermally excited in a low temperature flame and when the atoms return to the ground state they emit radiation, which lies mainly in the visible region of the spectrum. Each element emits that radiation which is specific to that element. E.g.:- Na = 589nm, K = 766.5, Ca = 622.

Over a certain range of concentration the intensity of the emit radiation is directly proportional to the number of atoms returning to the ground state. This in turn is proportional to the absolute quantity species volatized in the flame i.e., light emitted is proportional to the sample concentration. An optical filter isolates the light emitted by the element at its characteristic wavelength and a photon detector, which provides a signal proportional to the sample concentrations, measures the intensity of that light.

Such an electrical signal is processed with the help of analog to digital converter and the microprocessor.

➢ *Inductively Coupled Plasma – Atomic Emission Spectrophotometer (ICP - AES)*

Principle:

ICP atomic emission spectrometry is based on the fact that the atoms are promoted to higher electronic energy levels when heated to high temperatures. In fact, the plasma temperature is sufficient to ionize most atoms. For about three quarters of the elements amenable to the technique, the most sensitive line arises from an ion rather than an atom. As the excited species leave the high temperature region, the absorbed energy is released as ultraviolet and visible photons when the excited atoms decay to lower energy levels or the ground electronic state. Useful emission lines generally occur in the region between 160 and 900 nm. Atomic and ionic emission lines are very narrow, typically less than 5 ppm (17), and their wavelength s follow well understood selection rules.

General Uses of ICP-AES

- Determination of Environmental Protection Agency (EPA) priority pollutants metals in water, soils solid wastes or air samples.
- Quality Determination of wear metals in used lubricants.
- Quality control to ensure correct elemental composition of raw materials, intermediates, and finished products.
- Determination of catalyst poisoning elements.
- Elemental composition of unknown materials.
- Absorbing solutes that are encountered i.e. the solute concentrations.
- The absorption of light is exponentially related to the length of the light path through absorbing solution.

5. RESULTS AND INFERENCE

5.1 CHARACTERIZATION OF ARSENIC CONTAMINATED SOIL

5.1.1 Physico-chemical parameters of soil

Some of the physico-chemical properties of arsenic contaminated soil are listed in Table 1:

PROPERTIES	CONCENTRATION
pH	7.900
Electrical conductivity (mS/cm)	2.010
Cation Exchange Capacity (CEC) (meq/100g)	9.035
Free calcium carbonate (%)	8.700
Organic carbon (%)	1.080
Water holding capacity (%)	42.940
Bulk density (g/cm^3)	1.380
Porosity (%)	65.600

Table 1: Physico-chemical characterization of soil

Soil properties such as pH and soil organic matter (SOM) are principle factors that influence the mobility/availability of heavy metals and are considered to determine the retention capacity of soil **(Sposito, 1989).** EC is a measure of the total concentration of soluble salts in the pore water. High salinity can have a large osmotic influence on plant growth, as well as on soil organisms.

CEC refers to the degree to which soil can adsorb and exchange cations. It is the measure of fertility, nutrient retention capacity and the capacity to protect ground water from cation contamination. It is dependent on soil texture and organic matter content. A low value of CEC indicates less binding of the metal ions to the soil. Generally, the more the clay and organic matter content in soil the higher is its CEC.

The organic matter content of the soil was found to be 1.5%. Organic particles such as humics are ubiquitous in most natural environment and their colloidal size and high chemical reactivity make them extremely important in controlling the mobility and bioavailability of micronutrients and pollutants (Davies *et al*., 2001).

Free $CaCO_3$ content of soil (9.775 %) contributes to the alkalinity of the soil. Such soil is called chalky or calcareous soils. Calcium carbonate is an efficient neutralizer of acidic constituents of the soil sample. Significant contents of organic matter and free $CaCo_3$ are critical in binding metal ions to the soil indicating limited solubility and less mobility of the metal ions.

The results presented in Table 1indicated that the parameters such as pH, EC, organic carbon, water holding capacity etc. of As contaminated soil are higher which might be making the environment favourable for the growth of micro-organisms. This could be one of the reasons for the higher microbial count of organisms in soil. Also there are possibilities that the prevailing micro-organisms might have developed resistance against the particular concentration of arsenic.

