STUDIES ON THE ROLE OF AEROBIC SOIL BACTERIA IN ARSENIC TRANSFORMATION AND MOBILIZATION

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Aerobic bacteria
Arsenic
Bioaccumulation
Mobilization
Transformation
Tolerance

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The disposal of toxic heavy metals such as arsenic posed high risk to the environment. The toxicity of arsenic depends on the arsenic species and chemical form. Arsenite [As(III)], a reduced form of arsenic, is more toxic and more mobile than Arsenate [As(V)]. Role of microbial ecosystem for arsenic transformation have been recently discovered. Bacteria have developed a variety of mechanisms to avoid arsenic toxicity. Like, minimizing the uptake of arsenate through the system for phosphate uptake, by peroxidation reactions with membrane lipids or using the best characterized microbial arsenic detoxification pathway involving the ars operon. The aim of this work was to isolate arsenic tolerant bacteria from arsenic contaminated soil followed by screening these bacteria for their ability to transform arsenic. Strains have been isolated from arsenic contaminated zone of Nadia District, West Bengal, which was previously reported as arsenic prone zone. Three aerobic iron bacterial strains have been isolated (S₁₆, S₁, N₁) and among these S₁₆ strain was isolated from methopara, S₁ strain from Saontalpara and N₁ strain from Nischindipur which is the most arsenic contaminated site among all three sites. The S₁₆ strain is Gram positive, coccus in nature. S₁ strain is also Gram positive, coccus in nature. N₁ strain is Gram negative, coccus in nature. Cultural and biochemical characterization of isolated bacteria has been attempted. Biochemical tests revealed that three strains are separate from each other. Arsenic tolerance test were carried out by Minimum Inhibitory Concentration method and cup assay method All the three strains can tolerate higher concentration of arsenate and arsenite concentration. S₁₆ can tolerate both arsenate and arsenite upto 1000mgL⁻¹. Where as, S₁ and N₁ can tolerate arsenate upto 1000 mgL⁻¹ but arsenite upto 500 mgL⁻¹. The role of these bacteria in transformation of As (v) to As (III) or vice versa were analyzed in depth. All of them appeared as arsenite oxidizing bacteria.
INTRODUCTION

Arsenic is a toxic element released into the environment either by natural phenomena (weathering, volcanic activity) or by anthropogenic activities (Cullen et al., 1989). Native (elemental) arsenic occurs rarely, where as traces of toxic arsines can be detected in gases emanating from anoxic environments. Arsenic can exist in four oxidation states: As (-III), As (0), As (III), and As (V). The predominant form of inorganic arsenic in aqueous, aerobic environments is arsenate where as arsenite is more prevalent in anoxic environment. The accumulation of arsenic in groundwater is a serious problem in many parts of the world, notably Bangladesh, where arsenic- contaminated ground water is used for drinking by over 40 million peoples (Smedley et al., 2002).

Due to the natural abundance of arsenic in the environment, representatives from various bacterial genera have developed different resistance mechanisms for arsenic compound. While arsenate enters into the microbial cells via transmembrane phosphate transport proteins, arsenite enters at neutral pH via aquaglyceroporins (Wyssocki et al., 2001). The bacterial resistance with regard to reduction of arsenate or oxidation of arsenite can be divided into two basic categories consisting of either detoxification reactions that confer arsenic resistance, or redox reactions that conserve the energy gained by the reactions for cell growth (Silver et al., 2005). Arsenic can also be methyalated (Turpeinen, 2003). Arsenic may also be converted to arsenobetaine and arsenic found in high abundance in some marine animals and algae as well as terrestrial plants and animals (Oremland et al., 2002). The most well studied mechanism of detoxification and resistance mechanism in bacteria is the Ars C system (Widdel et al., 2002). Ars C, is a small molecular mass protein (13 to 16 KDa), which mediates the reduction of As (V) to As (III) in the cytoplasm. Although As (III) is much toxic, it can be excreted via an As (III) transporter, Ars B. The ars operon in Escherichia coli has both plasmid and chromosomal loci. The plasmid R733 comprises of ars A, ars B, ars C, ars D, and ars R, whereas the chromosomal locus has only ars B, ars C, and ars R (Silver et al., 2005). A cysteine residue near the N-terminal of Ars C binds the As (V), which is then reduced with electrons donated by the reduced glutathione. The As (III) is then expelled from the cytoplasm through an adenosine 5-triphosphate (ATP)-dependent arsenite transporter formed by Ars AB (Spliethoff et al., 2002). The ars operon in plasmid pI258 of Staphylococcus aureus contains only ars B, ars C, ars D. Reduced thioredoxin provides the electrons to reduce As (V), and As (III) is expelled from the cell via an ATP- independent Ars B. Although this process has been studied in detail in E. coli and Staphylococcus aureus, it is found in many other bacteria and occurs in strict anaerobes like Clostridium and Desulfovibrio. Arsenic reduction to As (III) has been noted in several aerobic bacteria isolated from As-contaminated soils and mine tailings (Wysocki et al., 2001; Cervantes et al., 2004), suggesting that As (V) resistance plays an important role in the biogeochemical cycling of this element in nature (Macy et al., 2000). The oxidation of arsenite to arsenate is used both for detoxification and energy generation. Over thirty strains representing at least nine genera of arsenite oxidizing prokaryotes have been reported (Spliethoff et al., 2002).

In this present study we describe the isolation of arsenic tolerant species along with their potential tolerance level and their transformation ability under aerobic environment.

