WORK PLAN

Isolation of Arsenite oxidizing Bacteria

Determination of MIC of arsenic hypertolerance in bacteria

Optimization of Growth Conditions

Determination of oxidation ability
Preliminary test: 1) AgNO₃ method 2) Microtiter plate assay
Confirmatory test: Molybdenum blue spectrophotometric method

16S rDNA sequencing and biochemical characterization of the isolated strains

Molecular analysis of Strain with highest oxidizing ability

PCR Amplification of aox

Localization of Arsenite oxidase

Microcosm studies of natural attenuation of Arsenite using Design Expert Statistical Software Version 9.0.4.1
The primary focus was to collect the soil samples from the sites impacted with heavy metal poisoning. The sampling from two states (Jaipur, Rajasthan and Tezpur, Assam) of India was conducted. The main aim was to explore the arsenic resistance potential possessed by bacteria taken from, soil having different contamination sources. The study focused on 1) the isolation of arsenite oxidizing bacterial strain, 2) to detect the molecular basis of oxidation and 3) to explore the applicability of potential bacterial strain in environment for arsenite detoxification.

3.1 Isolation and characterization of arsenite oxidizing bacterial isolates

3.1.1 Soil sample collection and physicochemical analysis:

Soil samples were collected from two different states on the basis of prior history of arsenic contamination. Soil sample from Rajasthan was taken from Sanganer area of Jaipur City which had Textile Industries discharging effluents in open field. Sample from open field was taken from the depth of 10-15 cm, in the month of December 2012. The second sample was collected from, Tezpur in Sonitpur district, Assam in reference with the prior studies of arsenic contaminated tube wells and rings wells by geogenic sources (Sabhapandit et al., 2010). So the soil near tube well was collected from depth (i) 6inch (15.24cm) and (ii) 8inch (45.72cm) in February 2013. All the three soil samples i.e. one from Jaipur and two from Tezpur were collected in sterile polypropylene zip lock bags. Fresh samples were then processed for bacterial isolation, after which the samples were passed through 2 mm sieve to remove sand gravel, plant debris and stored at 4°C for physicochemical analysis, metal estimation.

The soil quality of each soil sample was analyzed by estimation of physicochemical parameters such as pH, electrical conductivity(EC), water holding capacity, texture analysis, organic carbon, organic matter, total hardness, sodium, potassium concentration, Sodium Adsorption Ratio(SAR), Cation Exchange Capacity (CEC) using standard protocols(APHA 2005 and Maiti, 2003). The physical parameters include;
(i) **pH:**

pH is the measure of acidity and alkalinity in soil which is defined as the negative logarithm (base 10) of the activity of H\(^+\) ion and OH\(^-\) ion in soil suspension. The range of pH determines the nature and chemical processing of soil which effects the microbial and plant diversity and growth. According to Ramachandra *et al.*, 2012 optimum pH for crops is 6.5 to 8.5, while at pH lesser than 6.5 is considered acidic which requires liming while pH higher than 8.5 is alkaline for crops and require gypsum for its reclamation.

**Procedure:**

Soil suspensions were prepared in 1:2.5 ratio with water followed by stirring through Magnetic Stirrer (Eltek MS-203) for 30 minutes each. pH was measured by digital pH meter (Electronic India, digital pH meter model-III) calibrated with pH- 4 and 7 buffer (Maiti, 2003) to estimate the acidity or alkalinity of the soil as it indicates the suitability for crop plantation and availability of nutrients.

(ii) **Electrical Conductivity:**

Electrical Conductivity (EC) is a measure of soluble salts (ions) in the soil which depicts the health of soil affecting crop yield, crop suitability, plant nutrient availability and microbial activity. EC increases with the increasing content of soluble salts. Soil properties also vary with EC values as given in Table 1.

**Table 1: Soil state for crops in relation to Electrical Conductivity (EC)**

(Ramachandra *et al.*, 2012)

<table>
<thead>
<tr>
<th>Electrical Conductivity (mS/cm)</th>
<th>Nature of Soil</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;0.8</td>
<td>Normal</td>
</tr>
<tr>
<td>0.8 to 1.6</td>
<td>Critical for sensitive crops</td>
</tr>
<tr>
<td>1.6 to 2.5</td>
<td>Critical for salt tolerant crops</td>
</tr>
<tr>
<td>&gt;2.5</td>
<td>Injurious for many crops</td>
</tr>
</tbody>
</table>

**Procedure:**

Electrical conductivity of soil suspensions prepared in 1:2.5 ratio of soil and water, which is stirred in Magnetic stirrer (Eltek MS-203) at regular intervals of 1 hour. The EC in suspensions were determined by digital conductivity meter (Century
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CC- 601) prior calibrated with 0.956 cell constant. It is measured in mS/cm (Maiti, 2003).

(iii) Water Holding Capacity:
Water holding capacity is the maximum capacity of the soil to hold water to get saturated leaving no pores spaces left to hold water. It varies with the time and season, which measured by immersing unit weight of dry soil in water under standardized conditions. It also depicts the physical and chemical health of soil (Maiti, 2003).

Procedure:
Soil samples were oven dried at 105°C, from which 50g of each soil sample was taken in previously weighed circular aluminium containers. These containers were perforated from the bottom for the entrance of water; while a lining of whatman filter paper was applied into the base of container to avoid the seepage of soil from perforations. These soil containing, perforated containers were allowed to stand in water for 24 hours. The final weight was taken after draining excess water and wiping the containers. Water holding capacity was determined by the following formula (Maiti, 2003):

Calculation:

\[
\text{WHC (\%)} = \frac{(W_3 - W_2)}{(W_2 - W_1)} \times 100
\]

Where,

\[
W_1 (g) = \text{Weight of box + filter paper}
\]

\[
W_2 (g) = \text{Weight of box + filter paper + soil}
\]

\[
(W_2 - W_1) (g) = \text{Weight of Soil}
\]

\[
W_3 (g) = \text{Weight of water absorbed}
\]

(iv) Texture Analysis:
Texture of soil was determined by sieving method (Gee and Bauder, 1986) to find whether the soil is sandy, clay or silt in nature, as it depicts nutrient retention and water storage condition. Air dried samples were passed through Gyratory sieve shaker (MAC, Macro Scientific works ltd.) which contains seven standard sieves
with specific number and mesh size and size opening in micron (Table 2). These samples after processing in the gyratory sieve shaker were collected from each sieve and each fraction was weighed. The percentage of silt, clay and sand proportion was determined and the final texture was interpreted using soil texture triangle according to USDA (United States Department of Agriculture) guidelines (Table 3, Figure 1).