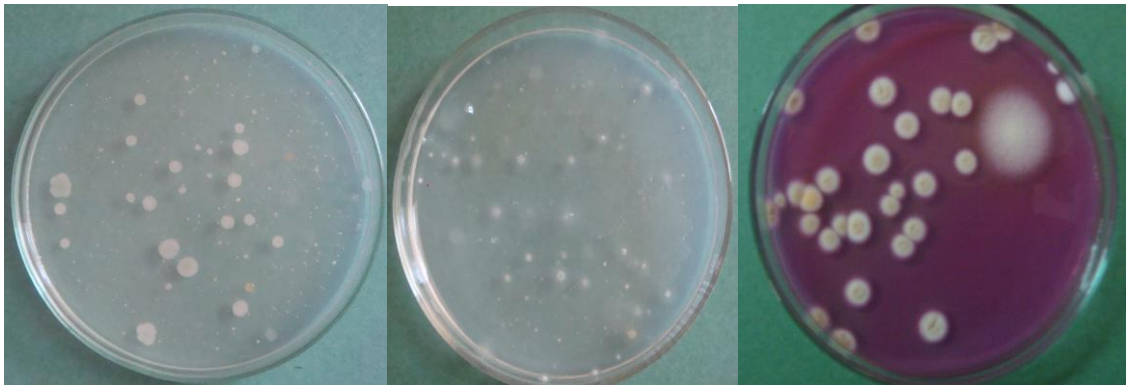
5.2 MICROBIOLOGICAL STUDY OF ARSENIC CONTAMINATED SOIL

The development and biochemical activities of soil micro-organisms undergo several alterations under stress conditions caused by adverse anthropogenic effects such as dissemination of chemical pollutants. Biological methods can measure the actual impact of contaminants on soil organisms; they show the growth and activity inhibition under stress conditions.

On incubation of arsenic contaminated soil on different media, growth at both 10^{-1} (plate 1) and 10^{-2} (plate 2) dilution was observed. It was also seen that colonies formed at 10^{-1} dilution are more as compared to colonies at 10^{-2} dilution (Table 2).

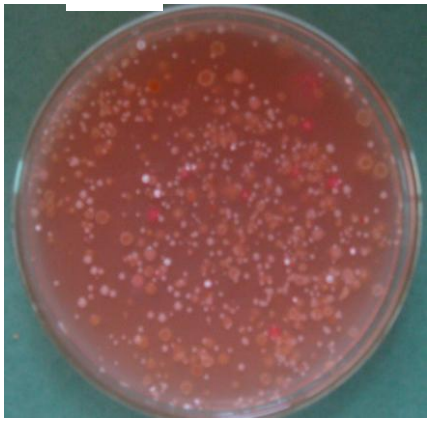
Dilution	***Bacteria (CFU/ml)***	***Azatobacter (CFU/ml)***	***Fungi (CFU/ml)***	***Actinomycetes (CFU/ml)***	***Rhizobium (CFU/ml)***
10^{-1}	29×10^2	4×10^2	21×10^2	9×10^2	19×10^2
10^{-3}	17×10^4	1×10^4	9×10^4	5×10^4	11×10^4

Table 2: Microbiological characteristics of arsenic contaminated soil



B C

A



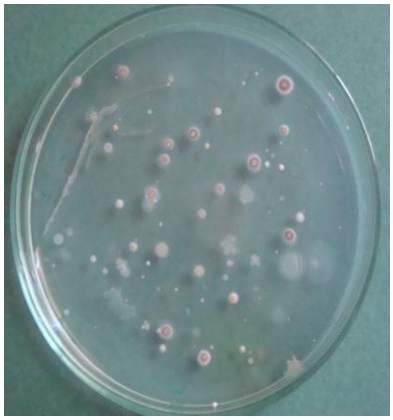


Plate 1: Microbiological characteristics of arsenic contaminated soil (10^{-1})

