Arsenic is the third element in Group V of the periodic table which is a natural toxic metalloid and is harmful to most of the organisms; also it is considered as a carcinogenic agent by the World health Organization. Arsenic is abundantly distributed on earth’s crust with small quantities found in soil, water, air and rock. The prevalence of arsenic is due to the continuous cycling of its different forms in the environment. The chief source of contamination is due to excess discharge of arsenic from both natural and anthropogenic processes. Natural resources contribute one-third of arsenic forms to the earth’s atmosphere (Green facts on health and environment: "Environmental Health Criteria for Arsenic and Arsenic Compounds (EHC 224) 2001). Natural or geogenic contamination occurs due to mining, smelting, combustion of fossil fuels, forest fires and volcanic activity sand other natural processes occurring in the soil and ground and surface water. (Mandal and Suzuki 2002; Donahoe et al 2004; Valenzuela 2009) while the anthropogenic activities include use of arsenic in fertilizers, pesticides, wood preservative manufacturing, mining processes, use in tobacco, Glass and textile industries etc. (Salam et al 2009; Neumann et al 2010 and National institute of Environmental health sciences fact sheet 2014).

The permissible limit for arsenic in drinking water as recommended by WHO (World Health Organization) and FAO (Food and Agriculture Organization According to Union) is 10ppb while in India the permissible limit according to Bureau of Indian standards (BIS) is 50ppb. The global average of arsenic level in soil is 10ppm and according to European Union the maximum acceptable arsenic limit for agricultural soil is 20ppm (Rahaman et al 2013). The maximum concentration of arsenic permitted by the U.S. Environmental Protection Agency (EPA) 1993 in the industrial sludge is 75ppm.

Arsenic contamination in groundwater has become a major problem worldwide. It’s toxicity 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 (Chowdhary et al., 2000; Smith et al., 2001; Anwar et al., 2002; Mitra et al., 2002; Pandey et al., 2002; McCarty et al 2011). In India the contamination of arsenic in water above
permissible limit (50 ppm) has been reported in Bengal delta plain sediments, (Islam et al., 2004), middle Ganga plains of Bihar, U.P (Chakraborty et al., 2003; Srivastava et al. 2012) the Malda district of West Bengal, (Rahaman et al., 2013) and in 18 out of 23 districts of Assam (UNICEF 2014). Although the presence of arsenic within permissible limit was also detected in groundwater in Rajasthan while high arsenic concentrations are reported in the mining areas of Rajasthan in western India, especially around the mining areas of Khetri Copper Complex and Zawar mines in Jhunjhunu and Udaipur districts respectively (Ministry Of Water Resources, River Development And Ganga Rejuvenation Occurrence Of High Arsenic Content In Ground Water Committee On Estimates 2014-15).

Many arsenic compounds have strong affinity with soil particles and the arsenic reaches soil and rocks by the percolation of arsenic from either groundwater or contaminated surface water which further extends the pollution in environment (National institute of Environmental health sciences fact sheet 2014). The contamination of surroundings with high concentration of arsenic leads to measurable health effects including cancer of skin, lung, liver, kidney and bladder (Chen et al., 1992; Col et al., 1999), and cardiovascular and neurological effects (Jack et al., 2002; Oremland et al., 2005; Basu et al., 2013).

Arsenic is found in its both organic as well as inorganic form, in which inorganic forms are more toxic than organic forms (Hopenhayn et al., 2006). The primarily stable oxyanionic inorganic forms found in the environment are Arsenite [As (III)] (As (III) as H_3AsO_3, etc.), and Arsenate [As (V)] (including As (V) as H_2AsO_4^−, HAsO_4^{2−}, etc.) (Jack et al., 2002; Jackson et al., 2003; Oremland et al. 2004; Branco et al., 2009; Chang et al., 2010). Arsenite is 100 times more toxic (Nakamuro and Sayato, 1981, Cullen and Reimer, 1989; Neff, 1997) than arsenate which is poorly soluble in water and, therefore, less bio available (Mandal and Suzuki 2002).