Table 2: Standard sieve set (mesh size and size opening in micron) for soil texture analysis

<table>
<thead>
<tr>
<th>S.No</th>
<th>Mesh size number</th>
<th>Size of opening</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No.8</td>
<td>2000µm (2mm)</td>
</tr>
<tr>
<td>2</td>
<td>No.16</td>
<td>1000µm (1mm)</td>
</tr>
<tr>
<td>3</td>
<td>No.30</td>
<td>500µm (0.50mm)</td>
</tr>
<tr>
<td>4</td>
<td>No.60</td>
<td>250µm (0.250mm)</td>
</tr>
<tr>
<td>5</td>
<td>No.150</td>
<td>100µm (0.10mm)</td>
</tr>
<tr>
<td>6</td>
<td>No.300</td>
<td>50µm (0.050mm)</td>
</tr>
<tr>
<td>7</td>
<td>No.500</td>
<td>Less than 38µm (less than 0.038mm)</td>
</tr>
</tbody>
</table>

Table 3: Soil separation as per International Society of Soil Science (ISSS system)

<table>
<thead>
<tr>
<th>S.No</th>
<th>Soil separating texture name</th>
<th>Diameter range mm (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Coarse sand</td>
<td>2.0-0.2(2000-200)</td>
</tr>
<tr>
<td>2</td>
<td>Fine sand</td>
<td>0.2-0.02(200-20)</td>
</tr>
<tr>
<td>3</td>
<td>Silt</td>
<td>0.02-0.002(20-2)</td>
</tr>
<tr>
<td>4</td>
<td>Clay</td>
<td>Less than 0.002(&lt;2)</td>
</tr>
</tbody>
</table>
(v) **Organic Carbon and Organic Matter:**

Soil carbon being the largest pool of organic carbon terrestrially, is highly contributed by human activities. Soil carbon was estimated using rapid titration method, by Walkley and Black, 1934. Soil organic fraction contains cells of organism like microbes, plant and animals which decompose resulting in formation of humus from their residues and finally get carbonized to form highly carbonized compounds such as charcoal, graphite and coal. The organic matter also known as humus in soil which is a stable substance which does not go for further decomposition. Soil carbon was estimated using Rapid titration method, given by Walkley and Black, 1934.

**Procedure:**

Oven dried soil was passed through 0.2 mm sieve, from which 0.50 gram of sample was taken for the analysis. To this amount of soil 10 ml of 1N potassium dichromate \((K_2Cr_2O_7)\) and 20 ml of conc. \(H_2SO_4\) (containing 1.25% \(Ag_2SO_4\)) was added, mixed properly and allowed to stand for 30 minutes. Distilled water(200ml) was added to this mix followed by the addition of 10 ml of Otho-phosphoric acid (85%) which helps in getting sharper end point during titration. 1ml Diphenyl indicator was added just prior to titration of suspension with ferrous ammonium sulfate. The end point resulted in conversion of blue-violet to green color which was compared with Blank. The organic Matter in soil gets oxidized by potassium...
dichromate and conc. H$_2$SO$_4$, the unreacted dichromate was determined by titrating with ferrous ammonium sulfate (redox reaction) (Maiti, 2003).

$$2\text{Cr}_2\text{O}_7^{2-} + 3\text{C} + 16\text{H}^+ \rightarrow 4\text{Cr}^{3+} + 3\text{CO}_2 + 8\text{H}_2\text{O}$$

Organic carbon and organic matter was calculated from the following formulas:

$$\text{Organic Carbon} \, (\%) = 10 \, (\text{B-T})/\text{B} \times 0.003 \times 100/\text{S}$$

Where,

- $\text{B} = \text{Volume of ferrous ammonium sulphate required for titration of blank in mL}$.
- $\text{T} = \text{Volume of ferrous ammonium sulphate needed for soil sample in mL}$.
- $\text{S} = \text{Weight of soil in g}$.

$$\text{Organic matter} \, (\%) = \% \, \text{Organic carbon} \times 1.724.$$  

(vi) **Exchangeable Calcium and Magnesium:**

Calcium (Ca$^{2+}$) and Magnesium (Mg$^{2+}$) both are abundant elements in soil and play an essential role as nutrient in plants. Availability of these nutrients improves water penetration in soil thereby increasing the favorable conditions in soil for the growth of plants and soil microbes. Both these cations share common chemical properties like carbonate, phosphate, sulfate and precipitate in common fashion. Their concentration in soil was detected through EDTA titration method given by Tucker and Kurtz, 1961.

**Procedure for estimation of Ca$^{2+}$ and Mg$^{2+}$ (Total hardness):**

Suspension of 30 g oven dried soil was prepared in 75 ml distilled water (1:2.5) by stirring the mix on magnetic stirrer. The solution was allowed to stand overnight followed by the filtering the same by Whatman filter No 1. The resultant aliquot was mixed with 10 ml of ammonium chloride-ammonium hydroxide buffer solution (it rises the pH to 10 which avoids precipitation of Magnesium) and then titrated with standard EDTA solution (0.01N) after adding 2-3 drops of Erichrome black T indicator. At the end point the purple color of the test sample changes to pure blue colour which is taken to calculate the hardness.

**Procedure for estimation of Ca$^{2+}$:**

10 ml of the test aliquot was taken in 100 ml flask to which 5ml of 4N NaOH was added for the precipitation of magnesium in form of Mg(OH)$_2$. Further the
contents were titrated against 0.01N EDTA solution after adding pinch of Murexide (ammonium purpurate) indicator. The end point was observed by the change of orange red color to purple or violet color (Maiti 2003).

**Procedure for estimation of Mg$$^{2+}$$:**

The results obtained from total hardness and calcium was used to determine amount of magnesium in soil. The amount of titrant volume obtained from total hardness was substracted by the titrant volume of Ca$$^{2+}$$.

$$\text{Mg}^{2+} = \text{Total hardness (Ca}^{2+} + \text{Mg}^{2+}) - \text{Ca}^{2+}$$

**Calculation:**

1mL of 0.01N EDTA = 0.4008g Ca$$^{2+}$$
1mL of 0.01N EDTA = 0.2342g Mg$$^{2+}$$

(vii) **Estimation of Sodium (Na):**

Sodium level in soil decides the sodic and sodic saline problem in soil which is widespread problem in arid and semiarid regions of world. It was estimated by Ammonium acetate method using flame photometer (Toshniwal, model TOF4S), Lal Singh, (2012).

**Procedure:**

Soil suspension of 5g soil was prepared with 25 ml of neutral ammonium acetate (1N) solution (1:5) which was stirred for 5min and then filtered using Whatman filter paper No 1. Sodium concentration was determined by flame photometer (Toshniwal, model TOF4S) by sodium filter.

**Preparation of standard curve:**

Stock solution of ammonium acetate was prepared from which different sodium standard solutions were prepared ranging from 10-60 ppm. In the flame photometer reading was adjusted to zero for blank (ammonium acetate), followed by the reading of different standards were obtained. The Standard curve was plotted from the above readings from different standards (10-60ppm). The amount of sodium in test sample was estimated directly from the standard curve (Maiti, 2003). Available sodium in soil was calculated as per following formula

$$\text{Sodium (mg of Na/g of soil) = A} \times \frac{V}{W} \times 100$$
(viii) **Estimation of Potassium (K):**

Plants utilize only exchangeable potassium from surface of soil particles. A relatively very less amount is available in exchangeable form (approx 1%) out of total potassium found in soil. Available potassium is the sum of exchangeable and water soluble form of potassium in the soil. It was estimated by Ammonium acetate method using flame photometer (Toshniwal, model TOF4S), Lal Singh, (2012).