A : Bacteria B : Azotobacter C: Fungi D:Rhizobium E:Actinomycetes

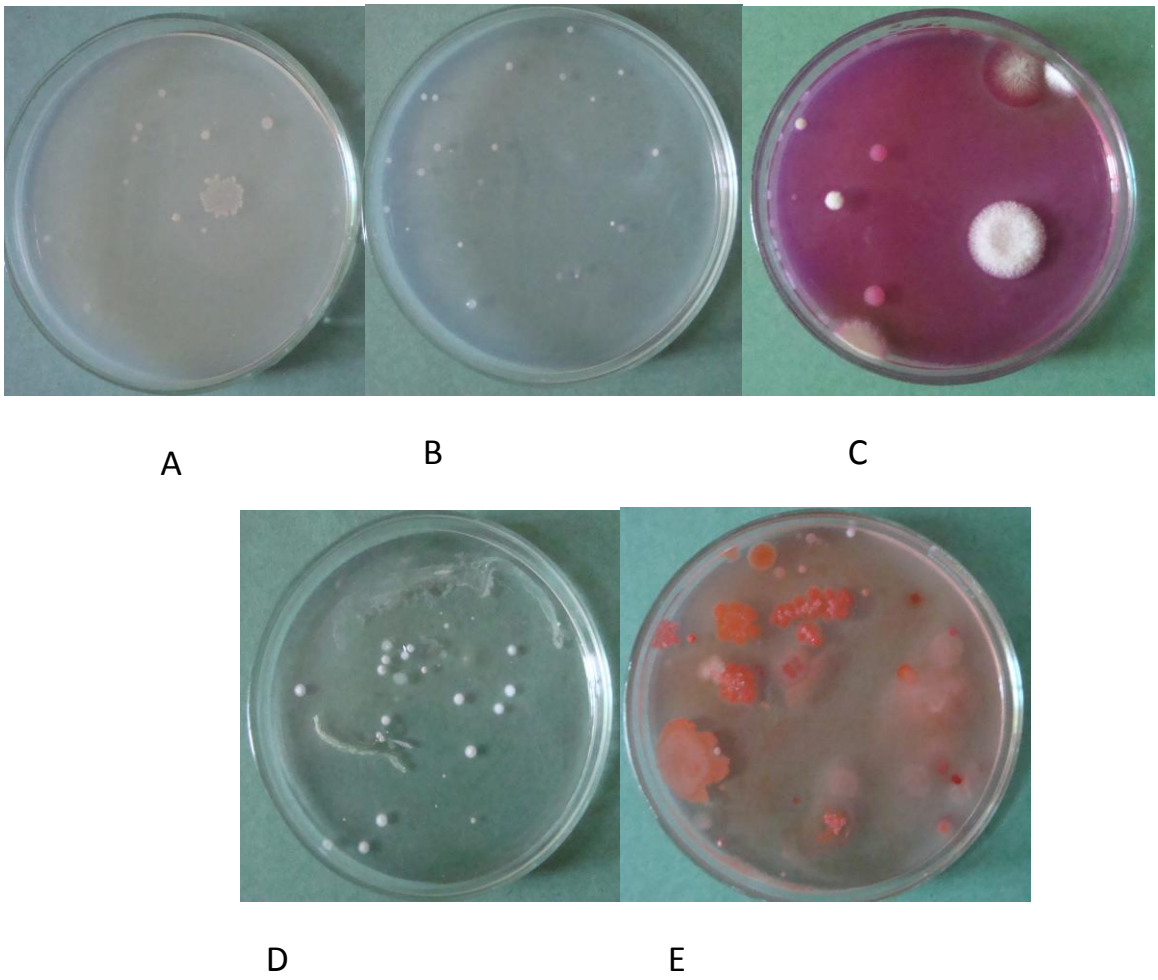


Plate 2: Microbiological characteristics of arsenic contaminated soil (10^{-3})

A : Bacteria B :Azotobacter C :Fungi D :Actinomycetes E :Rhizobium

5.3 ISOLATION OF RHAMNOLIPID PRODUCING BACTERIA

On plating the bacterial isolates on CTAB-MB MSM agar media dark blue colonies were observed which indicates the presence of rhamnolipid producing bacteria (plate 3).

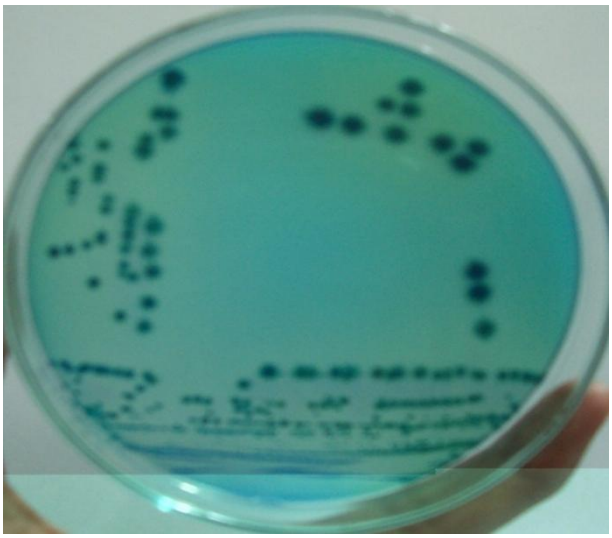


Plate 3: Dark blue colonies of rhamnolipid producing bacterial isolates on CTAB-methylene blue agar.

5. 4 BIOSURFACTANT

5.4.1 Production of biosurfactant

For production of rhamnolipid, strain of *Pseudomonas aeruginosa* (reported to produce rhamnolipid biosurfactant) was inoculated into sterile mineral salt medium (MSM) (Dubey *et al.*, 2001). The foaming developed after 96 hrs of continuous shaking on a rotary shaker and incubation indicates biosurfactant production (fig. 6).