The arsenic can be transformed into its various forms by native bacteria. Thus, the microbial approach of arsenic bioremediation is a promising strategy, as the use of microbes acts as an attractive green cure technology for the low cost and
efficient removal of arsenic from soil (Purakayastha, 2011). Bacteria has developed different strategies to transform arsenic including arsenite oxidation, cytoplasmic arsename reduction, respiratory arsename reduction, and arsenite methylation (Mukhopadhyya 2002; Oremland et al., 2002; Silver and Phung 2005; Bhattacharya et al., 2007; Purakayastha, 2011).

This study is based on exploring the potential of arsenite-oxidizing bacteria in conversion of most toxic form of arsenic which is arsenite to less toxic form i.e. arsename, which could significantly help in bioremediation of polluted soils and water. This arsenic biotransformation by bacteria is due to the production of enzyme arsenite oxidase.

Genes encoding arsenite oxidation enzyme (*aox*) are organized in an operonic structure. These genes have been identified and sequenced in several organisms, showing a common genetic organization of *aoxA*-*aoxB* genes, that encodes the small and large subunits of enzyme arsenite oxidase, respectively (Muller et al., 2003; Santini et al., 2004; Alvarez-Martinez et al., 2007; Muller and Medigue et al., 2007).

The bacteria bearing the capacity to transform arsenite to arsename by virtue of presence of arsenite oxidase enzymes can be further studied in bioremediation of artificial soil environment such as microcosm. Microcosm is an *ex-situ* bioremediation approach where soil is enriched to study the behavior of resident bacteria towards the toxicant or the augmentation of bacterial culture is done to remediate the contaminant. Bioremediation of crude oil and poly aromatic hydrocarbon by bacteria was conducted under controlled conditions by Mirdamadian et al. 2010 and Sathishkumar et al. 2008, which explore the potential of bacteria to oxidize toxicants in natural condition. Bioaugmentation and biostimulation are essential step in the microcosm approach. The former is the addition of specialized and concentrated form of bacteria to the contaminated soil while the later is addition of nutrients and adjustment of physical factors in the microcosm for the survival of augmented bacteria and enhancing the potential of resident bacteria in degradation of the contaminant (Abdulsalam et al 2009).
Thus, the importance of bioremediation of arsenite by biological oxidation using bacteria was understood. The isolation of the arsenite oxidizing bacterial strains from arsenic contaminated soil of two different regions (Jaipur, Rajasthan and Tezpur, Assam) was undertaken and genetic and molecular basis of characterization was conducted. The importance of microcosm approach was perceived in exploring the bioremediation capacity of the isolates in detoxifying the contaminant (arsenite) in the soil. The optimization and interaction of factors required for bacterial arsenite oxidation in microcosm were planned and concluded with the help of statistical software Design Expert Version 9.0.4.1, Stat-Ease Inc.

**Material and Method:**

1. **Isolation and characterization of arsenite oxidizing bacterial isolates**
   1. **Soil sampling and its physico-chemical characterization**
      
      Soil samples were collected from Assam and Rajasthan (Tezpur and Jaipur respectively) at various depths from a textile dyeing industry in Jaipur and soil near tube well in Tezpur in a previously ethanol cleaned polypropylene zip locked bags. Soil samples were physico-chemically characterized for pH, electrical conductivity, organic carbon, organic matter, exchangeable calcium, water holding capacity and metal content (Maiti, 2003; Standard methods for examination of water and waste water-APHA, 2005).

2. **Isolation of arsenite resistant bacteria**
   
   For isolation of arsenite tolerant bacteria from the contaminated soil samples serial dilution method was applied. Soil samples were serially diluted and then inoculated in nutrient broth supplemented with increasing concentration of sodium arsenite. Pure colonies were obtained with repeated spreading, streaking and Gram’s staining.