**Procedure:**

Soil suspension of 5g soil was prepared with 25 ml of neutral ammonium acetate (1N) solution (1:5) which was stirred for 5min and then filtered using Whatman filter paper No 1. Potassium concentration was determined using flame photometer (Toshniwal, model TOF4S ) by potassium filter.

**Preparation of standard curve:**

Stock solution of Potassium chloride was prepared from which different potassium standard solutions were prepared ranging from 10-60 ppm. On the flame photometer reading was adjusted to zero for blank (ammonium acetate), followed by the reading of different standards was obtained. The standard curve was plotted from the above readings from different standards (10-60ppm). The amount of potassium in test sample was estimated directly from the standard curve (Maiti, 2003).

Available potassium in soil was calculated as per following formula

\[
\text{Potassium (mg of Na/g of soil) = } \frac{A \times V}{W} \times 100
\]

Where,

- \(A\) = Potassium content of soil extract from standard curve, mg/l
- \(V\) = Volume of soil extract, ml
- \(W\) = Weight of air dry sample taken for extraction in g
(ix) **Cation Exchange Capacity (CEC)**

The cation exchange capacity explains the capacity of soil to hold positively charged ions thereby, it depends on the nature of soil especially texture. CEC is usually expressed in milliequivalents per 100g of soil. A soil having 1 meq CEC (1meq/100g of soil) is capable of absorbing 1mg of its equivalent per 100g of soil. From this absorption, cations become available to plants. CEC is calculated from the levels of potassium, magnesium, calcium, sodium and hydrogen as these cations are the ones with greatest concentration in most soils. Since the samples were alkaline so hydrogen was considered as zero, while for other cations the unit was converted from mg/l to meq/100g and substituted in the following formula (Rengasamy and churchman, 1999 and Maiti, 2003):

\[
\text{CEC (meq/100g)} = \text{Ca}^{2+} + \text{Mg}^{2+} + \text{K}^+ + \text{Na}^+ + \text{H}^+ 
\]

(x) **Sodium Adsorption Ratio (SAR):**

Sodium adsorption ratio (SAR) is the only factor which determines the suitability of water used for irrigation (Moasheri *et al*., 2012) of agricultural soil because sodium present in water greatly impacts permeability. It was estimated using sodium, calcium and magnesium values. Following formula is used where all the ions in milliequivalents/liter (Sposito and Mattigod, 1977).

\[
S.A.R. = \frac{N_{\text{Na}^+}}{\sqrt{\frac{1}{2}(\text{Ca}^{2+} + \text{Mg}^{2+})}}
\]

(xi) **Metal estimation:**

Various metals in the soil were determined and their concentration was interpreted. Metals like arsenic, copper, lead, zinc, nickel, cobalt, silver, cadmium and iron were estimated by Atomic Absorption Spectroscopy (AAS, model Chemito AA2013) at National Test House, Jaipur.

3.1.2 **Isolation of arsenite resistant bacteria and determination of Minimum Inhibitory Concentration (MIC):**

Isolation process was performed on fresh soil samples and the serial dilution method was used. The soil samples were subjected to serial dilution up to $10^{-5}$, from each dilution 100µl was spreaded on nutrient agar (HiMedia) (Peptic digest of
animal tissue -5g, Beef extract-1.5g, Yeast extract- 1.5g, Sodium Chloride-5g, Agar-15g, Distilled water-1liter, pH-7.4±0.02;) supplemented with 1g/l of sodium arsenite (Loba chemicals) and incubated for 48 hours at 37±2°C in incubator (MAC Macro Scientific works ltd, Model no:MSW231). After the growth was observed the colonies with different morphology were randomly selected and continuously spreaded and streaked to obtain pure colonies. On the basis of this preliminary screening colonies showing resistance towards arsenic were selected for further studies. These Selected colonies were subjected to microscopic screening by Gram Staining and Negative Staining to understand the colony morphology.

Isolated pure colonies were inoculated in nutrient broth medium supplemented with increasing doses of sodium arsenite (mM) to determine the lowest concentration, which completely inhibits bacterial growth after overnight incubation. The different doses of sodium meta arsenite (Loba chemicals) were externally added into the Nutrient broth (Hi Media, Peptone-10g, beef extract-10g, sodium chloride 5g, distilled water-1liter, pH-7.3±0.01) to which the bacterial colonies were inoculated and kept in shaker incubator (Orbital Shaking incubator, Genei) at 37±2°C for 48 hours and at 120rpm. The increasing doses of arsenite ranged from 0-6g/l. The colonies were simultaneously streaked on the nutrient agar plates to check and maintain the purity of the culture. The growth after 48 hours in each dose was judged on the basis of optical density at 600nm which was determined by UV-Vis spectrophotometer (Systronics UV-Vis. Spectrophotometer-106). The strains (SE-3,TB-1) possessing the highest MIC i.e. the ability to tolerate maximum dose of arsenite among the isolates were selected for further studies.

(i) **Effect of arsenite on Bacterial Growth:**

The Growth pattern was also examined in the selected strains (TB-1 and SE-3) by plotting the growth pattern and dose curve for which both the strains were grown with and without arsenic respectively. The growth of bacteria was determined in nutrient broth. From the overnight culture, 5% inoculum was inoculated into the fresh autoclaved nutrient broths at different doses of 0, 1, 2, 3, 4, 5, 6g/l of sodium arsenite and for each dose separate set of broth was inoculated for 0, 24, 48, 72, 96 hour. The cultures were incubated at 37±2°C in shaker incubator at 120rpm. The
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growth of isolates was monitored by determining the optical density at 600nm in triplicates for each set from UV-Vis Spectrophotometer (Systronics UV-Vis. Spectrophotometer-106). The obtained optical density was used to plot a dose curve which was compared with the control (Growth curve) which did not contain arsenic.