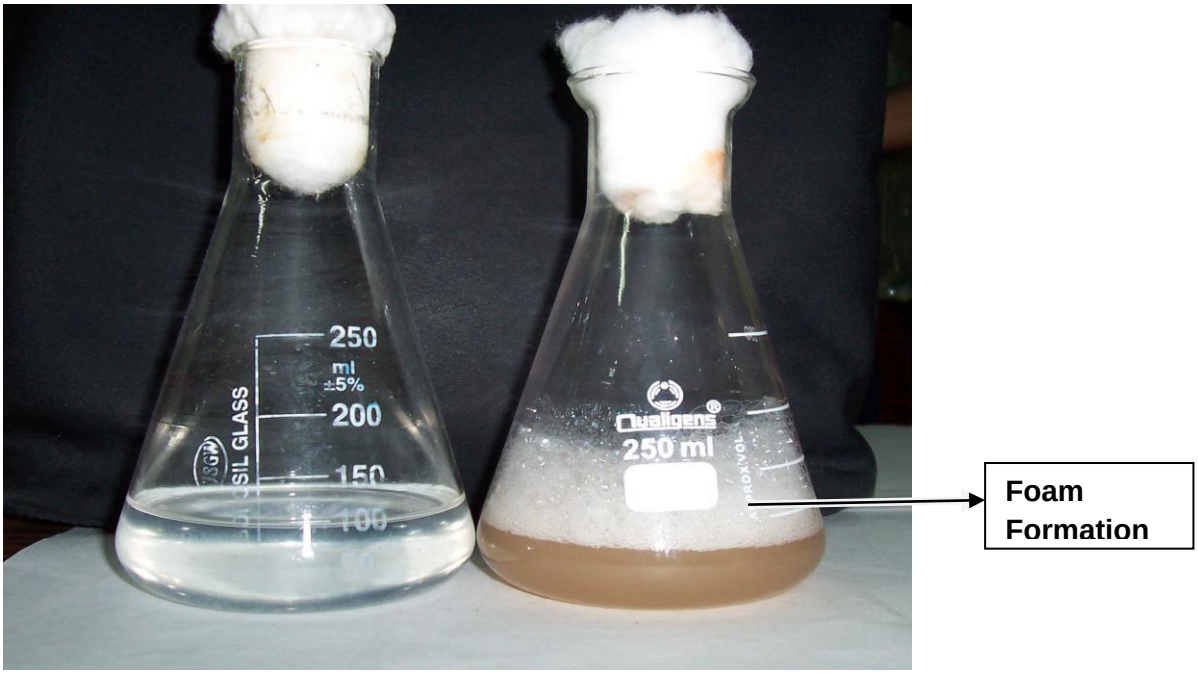


Figure 6: Biosurfactant produced by *Pseudomonas aeruginosa*

5.4.2 Quantification of Biosurfactant

Production of rhamnolipid biosurfactant was investigated by many workers using different carbon sources. The concentration of biosurfactant produced(using glucose as the sole carbon source) was found to be 0.45g/l (fig. 7).

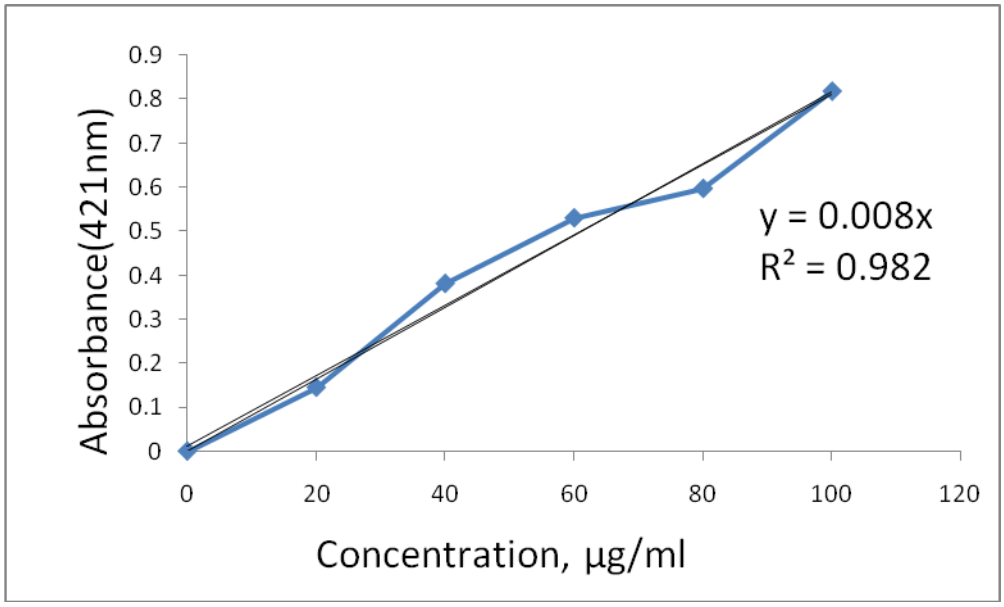


Figure 7: Standard graph for rhamnose using orcinol assay.

5.4.3 Emulsification stability of Biosurfactant

The emulsion stability assay was conducted for 30 days. Stable emulsion of kerosene and biosurfactant was formed after preliminary vortexing (fig. 8). The stability of emulsion decreased drastically during the initial days and later was found to be almost stable (fig. 9). Thus, this study suggests greater is the emulsion stability of biosurfactant, more is the complexation durability.