3. **Determination of Minimum Inhibitory Concentration (MIC)**
   
   The Minimum Inhibitory Concentration (MIC) is defined as the lowest concentration that completely inhibits bacterial growth (Courvalin et al., 1985; Muller et al., 2003). MIC for purified bacterial strains against arsenite was determined in nutrient broth amended with different concentrations of sodium
arsenite. Cell density was measured by measuring the culture turbidity using a spectrophotometer (Systronics UV-Vis. Spectrophotometer-106) at 600nm.

4. **Study of metal and antibiotic sensitivity for the selected strains**

The disc diffusion method of Huysmans and Frankenberger (1990) was used to screen the isolated strain for resistance to various metals like Cadmium chloride, Cobalt (III) nitrate, Lead nitrate, Nickel chloride, Zinc sulphate, Mercuric chloride, Chromium (III) chloride, Sodium selenate, Stannous chloride and Antimony (III) chloride at the concentration of 100 μg/ml. Isolated strains were also screened for resistance to various antibiotics such as: Erythromycin (15 mcg); Penicillin G (10 μg); Oxacillin (1 μg); Cephalothin (30 μg); Clindamycin (2 μg); Amoxyclav (30 μg); Tobramycin (10 μg); Co-Trimoxazole (25 μg); Cephotaxime (30 μg); Ampicillin (10 μg) and Gentamicin (10 μg). Antibiotic sensitivity was established on the basis of zone of inhibition around the disc.

5. **Identification of strains using 16S-rDNA sequencing and biochemical test**

The strain capable of arsenite oxidation was further identified by 16S rDNA sequencing. DNA was isolated from the culture using QIAamp DNA Purification Kit (Qiagen). The 16S rDNA gene fragment was amplified by PCR from genomic DNA using 16S rDNA gene universal primers: 8F and 1492R (Maniatis *et al*., 1989; Sacchi *et al*., 2002) 8F: (5’ AGA GTT TGA TCC TGG CTC AG 3’), 1492R: (5’ ACG GCT ACC TTG TTA CGA CTT 3’) and a single discrete PCR amplicon band of 1500 bp was observed when resolved on agarose gel. The concentration of the purified DNA was determined and was subjected to automated DNA sequencing on ABI 3730x1 Genetic Analyzer (Applied Biosystems, USA). The 16S rDNA gene sequence was used to carry out BLAST with the non redundant database of NCBI genbank database. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al*., 2007). The sequencing results were supported by biochemical characterization from Bergey’s Manual of Determinative Bacteriology (Holt *et al*., 1994).
II. Optimization of growth conditions and analysis of oxidizing ability

1. Optimization of growth conditions (temperature, pH, media) for the selected strains:

For optimization of growth conditions isolates with highest MIC were studied at different temperature (4°C, 30°C, 37°C, 45°C and 60°C), pH (5, 6, 7, 8, 9) in nutrient broth and media optimization in chemical defined media and minimal media was conducted.

2. Analyze oxidizing abilities by a preliminary test (AgNO₃ and microtiter plate method):

Isolated strain showing the maximum MIC was subjected to the following preliminary tests to study its ability of oxidizing arsenite to arsenate using silver nitrate test and Microplate Screening Assay. Development of brown color precipitate in the well showed the presence of arsenate (Mokashi and Paknikar, 2002; Simeonova et al., 2004; Krumova et al., 2008).

Confirmation of oxidizing potential by determination of bacterial arsenite oxidation rate by Molybdenum blue spectrophotometric method.

The basic principle involved in this method is that initially arsenate can react with molybdate to form a complex and then gets reduced by ascorbic acid to produce blue color under conditions of certain acidity and temperature, while arsenite does not react under the same conditions. The blue complex has an absorbance peak at 838 nm and can be measured by spectrophotometric method (Cai et al., 2009b). The strains were confirmed about arsenite oxidation potential using this method.