3.1.3 Analysis of multi-metal and antibiotic resistance for the selected strains

To study the sensitivity or resistance towards metals, experiments were conducted according Huysmans and Frakenberger (1990) method in which round discs were punched from whatmann No1. These discs were soaked into separate solutions containing metal salts in 100µg/ml concentration. These salts included Cadmium Chloride (CdCl₂.5H₂O), Cobalt Nitrate (Co(NO₃)₂.6H₂O), Lead Nitrate (Pb(NO₃)₂), Nickel Chloride (NiCl₂.6H₂O), Zinc Sulphate (ZnSO₄.H₂O), Chromium Chloride (CrCl₃.6H₂O), Sodium Selenate (Na₂SeO₄), Stannous Chloride (SnCl₂.2H₂O), Antimony Chloride (SbCl₃) and Mercuric Chloride (HgCl₂). Both the cultures TB-1 and SE-3 were spreaded on nutrient agar plates to which these metal discs were placed. After incubation of 48 hours at 37±2°C, the result was deduced. For antibiotic sensitivity test the bacterial lawns spreaded on nutrient agar plate were used on which the antibiotic hexadiscs (HiMedia) were placed. These discs contained Penicillin-G (10µg), Oxacillin (1 µg), Cephalothin (30µg), Clindamycin (2µg), Erythromycin (15µg), Amoxyclyav (30µg), Ampicillin (10µg), Cephotaxime (25µg), Co-trimoxazole (30µg), Gentamycin (10µg), Tobramycin (10µg) antibiotics. Results were obtained in the same manner as seen in metal sensitivity test after 48 hours incubation at 37 degree.

No Zone around the discs describes the resistance for the particular metal and antibiotic, while sensitivity against each was interpreted by the diameter (mm) of the zone around the disc (Birnboin et al., 1979) measured using scale (HiMedia).

3.1.4 Identification of strains using 16S-rDNA sequencing and biochemical test:

The two strains were selected for their high MIC and were further identified by their morphological features and biochemical properties. Biochemical properties
of the strains were tested according to Bergey’s Manual of Systematic Bacteriology (Holt et al., 1994), while procedures of these tests were taken from Cappuccino and Sherman (2002). Biochemical tests included utilization of various sugars, H₂S production, IMViC test, oxidase, catalase activity, starch hydrolysis, nitrate reduction and urease activity. On the basis of biochemical properties of both the strains, genus of each was identified. The determined genus was confirmed with 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 3730xl 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). 16s rDNA sequencing was conducted by Xcelris Labs Ltd., Ahmedabad, one of the pioneers for Next Generation Sequencing in India. DNA was isolated from the culture using QIAamp DNA Purification Kit (Qiagen) and BioAxis DNA Research Centre Private Limited, Hyderabad by Sanger Dideoxy sequencing technology. The sequences obtained were submitted in Genebank as *Pseudomonas sp. SE-3* (accession no. KP730605) and *Bacterium TB-1* (accession no KP866680).

3.2 Optimization of growth conditions and analysis of oxidizing ability:

For optimum growth, bacteria require specific conditions which also affect their metabolic processes. Arsenite oxidation is a very essential process for bioremediation purpose thus it becomes necessary to understand those specific conditions which are required for both the bacteria to survive and grow vigorously.
3.2.1 Optimization of growth conditions at various (temperature, pH, media) for the selected strains (SE-3 and TB-1)

(i) Study of temperature tolerance:

Temperature plays a very important role on the growth and bacteria are also grouped on the basis of tolerance level into Psychrophiles, Mesophiles and Thermophiles. To determine nature of isolates, they were grown in varied temperature ranges like 4°C, 25°C, 37°C, 45°C, 60°C celsius. Each 50 ml fresh culture was inoculated in triplicates on nutrient broth medium without arsenic and was incubated at different temperatures at 7.4±0.2 pH at 120 rpm in shaker incubator. The optical density was taken at 0, 24, 48, 72, 96 hour interval in triplicates at 600nm using UV-Vis spectrophotometer.

(ii) Study of pH Tolerance:

For the optimization of pH different nutrient broth medium without arsenic were adjusted to different pH range i.e. 5, 6, 7, 8, 9 using 1N NaOH and 1N HCL. To these different ranges of pH medium bacterial culture were inoculated in triplicates and incubated at 37±2°C, 120 rpm in shaker incubator. The observation of optical density in each medium was taken at 0, 24, 48, 72, 96 hour interval at 600nm from UV-Vis spectrophotometer.

(iii) Media optimization:

Growth pattern was observed for SE-3 and TB-1 in three medium 1) Minimal Media 2) Chemical defined medium 3) nutrient Broth. Isolated strains were grown in minimal media composition(Table 4) as suggested by Santini et al., 2000 and Lugtu et al., 2009 in the presence of 1g/l sodium arsenite, pH=8 and temp 37°C at 120 rpm.

Chemical defined media(Table 4) was used for growing bacterial cultures with sodium arsenite (1g/l NaAsO₂) and without arsenic at pH=7.2, 37°C at 120 rpm as suggested by Weeger et al., 1999 and Liao et al., 2011.

Culture was also subjected to grow in nutrient broth with (1g/L) and without arsenic. The growth was plotted to interpret the optimum media for ideal growth of the isolated strains.
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Table 4: Media compositions used in the optimization of growth conditions (Different Media constituents)

<table>
<thead>
<tr>
<th>S. No</th>
<th>Minimal Media Constituents</th>
<th>Chemical Concentration</th>
<th>Chemically Defined Media</th>
<th>Nutrient Broth</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Na₂SO₄ (Sodium Sulfate)</td>
<td>0.07 g/L</td>
<td>MgSO₄ (Magnesium Sulphate)</td>
<td>2.0014176 g/L</td>
</tr>
<tr>
<td>2.</td>
<td>Na₃SeO₃·5H₂O (Sodium Selenite Pentahydrate)</td>
<td>0.000017 g/L</td>
<td>NH₄Cl (Ammonium Chloride)</td>
<td>1.000263 g/L</td>
</tr>
<tr>
<td>3.</td>
<td>Na₂WO₄·2H₂O (Sodium Tungstate Dihydrate)</td>
<td>0.00003 g/L</td>
<td>Na₂SO₄ (Sodium Sulfate)</td>
<td>0.99428 g/L</td>
</tr>
<tr>
<td>4.</td>
<td>KH₂PO₄ (Potassium dihydrogen phosphate)</td>
<td>0.17 g/L</td>
<td>K₂HPO₄ (Di-Potassium Hydrogen Phosphate)</td>
<td>0.00999334 g/L</td>
</tr>
<tr>
<td>5.</td>
<td>KCl (Potassium Chloride)</td>
<td>0.05 g/L</td>
<td>CaCl₂·2H₂O</td>
<td>0.0671837 g/L</td>
</tr>
<tr>
<td>6.</td>
<td>MgCl₂·6H₂O (Magnesium Chloride Hexahydrate)</td>
<td>0.04 g/L</td>
<td>Sodium Lactate</td>
<td>16.070272 g/L</td>
</tr>
<tr>
<td>7.</td>
<td>CaCl₂·2H₂O (Calcium Chloride Dihydrate)</td>
<td>0.05 g/L</td>
<td>Fe₂SO₄·7H₂O (Ferrous Sulfate Heptahydrate)</td>
<td>0.00333624 g/L</td>
</tr>
<tr>
<td>8.</td>
<td>KNO₃ (Potassium Nitrate)</td>
<td>0.15 g/L</td>
<td>NaHCO₃</td>
<td>0.6821612 g/L</td>
</tr>
<tr>
<td>9.</td>
<td>(NH₄)₂SO₄ (Ammonium Sulphate)</td>
<td>0.1 g/L</td>
<td>pH</td>
<td>7.2</td>
</tr>
<tr>
<td>10.</td>
<td>NaHCO₃ (Sodium Bicarbonate)</td>
<td>0.5 g/L</td>
<td>Temp</td>
<td>37°C</td>
</tr>
<tr>
<td>11.</td>
<td>Biotin</td>
<td>0.0001 g/L</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>12.</td>
<td>Ascorbic acid</td>
<td>0.0001 g/L</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>13.</td>
<td>Vitamin B₆</td>
<td>0.0001 g/L</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>14.</td>
<td>Nicotinic acid</td>
<td>0.0001 g/L</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>15.</td>
<td>Vitamin A</td>
<td>0.0001 g/L</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>16.</td>
<td>Lactose monohydrate</td>
<td>As required</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>17.</td>
<td>pH</td>
<td>8</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>18.</td>
<td>Temp</td>
<td>37°C</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>
3.2.2 Analysis of oxidizing abilities of the selected strains