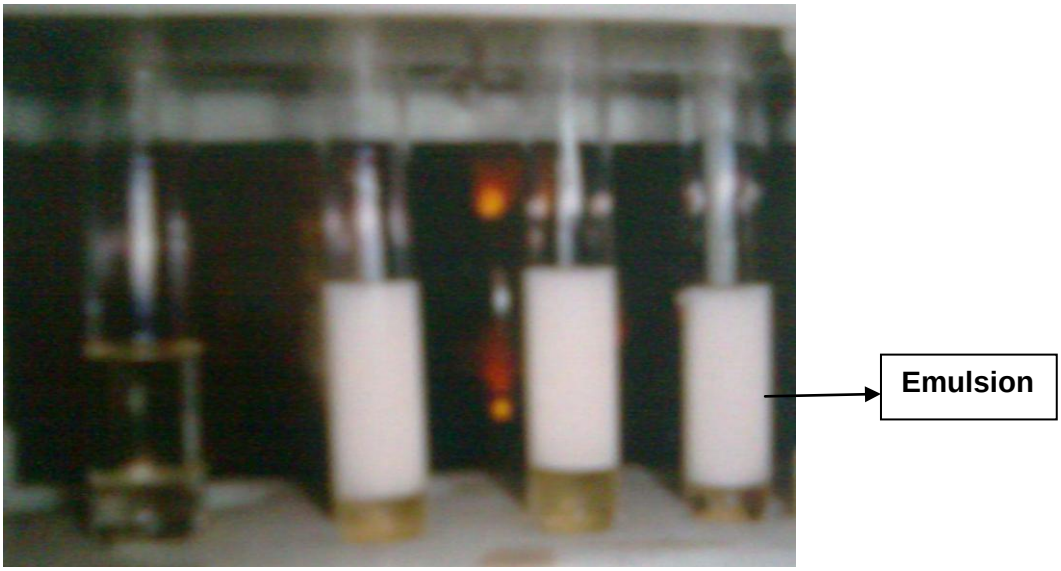


Fig 8: Emulsification stability test of rhamnolipid biosurfactant

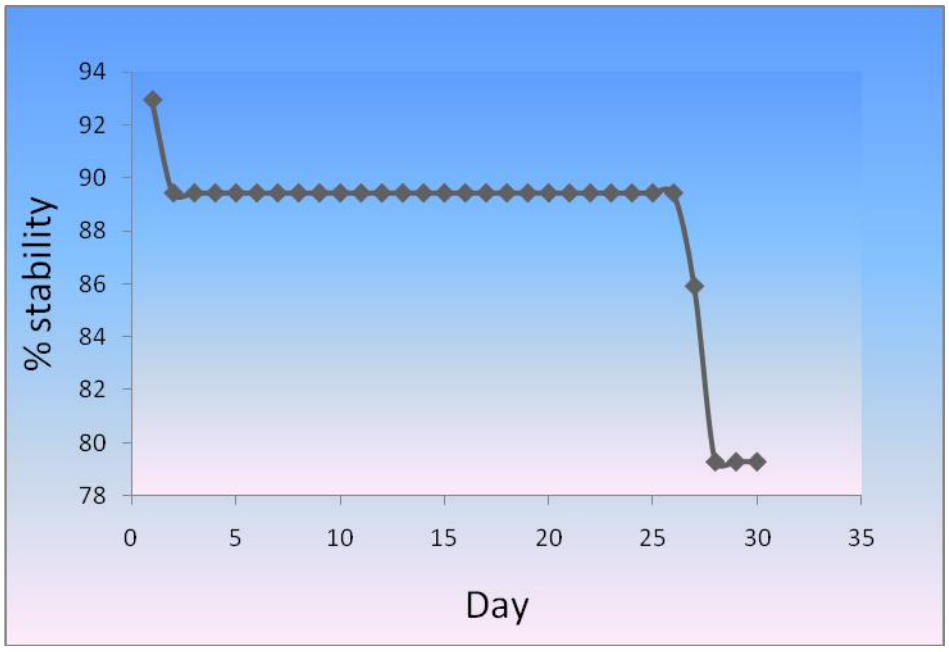


Fig 9: Emulsification stability of biosurfactant

5.5 ESTIMATION OF ARSENIC CONCENTRATION IN CONTAMINATED SOIL

5.5.1 Total Arsenic concentration in soil

The concentration of arsenic in the contaminated soil sample was found to be 566 ppm. However, the maximum permissible arsenic concentration in soil, as per the guidelines given by the USEPA (United States Environment Protection Agency) is known to be 20 ppm

5.5.2 Fractionation of Arsenic in soil:

Maximum amount of arsenic was found in residual fraction followed by organic bound fraction and oxide bound fractions (fig. 10). These three fractions confined more than 80% of total Arsenic found in soil. The remaining arsenic was bound as carbonate fraction exchangeable fraction and water extractable fractions. Metals bound to carbonates can be immobilized by change in pH whereas exchangeable metals are most readily available to biota.

As maximum arsenic concentration occurs as the residual or non-extractable fraction, it can be concluded that these metals are generally retained within the crystal lattices of minerals and hence these metals are usually not expected to be released over short period of time under the natural conditions and therefore cannot be easily removed. Therefore, fractionation assay is an effective means to ascertain the fraction harboring the maximum amount of metal and hence help to channelize remediation strategies towards that fraction to achieve better results in the form of maximum removal.