III. Molecular analysis of strain with highest oxidizing ability

1. PCR amplification of aox gene using specific primers in strain with highest oxidation rate.

The isolated strain was checked for the presence of aox gene which is responsible for arsenite oxidation at molecular level. This was performed by PCR amplification of aox genes using specific primers (Quemeneur et al., 2008; Chang et al. 2010).
2. Localization of enzyme arsenite oxidase responsible for arsenite oxidation

Spheroplast preparation was done for the localization of arsenite oxidase as it is the enzyme responsible for arsenite oxidation in the bacterial system. In order to locate the arsenite oxidase activity the Spheroplast was prepared according to Anderson et al 1992 and Prasad et al 2009 with slight modification.

IV. Ex-situ application of arsenite oxidizing strain Pseudomonas sp. SE-3 in microcosm studies

An automated microcosm experiment was conducted to explore bioremediation potential of Pseudomonas sp. SE-3 in natural conditions in transformation of arsenite to arsenate in contaminated soil. This is an ex-situ application of the bacterial capacity to detoxify arsenic which is less costly and effective. Uncontaminated soil was taken and then the desired amount of arsenic (contaminant) was added for the accuracy on analysis of conversion of arsenite before and after the experiment. In this method bioaugmentation and biostimulation (N: P: K inorganic fertilizer, moisture) was done, the former is the addition of specialized and concentrated form of bacteria to the contaminated soil while the later is addition nutrients and adjustment of physical factors in the microcosm for the survival of augmented bacteria (Pseudomonas sp. SE-3 in this case) and enhancing the potential of resident bacteria in degradation of the contaminant (Abdulsalam et al 2009).

Design of Experiment:

DOE helps in understanding and identify interactions between factors and use contour plots to give a proper understanding of interpretations. The experiment Design Expert Software Version 9.0.4.1 was used, it uses regression model for designing the experiment. The Box-Behnken factorial experimental design employed had four independent variables viz., Arsenic concentration, Biomass, NPK (19:19:19) fertilizer, and moisture. Each of the independent amendment variables was studied at three levels (1, 0, and +1), with 27 experimental runs.

Results:

From this extensive study on contaminated soil from two different regions helped in isolating diversified bacterial species having common potential of oxidizing
the arsenite toxicant to its 100 times less toxic arsenate species. Following findings were achieved:

1. Both soil samples were found to be contaminated with arsenic. This contamination is due to water containing arsenic through textile discharges flowing in the open field in jaipur while tube well water seeping in the soil in Tezpur. Tube well water contamination in Assam was reported by many researchers (Sabhapandit et al 2010; Chetia et al 2011; Bhuyan 2011) which stated it as very serious problem as 80% of rural population still depends on tube well water. This problem encouraged to study soil near the tube well area. The physicochemical nature of both the soils was entirely different.

2. Eight bacterial isolates were achieved i.e. four from each sample. The MIC of the bacterial strain obtained was in range from 1-6g/l As(III), which shows the hypertolerance against arsenic toxicity.

3. The two strains SE-3, TB-1 from Jaipur and Tezpur with highest MIC were further studied and SE-3 showed sensitivity to stannous chloride, mercuric chloride and antimony chloride, while TB-1 was sensitive towards Zinc sulphate, stannous chloride and mercuric chloride. Both the strains were found to be sensitive towards antibiotics like Erythromycin (15 mcg); Penicillin G (10 μg); Oxacillin (1 μg); Cephalothin (30 μg); Clindamycin (2μg); Amoxyclov (30 μg); Tobramycin (10 μg); Co-Trimoxazole (25 μg); Cephotaxime (30 μg); Ampicillin (10 μg) and Gentamicin (10 μg). These tests were based on zone inhibition around the disc containing metal and antibiotics.