1. **Analyze oxidizing abilities by a preliminary test (AgNO₃ and microtiter plate method).**

   **Preliminary determination of oxidizing ability in strains:**
   The two strains with maximum MIC were further carried for testing their oxidizing ability by:
   
   (i) **Silver nitrate test:**
   The silver nitrate test was performed as described by Simenova *et al.* (2004). Sodium arsenite (1g/l) supplemented nutrient agar plates were streaked with the isolated strains separately followed by their incubation at 37±2°C for 24 hours. After incubation these plates were flooded with a solution of 0.1M AgNO₃ along with the control plates and were again incubated for next 24 hours. The presence of brown precipitate on the plates indicates the presence of arsenate which is the result of oxidation by arsenite oxidizing bacteria while the presence of bright yellow color indicated the presence of arsenite.

   (ii) **Microplate screening assay:**
   Microplate screening was conducted to observe the qualitative arsenic species transformation in microtiter 96 well plate. 10ml of 48 hour cultures was taken for this study, which was centrifuged at 5000 rpm for 10 min. Supernatant was separated and pellet was suspended in water (2ml deionized water). Four wells of the standard 96 well microtiter plate was used for each culture. These four groups contained: a) Control (Water 250 μl+ 0.1M AgNO₃ (150μl). b) Supernatant of the culture (150μl) + 0.1M AgNO₃. C) Pellet suspension (150μl) + 0.1M AgNO₃. d) Culture without centrifugation (150μl) + 0.1M AgNO₃. The microplate was then incubated at 37±2°C for 96 hours and observed for the appearance of brown precipitate in the wells. The brown color depicts the presence of complex Silver-orthoarsenate showing the presence of arsenate while yellow color shows arsenite which forms Silver-orthoarsenite (Diliana *et al.*, 2004; Simeonova *et al.*, 2004).

2. **Confirmatory examination of oxidizing ability in strains:**
   (i) **Molybdenum blue method:**
   Molybdenum blue method is based on the quantification of the arsenate-molybdenum complex formed as a result of arsenite oxidation. In this method the arsenite converted (oxidized) form arsenate shows its presence by binding with
molybdenum in the reaction mix and which in turn reacts with ascorbic acid to form blue color complex. The amount of the complex can be quantitated at 838nm on UV-Vis Spectrophotometer. While arsenite does not have this ability thus both the As species can be differentiated and can be measured (Johnson, 1971; Johnson and Pilson, 1972; Cai et al., 2009a; Hu et al., 2012).

Procedure:

For executing this method, both cultures were grown in 10ml and 20 ml nutrient broth respectively. After 24 hour optimum growth was attained, the cultures were then pelleted out and pellet weight was recorded. SE-3 had pellet weight of 0.154g ±0.01 (10ml) and 0.433g ±0.01 (20ml), while TB-1 showed 0.184g ±0.01 (10ml) and 0.447g ±0.01 (20ml). These pellets were then suspended in sodium arsenite (0.1g/800µM) water, and incubated the sets for 0,24,48,72 hours respectively with the controls for each our at 120rpm, 37°C in shaker incubator.

For analysis, sample from both the cultures were withdrawn each hour and was added to the reaction mix which contain deionized water, sulphuric acid, sodium molybdenum, ascorbic acid and sample in the following quantity:

Reaction mix(10ml)=(4ml deionized water+0.4ml, 50% H2SO4+0.4ml, 3%Na3MoO4+0.2ml, 2% Ascorbic acid +0.3ml Sample).

The reaction mixture for each drawn sample was prepared in volumetric flasks (10ml) and was then boiled for 20 minutes in water bath at 90°C. The volume was made up to 10ml using deionized water and reading was taken at 838nm on UV-Vis spectrophotometer to quantify arsenite oxidation.

Statistical analysis: All above experiments were performed in triplicates and results were represented as mean± standard error. The results were compared using student’s t-test at the significance levels of p<0.05, p<0.001, p<0.01. The statistical analysis was performed using Microsoft Excel (Version 2007).

Selection of ideal strain from the isolates for further study:

After observing the results for higher MIC and arsenic oxidizing ability, it was interpreted that the best strain among the two strains was Pseudomonas sp. SE-3. This particular strain was selected for the further study at molecular level and was subjected for application based study creating microcosm environment.
Materials and Methods

Pseudomonas sp. SE-3 was further explored on sequence level and its phylogeny was obtained. BLAST was performed by 16s rDNA sequences on NCBI genebank database at NCBI website (www.ncbi.nlm.nih.gov). On the basis of maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA4. Information about other close homologs for the microbe was found from the Alignment View table.

3.3 Molecular analysis of strain with highest oxidizing ability

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

1) PCR amplification of aox gene:

 Genetic basis of arsenite oxidation lies in aox genes which encodes arsenite oxidase enzyme. The arsenite oxidation is supported on molecular level by showing the presence of aox gene in Psuedomonas sp. SE-3 by PCR amplification. For this pure bacterial genomic DNA was isolated using phenol chloroform method.

Procedure of DNA isolation:

The culture was grown in the presence of 1g/l sodium arsenite and incubated for 24 hours at 37±2°C at 120rpm. Bacterial culture was centrifuged at 10,000rpm for 15 min and obtained pellet was washed with TE buffer and suspended in 400µl TE buffer followed by 5 µl of lysozyme treatment and incubated at 56°C for 45 min. 45 µl of 10% SDS was added, which aids in destruction of cell and protein conformation. The vial was vortexed and incubated again at 37° for one hour after which 445 µl of saturated phenol was added for extraction of protein and lipids. Vial was centrifuged at 10,000 rpm for 10 min, followed by the separation of supernatant to other vial. To this fresh supernatant equal volume of chloroform: isoamylalcohol and 10% SDS was added and vortexed. This separated proteins and polysaccharides in denser section after centrifugation at 10,000rpm for 10 min, and supernatant containing DNA was taken, to which again chloroform: isoamyl alcohol was added. This was repeated until clear supernatant was achieved. 0.1 volume of 3M sodium acetate was added to the clear fraction achieved to remove remaining cellular and histone proteins. Twice the volume of alcohol was then added followed by the incubation of mix at -20° for 24 hours. After 24 hours the mix was centrifuged at
10,000 rpm for 15 min, supernatant was discarded and pellet was washed with 70% ethanol. Finally 100µL of ethanol was added to the suspension and again centrifuged 10,000 rpm for 15 min. Resultant supernatant was discarded and pellet was dried, which was then dissolved in TE buffer which prevents DNA degradation. Isolated DNA was quantified by electrophoresis on 1% agarose gel with ethidium bromide dye while concentration was determined by Nanodrop (ND-1000) Spectrophotometer at 260nm. This DNA was then used in PCR amplification.