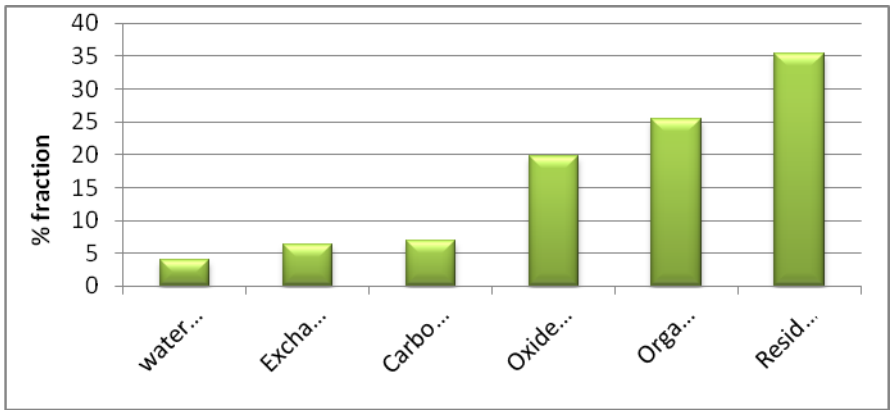


Fig10: Fractionation of arsenic in contaminated soil

5.6 CHARACTERIZATION OF ROOT EXUDATES

5.6.1 Total carbohydrate content:

According to the results decrease in total carbohydrate content of root exudates was observed in presence of arsenic salts. In overnight incubation procedure, trivalent salt of arsenic caused lesser decrease in carbohydrate content of root exudates as compared to pentavalent salts of arsenic (fig. 11). In shock treatment procedure, decrease in carbohydrate content of root exudates was comparable in presence of trivalent and pentavalent salt of arsenic (fig. 12).

The total carbohydrate content present in root exudates is presented in Table 3.

Total Carbohydrate content (mg/mL)	As(+3) Overnight incubation	As(+5) Overnight incubation	As(+3) Shock Treatment	As(+5) Shock treatment
0 ppm As	3.209	3.238	4.766	4.872
5 ppm As	2.416	1.213	1.696	1.903
15 ppm As	1.982	1.008	1.144	1.565
25 ppm As	1.504	0.948	1.005	1.267

Table 3: Carbohydrate content in root exudates at different concentration of arsenic

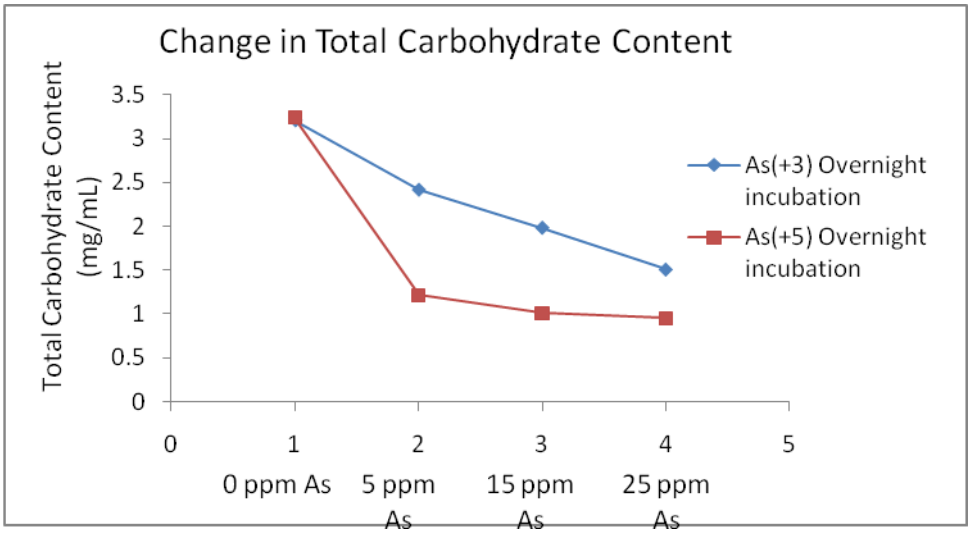


Fig 11: Change in cabohydrate content during overnight incubation

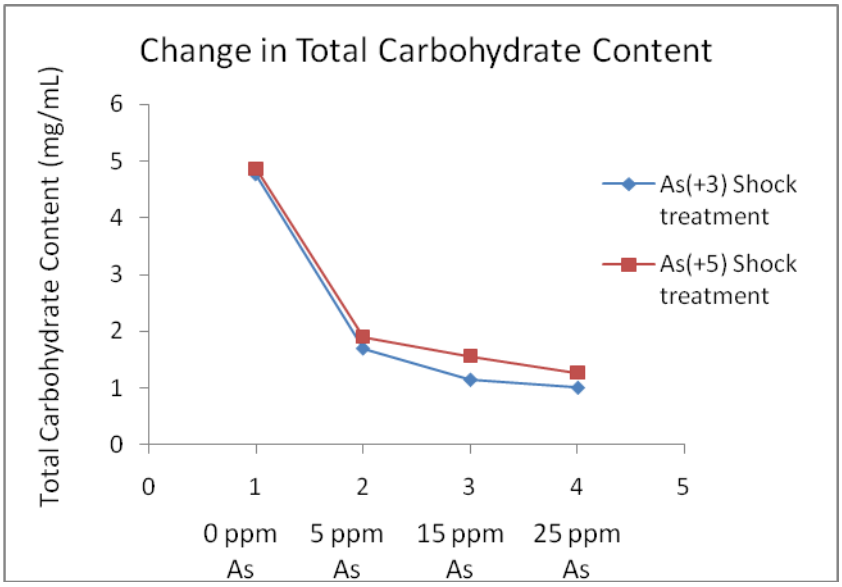


Fig 12: Change in carbohydrate content during shock treatment

5.6.2 Total protein content:

From the results no significant change in total protein content of root exudates was observed in both overnight and shock treatment in presence of different concentration of arsenic (fig. 13).