4. The Identification of strains using 16S-rDNA sequencing and biochemical test resulted in identifying the strains as showing similarity with Pseudomonas species and with uncultured bacterial clone. These two bacterial sequences thus obtained were submitted in NCBI genebank under the name Pseudomonas sp. SE-3(accession no KP866680) and Bacterium sp. TB-1(accession no.KP730605) respectively.

5. Optimization of growth conditions were approximately similar for both the strains and were able to grow best at 37°C, pH 7-8 and in nutrient broth.
6. Analysis of oxidizing abilities in both the strains SE-3 and TB-1 was established by a preliminary test (AgNO$_3$ and Microtiter plate method) and confirmation of oxidizing potential and determination of bacterial arsenite oxidation rate by Molybdene blue spectrophotometric method was proved.

7. PCR amplification of $aox$ gene using specific primers in strain with highest oxidation i.e $Pseudomonas$ sp. SE-3 rate was studied which revealed the presence of $aox B, aox C$ genes.

8. The presence of enzyme responsible for oxidation arsenite oxidase was established in pellet fraction by DCPIP enzyme assay method by spheroplast preparation.

9. Single-variable optimization methods are time consuming, monotonous and they can also lead to misinterpretation of results as they select the precise factors that influence the process. Thereby in this study, Response Surface Methodology (RSM) was used in designing experiments for microcosm approach. This method in Design Expert Statistical software version 9.0.4.1, analyzed the interaction between different factors (biotic and abiotic) and optimized the factorial conditions which are best for arsenite oxidation in the microcosm.

10. Microcosm studies of natural attenuation of arsenite were planned by Design Expert Version 9.0.4.1. The uncontaminated soil augmented by $Pseudomonas$ sp. SE-3 explored the potential of bacterium of converting of arsenic natural condition under controlled parameters. The controlled parameters were arsenic concentration, biomass and moisture and NPK fertilizer. Oxidation in microcosm setup with varying parameters according to experimental run was studied for 28 days. Oxidation in samples was studied by Molybdene method and observation of 14$^{th}$ day was analyzed using design expert.

11. By the design expert it was concluded that model was significant and experiment suggested that increasing concentration of arsenic requires high amount of biomass and NPK fertilizer (inorganic nutrient). While it was found that increase in moisture reduced the rate of oxidation.

12. The correlation between colony forming unit and rate of oxidation between experiment run having same variables (8, 12, 19) and Control (32, 34) during the microcosm setup for 28 days was also seen.
13. The synergic association of introduced *Pseudomonas sp.* SE-3 (Allochthonous) and native soil micro flora (Autochthonous) during arsenite oxidation in microcosm setup was also concluded by this research.

**Conclusion**

From the above investigation it can be concluded that the soil samples from both the sites contain arsenite hypertolerant bacteria with the capability of oxidizing the arsenite playing a key in bioremediation strategy.

The molecular approach of arsenite oxidation in the isolated bacterial strains was analyzed by amplifying the genes responsible for biotransformation of arsenite to arsenate. Further the activity of enzyme in pellet fraction confers its potential role in bioremediation. The potential of the bacterium with highest MIC and oxidation rate was exposed to the natural conditions through microcosm revealing its application in future prospective of arsenite bioremediation of contaminated sites. This is an *ex-situ* application of the bacterial capacity to detoxify arsenic which is less costly and effective.

Thus, the investigation in the present research revealed, the arsenite oxidizing potential of the strains isolated from contaminated soil. The application of this study in natural environment is also promising by the microcosm approach as it suggested the optimum conditions for arsenite oxidation. Thereby, soil conditions of the contaminated site can be adjusted carefully to enhance the potential of inherent as well as introduced bacteria for reducing toxicity of arsenite from the environment. The synergy between the *Pseudomonas sp.* SE-3 and the native soil microflora proved to be predominant feature of this study as both utilize their inherent potential of arsenite oxidation. The introduced bacteria act as a “specialist” in the contaminated soil, which oxidizes arsenite and reduces time necessary for biotransformation of arsenite in the environment.