**Table 5: Sequence of aox primers for PCR amplification**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>Reference</th>
</tr>
</thead>
</table>
| aox A | **aox A 0415**  
S (Sense) 5'-ATAAAGTAAGTCGTCGCAATT-3' | Chang *et al.*, 2010 |
|      | **aox A 1106**  
A (Antisense) 5'-GAGCTGAATCTGAGGCAGATT-3' | Chang *et al.*, 2010 |
| aox B(a) | **aox B 0125**  
S (Sense) 5'-TGCGGCTACCACGCCTACACC-3' | Chang *et al.*, 2010 |
|      | **aox B 1022**  
A (Antisense) 5'-TGCCCCAGGTTTTTCGTAAC-3' | Chang *et al.*, 2010 |
| aox B(b) | **aox B M1-2F**  
S (Sense) 5'-CCACTTCTGCATCGTGGGNTGYGGNTA-3' | Quemeneur *et al.*, 2010 |
|      | **aox B 1022R**  
A (Antisense) 5'-TGTCGTTGCCCCAGATGADNCCYTTYTTC-3' | Quemeneur *et al.*, 2010 |
| aox C | **aox C 0710**  
S (Sense) 5'-TGGCATCGGGAGGAGGAT-3' | Chang *et al.*, 2010 |
|      | **aox C 1118**  
A (Antisense) 5'-TAGCCTGGGAAGTATGGC-3' | Chang *et al.*, 2010 |
| aox D | **aox D 1025**  
S (Sense) 5'-ATATGCCATTGTATTTTG-3' | Chang *et al.*, 2010 |
|      | **aox D 1124**  
A (Antisense) 5'-TACTTGCTCAGCCGCAAT-3' | Chang *et al.*, 2010 |
| aox R | **aox R 0725**  
S (Sense) 5'-AATCGCTCATCCAGCGACTTTGC-3' | Chang *et al.*, 2010 |
|      | **aox R 1103**  
A (Antisense) 5'-TGCGGTCTCGCGCAAGCCTACTGA-3' | Chang *et al.*, 2010 |
| aox S | **aox S 0507**  
S (Sense) 5'-TTCATCATCTCCGAGCGTATTG-3' | Chang *et al.*, 2010 |
|      | **aox S 0705**  
A (Antisense) 5'-TTGCGGTACGACGGGTTCAAAA-3' | Chang *et al.*, 2010 |
**Materials and Methods**

**Procedure of PCR amplification:***

The primers used for \textit{aox} gene amplification were \textit{aox A}, \textit{aox B1}, \textit{aox B2}, \textit{aox C}, \textit{aox D}, \textit{aox R}, \textit{aox S} (Table 5). The constituents of the PCR are summarized in Table 6. The PCR for each gene amplification consisted of 5 min of pre-denaturation at 95°C, 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 55°C, and 1.5 min extension at 72°C, and final 1 cycle of extension at 72°C for 7 min (Chang \textit{et al} 2010) with a negative control without culture was also setup. All the vials were stored at -20°C until loaded into PCR thermal cycler (MJ Research, USA). PCR product (10µl) was analyzed by electrophoresis in electrophoretic apparatus (biorad) 1.5% agarose gel with ethidium bromide (0.5µg/ml) and the tank was flooded with 1X TBE buffer. Now the PCR product (10µl) with gel loading dye (1µl) were loaded in the wells made by the combs, along with the PCR product, negative control and 1µl of DNA ladder (500bp, 50 µg; Merck Genei) with gel loading dye was loaded in separate wells. DNA ladder estimates the size of PCR product obtained. After the run the gel was observed under Gel documentation machine (Bio-rad).

**Table 6: Reaction mixture used in PCR amplification of \textit{aox} genes**

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Stock Concentration</th>
<th>Working Concentration</th>
<th>Volume required for 10.0µl</th>
<th>Volume required for 25.0µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR buffer</td>
<td>10X</td>
<td>1X</td>
<td>1µl</td>
<td>2.5µl</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>25mM</td>
<td>1.5mM</td>
<td>0.6µl</td>
<td>1.5µl</td>
</tr>
<tr>
<td>dNTPs</td>
<td>10mM</td>
<td>200µM</td>
<td>0.4µl</td>
<td>0.5µl</td>
</tr>
<tr>
<td>Primers (Forward &amp; Reverse)</td>
<td>100pmol</td>
<td>10pmol</td>
<td>0.4µl ([0.2(F)+0.2(R)])</td>
<td>2.5µl ([1.25(F)+1.25(R)])</td>
</tr>
<tr>
<td>Taq DNA Polymerase</td>
<td>3U/ µl</td>
<td>1U/ µl</td>
<td>0.3 µl</td>
<td>0.3 µl</td>
</tr>
<tr>
<td>DNA template</td>
<td>-</td>
<td>50ng/ µl</td>
<td>0.7 µl</td>
<td>1 µl</td>
</tr>
<tr>
<td>Ultrapure Water</td>
<td>-</td>
<td>-</td>
<td>6.6 µl</td>
<td>16.7 µl</td>
</tr>
</tbody>
</table>
3.3.2 Localization of enzyme arsenite oxidase responsible for arsenite oxidation:

Arsenite oxidase 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. The bacteria was grown in nutrient broth in the presence of 1g/l arsenite and incubated for 48 hours for optimum growth. Arsenic was supplemented in order to induce the operon which is essential for expressing the arsenite oxidase enzyme. The culture was pelleted by centrifugation at 4,500g for 10 min at 4°C. The resulting pellet was suspended in 20mM Tris HCl, 0.1 mM Phenyl Methyl Sulfonyl Fluoride (PMSF) 10 mM EDTA (pH 8.4) containing 20% sucrose. To isolate spheroplast the outer membrane was lysed by the treatment of 0.5 mg/ml of lysozyme for 40 min at 25°C, which was further centrifuged to obtain supernatant supernatant and pellet (Anderson et al., 1992). The presence of arsenite oxidase was determined by its activity in both the fractions i.e. the pellet and supernatant by the 2,4-dichlorophenolindophenol (DCIP). DCIP is a blue chemical compound (redox dye), which loses its color on getting reduced and can be measured by spectrophotometer at 600nm. The Arsenite oxidation by arsenite oxidase activity was thereby judged by the reducing equivalent from arsenite to 2,4-Dichlorophenolindophenol in the presence of 200µM sodium arsenite in 50mM MES (Morpholinoethane sulfonic acid),pH 6.0 at 25°C. Reaction was compared with the control which was boiled bacterial culture having denatured protein (Muller et al., 2003 and Prasad et al., 2009).