The total protein content present in root exudates are shown in Table 4.

Total Protein Content (mg/mL)	As(+3) Overnight incubation	As(+5) Overnight incubation	As(+3) Shock treatment	As(+5) Shock treatment
0 ppm As	1.879	1.969	2.069	2.039
5ppm As	1.865	2.027	1.989	2.081
15ppm As	1.923	2.005	2.011	1.989
25 ppm As	1.901	2.069	1.959	1.989

Table 4: Protein content in root exudates at different arsenic contamination

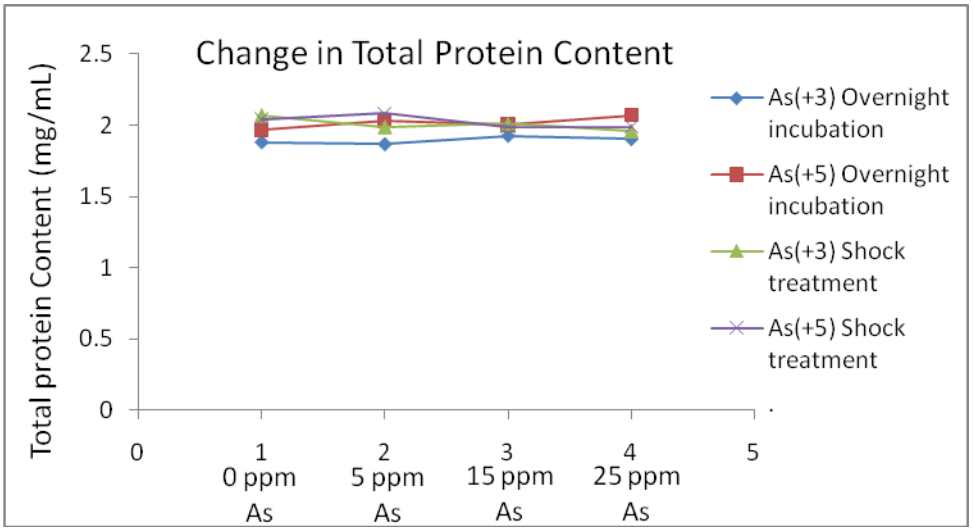


Fig 13: Change in protein content during shock and overnight treatment

5.6.3 Emulsification activity:

From emulsion activity results it is clear that root exudates of vetiver plant produces surfactant in both shock treatment and overnight incubation (fig. 14). Emulsification stability results shows that Triton (artificial surfactant) degrades rapidly with time whereas plant surfactant and microbial surfactant remains stable for longer time (fig. 15).