3.4 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 technique of the bacterial capacity to detoxify arsenic which is less costly and effective. The prior arsenic contamination in the soil would result in false interpretations about the arsenite oxidized and it would become difficult to judge arsenic concentration before
and after the experiment. Thus, the soil free from arsenic (uncontaminated soil) was taken in this experiment, to which the desired amount of arsenic (contaminant) was added.

In this method bioaugmentation and biostimulation 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 and enhancing the potential of resident bacteria in degradation of the contaminant (Abdulsalam et al., 2009). Following steps were conducted:

3.4.1 Soil Sample Collection and Physicochemical analysis:
Sample was collected from Sanganer area which was near the textile industrial effluent discharge. The samples were collected in February 2015, from the depth of 10-15 cm. The soil samples were then analyzed for arsenic contamination from CEG test house and research center private Ltd, Jaipur by Atomic Absorption Spectroscopy (AAS) using EPA 3050B testing method. Sample was analyzed for their physiochemical parameters and the rest amount of the sample was passed through 2 mm sieve to remove sand gravel, plant debris. Earthen pots with depth of 6-7 cm and 18-19.5 cm diameter were weighed and soaked in water to saturate its pores with water so that pots don’t absorb soil moisture after the experimental setup.

Figure 2: Soil sampling site for microcosm study
(i) **Physicochemical analysis of soil:**

Fresh soil sample was taken to determine moisture content of the soil in within 24 hours of sample collection for accuracy of the content of water in the soil, according to which moisture parameter of experimental setup was decided.

**Procedure for determining moisture content:**

Moisture was determined by gravimetric method for which an empty container was weighed \((m_1)\), into it 100g fresh sample was taken and wet weight \((m_2)\) of the container and was placed in oven at 105°C-110°C. After 24 hours drying container was again weighed \((m_3)\). Finally the weights were substituted in the following formula and moisture content was determined (Maiti, 2003).

- Weight of empty moisture can = \(m_1\)
- Weight of moisture can+ moisture Soil = \(m_2\)
- Weight of moisture can+ oven dry soil = \(m_3\)

**Calculation:**

\[
\text{Moisture Content (\%) = \frac{\text{Loss of Moisture (m}_2 - m_3)}{\text{Weight of oven dried sample (m}_3 - m_1)} \times 100}
\]

The soil was also subjected for other physiochemical parameter testing like pH, Electrical conductivity, Water holding Capacity, Organic Carbon, organic matter, Calcium ,Magnesium, Total hardness by the EDTA titration method as described earlier. Determination of Nitrogen, Phosphorus, and Potassium (N:P:K) in soil was analyzed by the standardized protocol at Govt. of Rajasthan, Department of agriculture, soil analysis laboratory, Durgapura, Jaipur. The concentration detected helped in deciding the supplementation dose of N:P:K (nutrient) in the soil.

(ii) **Soil microbial analysis:**

The total bacterial count or the soil microbial condition was explored using serial dilution method (Ogunmwonyi et al., 2008). 1g of soil was taken in 10 ml sterile water and stirred on magnetic stirrer for 15 min. This soil suspensions was then filtered and 1ml filtrate was taken to make serial dilution from \(10^{-1}\) to \(10^{-12}\). dilutions were inoculated (100\(\mu\)l) on agar plate and spreaded followed by incubation
of 24 hours at 37°C for the enumeration of bacteria in CFU/ml per gram of soil in comparison with control plate (no inoculum).

3.4.2 Selection of parameters by preliminary experimental setup (Biougmentation and Biostimulation):

Parameters to be kept in consideration during the experiment were based on various demands of the experiment. The optimum amount of factor to be amended was analysed which was considered as value 0 for the design expert to which above and below two values were also selected by the software creating three independent variables (-1,0,+1) for each factor.

Preliminary experiment:

i) Arsenic dose supplemented (1g/kg): Arsenic plays the role of contaminant in the experiment so its dose was decided on the basis of feedback attained from culture in natural condition in a preliminary setup and the amount of transformation achieved without the presence of any nutrient supplement.

ii) Biomass augmentation (200ml): Pseudomonas sp. SE-3 is the micro-organism in this study on which the whole bioremediation process relies on; in this experiment bacterial culture was augmented in specific amount to explore its capabilities. The prior experimentation was done with different amount of biomass of culture inoculated (10ml, 20ml, 30ml) in the presence of 1g/l of arsenite per 100g of soil. The rate of oxidation by this different biomass suggested the optimum amount of biomass to be used in 1kg of soil for best output.

iii) Moisture content (30%): Moisture acts as a supplement which fulfills the balance of water in the microcosm, which helps in growth of the bacteria. It was decided on the basis of the moisture content determined in the fresh sample that persisted in natural condition of soil.

iv) Nitrogen: Phosphorus: Potassium concentration (19:19:19/4g): As these three nutrients being most important in soil for growth, are supplied in agricultural field in form of N:P:K fertilizers for plants. Bacterial isolation is also from a place where N:P:K fertilizers were supplemented (agricultural field), due to which the prescribed amount of fertilizers which are used in Rajasthan were procured (Chambal chemicals and fertilizers Ltd) and different concentration of similar ratio (19:19:19) of this fertilizer were
added in the microcosm. This acted as the nutrient content in the microcosm in addition to the N, P, K which is present naturally in the soil (Agarry et al., 2012; Amenaghawon et al., 2014).

### 3.4.3 Designing of Experiment Using Design Expert Statistical Software Version 9.0.4.1 and quantification of oxidizing product (arsente)

DOE helps in understanding and identify interactions between factors and use contour plots to give a proper understanding of interpretations. It uses regression model for designing this experiment design expert software version 9.0.4.1 was used. This is a statistical technique which provides information about direct effects, pairwise interaction effects and curvilinear variable effects of the factors undertaken in the study. This design expert statistical software finds optimum combination of factors which yields good result. In this experiment the statistical approach of response surface methodology was used. The Box-Behnken factorial experimental design employed had four independent variables viz., Arsenic concentration, Biomass, NPK (19:19:19) fertilizer, and moisture (Table 7). Each of the independent amendment variables was studied at three levels (1, 0, and +1), with 27 experimental runs (Table 8).

#### Table 7: Range of four independent variables in terms of coded levels in Box-Behnken design experiment for evaluation of arsenite oxidation in the microcosm study

<table>
<thead>
<tr>
<th>Variable(Parameters)</th>
<th>Range and Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(-1)</td>
</tr>
<tr>
<td>Arsenite concentration(g/kg)</td>
<td>0.5</td>
</tr>
<tr>
<td>Biomass(ml)</td>
<td>100</td>
</tr>
<tr>
<td>N:P:K fertilizer(19:19:19) (g)</td>
<td>2</td>
</tr>
<tr>
<td>Moisture (%)</td>
<td>20</td>
</tr>
</tbody>
</table>
Table 8: Box-Behnken experimental runs for analysis of arsenite oxidation in microcosm study using Response Surface Methodology (RSM) in Statistical software Design expert version 9.0.4.1

<table>
<thead>
<tr>
<th>Run No</th>
<th>Factor A (As(III) conc.)</th>
<th>Factor B (Biomass)</th>
<th>Factor C (Moisture)</th>
<th>Factor D (NPK(19:19:19))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Code</td>
<td>Value (g/kg)</td>
<td>Code</td>
<td>Value (ml)</td>
</tr>
<tr>
<td>1</td>
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</tr>
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<td>27</td>
<td>-1</td>
<td>0.5</td>
<td>0</td>
<td>200</td>
</tr>
</tbody>
</table>
**Materials and Methods**

**Microcosm preparation and experimental setup:**

Sieved soil was equally weighed (1kg) in all the pots numbered according to the experimental run (27 experiment). Soil in control experiment was first treated according to the setup. Following Controls were planned (Table 9):

**Table 9: Control runs for analysis of arsenite oxidation in microcosm study**

<table>
<thead>
<tr>
<th>S.No</th>
<th>Run No</th>
<th>Factor 1: As conc(g/kg)</th>
<th>Factor 2: Biomass(ml)</th>
<th>Factor 3: Moisture (%)</th>
<th>Factor 4: N:P:K(g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30</td>
<td>1</td>
<td>Formaldehyde treated Soil (no augmentation)</td>
<td>30</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>31</td>
<td>1</td>
<td>Oven dried (no augmentation)</td>
<td>30</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>32</td>
<td>1</td>
<td>Soil microbes (no augmentation)</td>
<td>30</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>37</td>
<td>1</td>
<td>Autoclaved Soil</td>
<td>30</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>33</td>
<td>1</td>
<td>Oven Dried+100ml SE-3</td>
<td>30</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>34</td>
<td>1</td>
<td>Oven Dried+200ml SE-3</td>
<td>30</td>
<td>4</td>
</tr>
<tr>
<td>7</td>
<td>35</td>
<td>1</td>
<td>Oven Dried+300ml SE-3</td>
<td>30</td>
<td>4</td>
</tr>
<tr>
<td>8</td>
<td>36</td>
<td>1</td>
<td>Glucose augmented +200ml SE-3</td>
<td>30</td>
<td>4</td>
</tr>
</tbody>
</table>

The bacterial culture of *Pseudomonas sp.* SE-3, was inoculated in 1g/l As (III) nutrient broth and incubated for 48 hours for achieving optimum growth for further inoculation. Arsenic at this stage was added to induce the operon governing arsenic oxidation system in bacteria. This culture was inoculated for different set of runs suggested by design expert containing 100, 200, 300ml culture to be augmented. These cultures of different volumes were allowed to grow for 48 hours in shaker incubator at 120rpm and at 37°C. Optical density and pellet weight was determined after 48 hours and before augmentation to assure the uniformity in culture growth conditions. Centrifugation of cultures was performed in cooling centrifuge (REMI) at 10,000 rpm for 10 min and the resultant pellet was suspended in water to make culture ready for augmentation. Each experimental run experiment was set according to the Table 8 prescribing specific amount of factors in combination with each other. Each experimental run was homogenized properly to mix all the contents of prescribed factors properly. Then the pots were kept away from sunlight in room where temperature was maintained in range of 30°C-37°C (optimum for bacterial growth of SE-3 and TB-1).
Materials and Methods

Once the experiment was setup, the pots were finally weighed and moisture was maintained daily according to the loss in weight. All the microcosm sets were mixed properly thrice a week for four weeks. The soils were sampled each week from all the experimental and control runs (No. 1-37), and 1g was suspended in 10ml deionized autoclaved water and stirred on Magnetic stirrer for 15 min and then allowed to stand overnight. After which the soil suspension was filtered and the filtrate was used to analyze arsenite oxidation by molybdenum blue spectrophotometric method as described earlier.

Simultaneously 1g soil samples were suspended in 10 ml deionized autoclaved water and after stirring the suspension (1ml) was withdrawn from each run for microbial analysis. Serial dilution method and spread plate technique were used to analyze the microbial count in the microcosm experiment.

Results obtained for each run on 7, 14, 21, and 28 were analyzed and the day 21st of each experiment where maximum conversion took place was fed into the software and graph were generated on which the experimentation interpretation were made. The software Design Expert (Version 9.0.4.1, State-Ease Inc., Minneapolis, USA) was used for the experimental design, data analysis, quadratic model buildings, and graph (three-dimensional response surface and contour) plotting. The
fitted response graph was generated using statistically significant above model by Design Expert program to understand the interaction to get the maximum oxidation. The shape of the contour plots (circular or elliptical) indicates whether the mutual interactions between variables are significant or not. A circular contour plot indicates that the interactions between related variables are negligible. An elliptical contour plot indicates that the interactions between related variables are significant (Muralidhar et al., 2001). Contour graphs and three dimensional graphs were then plotted which explains the interaction of two parameters on the X and Y axes with the oxidation rate in Z axis, while other parameters are kept on central point.

3.4.4 Relation of bacterial count and rate of oxidation during the microcosm study:
For analyzing the differences in CFU/g of each run during 7th, 14th, 21st and 28th day of the study, one way ANOVA was performed using IBM SPSS statistical software version 20. As no significant difference was found among the runs, a separate analysis by one way ANOVA was performed on three experimental set (no.8, 12, 19) and on the control run (no.34). The experimental run in the sets (run no 8, 12, 19) have common factor variables (0) i.e. 1g/kg Arsenic, 200ml (4g) Biomass, 300ml moisture and 4g N:P:K. While the control no 34 contains all the same parameters as experimental group, except the soil taken was prior oven dried at 150°C-200°C. The oxidation in this control setup was assumed to be due to the test bacteria Pseudomonas SE-3 as native bacterial colonies were killed by oven drying, which was also studied statistically. The significant difference of CFU among these runs was further used to study the correlation between CFU/g and rate of oxidation in the runs. The oxidation rate and CFU/ml observations of this experimental group on 7th, 14th, 21st and 28th days were compared statistically for each week.

3.4.5 Association of introduced Pseudomonas sp.SE-3 and native soil microflora:
To understand the association of Pseudomonas sp.SE-3 with native soil micro flora and its effect on rate of oxidation of arsenic, further analysis was conducted. Level of arsenite oxidized in experimental run 8, 12, 19 (Pseudomonas sp.SE-3 and native soil micro flora) was compared with control run 32 (only native soil microflora) and control run 34 (Pseudomonas sp. SE-3).