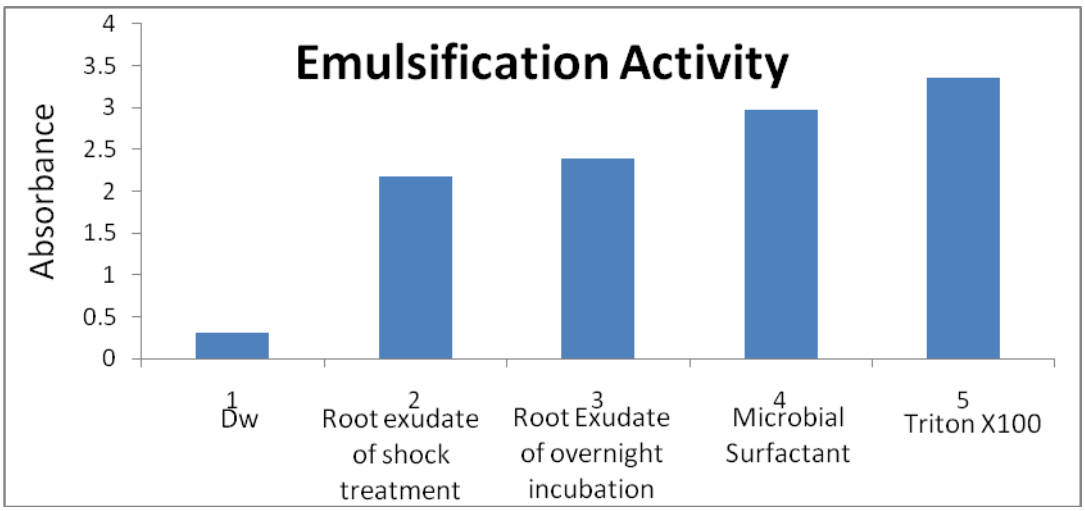


Fig 14: Emulsification activity of root exudates

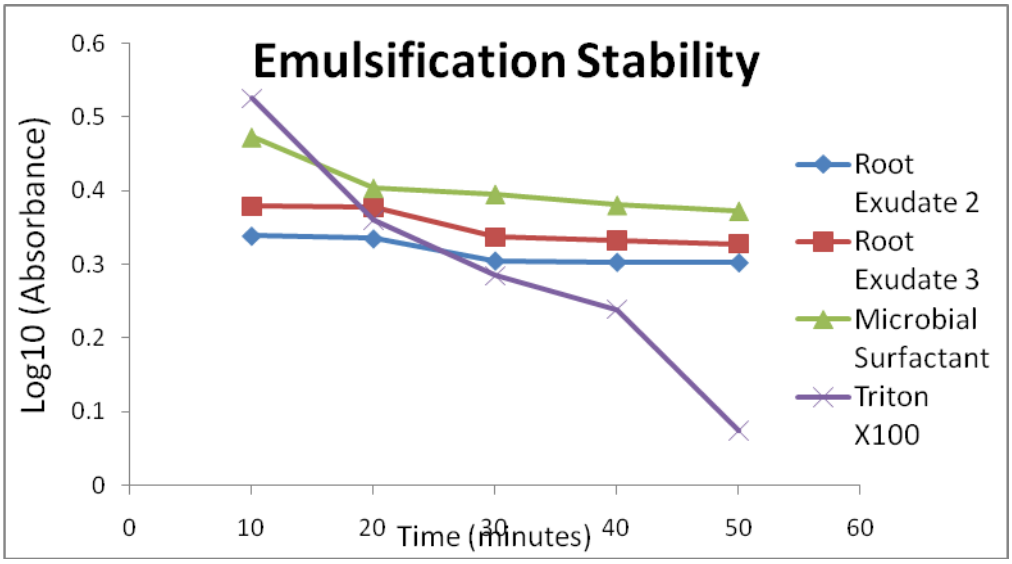


Fig 15: Emulsification stability of surfactant from root exudates.

The present study, in conclusion, summarizes that biosurfactant mediated phytoremediation is one of the novel approach that can be used in the remediation of metal contaminated soil. Use of this technique encompasses a twin approach since they render mobility of metal, which prevent them from accumulating in soil, thus enhancing metal removal and uptake by plants into their root and shoot. Moreover, these treatments are eco-friendly and biodegradable. This eventually leads to restoration of the normal soil physico-chemical properties of metal contaminated soil. The present study shows that biosurfactant assisted phytoremediation are exceptionally efficient in thwarting away detrimental environmental impacts by remediating metals from contaminated soil and help in restoring the integrity of nature.

6. SUMMARY AND CONCLUSION

Metal contamination of soil due to metal mining, metal plating, agricultural activities and industrial waste disposal has increased considerably in recent years leading to contamination of the environmental reservoirs such as water bodies and soils. Soil usually acts as a sink for harboring metals. These metals being immobile in soil accumulate and influence the physical, chemical and biological properties of soil adversely. Treatment of the contaminated soil for removal of metal with metal chelators of microbial origin like rhamnolipid biosurfactant is a novel approach in remediation technology.

The present study focuses on the production of rhamnolipid biosurfactant by *Pseudomonas aeruginosa* and assessing its potential for removal of arsenic from arsenic contaminated soil. The results of the studies conducted on metal removal efficiency of biosurfactant-plant system are presented below:

- ✓ The arsenic contaminated soil was characterized with respect to its physico-chemical properties. From the physico chemical parameters it can be concluded that significant contents of organic matter and free $CaCO_3$ are critical in binding metal ions to the soil indicating limited solubility and less mobility of the metal ions.
- ✓ The initial metal concentration in arsenic contaminated soil was found to be 566 ppm which is known to be beyond the permissible guideline limits.
- ✓ Bench scale experiment on fractionation of arsenic in arsenic contaminated soil was conducted where it was reported that maximum amount of arsenic was found to be in the residual fraction, followed by organic bound fraction, oxide bound fractions, carbonate fraction, exchangeable fraction and water extractable fractions. This indicated that arsenic is mainly present in non-available form.
- ✓ Contaminated soil was studied with respect to microbial characterization a total number of 22 isolates were isolated and were screened for bacteria having biosurfactant producing ability by CTAB- methylene blue MSM agar method.
- ✓ Biosurfactant was produced by *Pseudomonas aeruginosa* in mineral salt medium. The concentration of biosurfactant produced was found to be 0.45g/L after 96 hours of incubation.
- ✓ The biosurfactant produced was of Rhamnolipid type.
- ✓ The rhamnolipids biosurfactant produced was found to have better emulsion stability.

- ✓ Characterization of root exudates shows that vetiver plant produces surfactant in both shock treatment and overnight incubation with $CaCl_2$ and the produced surfactant has more emulsion stability as compared to artificial surfactant.
- ✓ Presence of arsenic in solution results in decrease in carbohydrate content of root exudates of vetiver whereas protein content is almost same.
- ✓ Also, the present study indicated that rhamnolipid biosurfactant is effective in mobilizing the soil bound arsenic to plant available form and hence can be utilized for remediation of metal contaminated site soil.

Hence, it can be summarized that biosurfactant mediated phytoremediation is one of the novel approach that can be used in the remediation of metal contaminated soil. Use of this technique encompasses a twin approach since they render mobility of metal, which prevent them from accumulating in soil, thus enhancing metal removal and uptake by plants into their root and shoot. Moreover, these treatments are eco-friendly and biodegradable. This eventually leads to restoration of the normal soil physico-chemical properties of metal contaminated soil. The present study shows that biosurfactant assisted phytoremediation are exceptionally efficient in thwarting away detrimental environmental impacts by remediating metals from contaminated soil and help in restoring the integrity of nature.

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