MATERIALS AND METHODS

Study area and sampling

Surface soil samples (0-15 cm) were collected in three replicates from six different location of Nadia district. They are Dogachinagarghata of Krishnanagar, Johura Kalibari of Ranaghat, Methopara and Panchpota of Chakdah, Saontalpara and Nischindipur of Haringhata and Dhakapara of Shantipur. In previous studies these locations are reported as arsenic prone zone, as it appears that the groundwater of entire seventeen blocks of Nadia district contains arsenic above WHO guideline value of arsenic in
drinking water (10 μg/L) and Indian Standard value (50 μg/L) (Majumdar et al., 2002).

**Soil analysis**

pH of soil sample was measured by Electrometric method with the help of a pH meter using combination Glass electrode. Organic Carbon was done by Walkley and Black method (Walkley et al., 1934). Chloride, Nitrate, Phosphate, Sodium and Potassium content were estimated by methods prescribed by APHA (APHA, 1992). The total nitrogen content was estimated by Kjeldahl method (APHA, 1992). For Arsenic measurement the samples were digested following heating block digestion procedure (Humayun, 2003). The amount of arsenic was estimated by Atomic absorption Spectrophotometer (Perkin Elmer, Analyst 400) accompanied with hydride generation system.

**Biological Characteristics**

**Isolation and Characterization of bacteria**

Bacterial strains were isolated in specific media. Iron Bacteria was isolated in iron bacteria isolating media containing Diammonium sulphate (0.5g), glucose(0.15g), Calcium Carbonate (0.1g), Dipotassium hydrogen phosphate (0.05g), Magnesium sulphate, 2H₂O (0.05g), Potassium chloride (0.05 g), Calcium nitrate (0.01 g), thiamine (0.4 mg/5mL), Cyanocobalamin (0.01mg/5mL), Agar (15.0 g) in 1000 mL of distilled water, pH 7.0. 1 ml of 10⁻³ time dilutions was added to sterilized media and the plates were allowed to incubate at 37°C for 24 hr. Pure cultures were obtained by subculture and pure culturing technique from the isolated colonies. For each cultures smears were prepared prior to Gram staining and cell morphology was investigated under microscope (1000x) (Dhar et al., 2004). Biochemical properties of the isolates were tested by following, Catalase test, Citrate utilization test, Methyl Red test, hydrogen sulfide test, Starch hydrolysis test, Carbohydrate Fermentation test, and Vogues Proskauer test (Krieg, 1984) for identification.

**Determination of Minimum Inhibitory Concentration of Arsenic**

Cup assay method was used to determine the minimum tolerable concentration of the strains for both arsenate and arsenite (Dhar et al., 2004). The bacteria were grown in both arsenate and arsenite containing broth media. The media contained 20 ppm, 50 ppm, 100 ppm, 500 ppm and 1000 ppm concentrations of arsenate and arsenite within their tolerable limit. It was incubated at 37°C for 24 hr. Optical density was measured at 420 nm at alternate days for at least 7 days (Dhar et al., 2004).

**Determination of Transforming Ability of the isolated Bacteria**

The verification of the transforming ability of the isolated bacteria was carried out by the usage of AgNO₃ method. Agar plates were flooded with a solution of 0.1M AgNO₃. A brownish precipitate revealed the presence of arsenate in the medium (arsenite oxidizing bacteria), while the presence of arsenite was detected by a bright yellow precipitate (arsenate reducing bacteria) (Krumova et al., 2001).

**RESULTS AND DISCUSSION**

**Characterization of soil**

A total of nine samples were collected from different sampling sites. Then several parameters of soil were determined. The pH of the soil is neutral to alkaline (Table 1). Soil contains large amount of organic carbon except sample no-1(Saontalpara) and sample no-2. (Nischindipur) (Table 1).Chloride content is high. Chloride content ranges from 2.84 to 11.36, sample no 6(Dhakapara-I) contains highest amount chloride (Table 1). Whereas nitrate, phosphate and total nitrogen content is low (Table 1). Arsenic content is moderate. Sample no.2 (Nischindipur) contain high amount of arsenic (Table 1). Arsenic concentration and species are generally influenced by pH and redox potential of soil, soil texture, type of parent rock, organic matter and cation exchange capacity of soil (Mandal et al., 2002).

**Characterization of Aerobic soil bacteria from arsenic contaminated soil**

Three aerobic iron bacterial strains have been isolated. They were named as \(S_{16}, S_1, N_1\). The \(S_{16}\) strain is
isolated from methopara, S<sub>1</sub> strain from Saontalpara and N<sub>1</sub> strain from Nischindipur which is the most arsenic contaminated site among these three (Table 2). The S<sub>16</sub> strain is Gram positive, coccus in nature (Table 2). S<sub>1</sub> strain is also Gram positive, coccus in nature (Table 2). N<sub>1</sub> strain is Gram negative, coccus in nature (Table 2). N<sub>1</sub> had cream colour, round shaped and slimy surface. S<sub>16</sub> and S<sub>1</sub> had round shaped opaque surface, but S<sub>16</sub> was white colour and S<sub>1</sub> was cream colour (Table 2).

Catalse test is performed to detect the presence of enzyme catalase in bacteria which protect the cell from hydrogen peroxide. Catalase test shows positive result for all three bacteria, which suggests that all these strains belong to Staphylococci or Micrococcci family. Citrate test, and MR-VP test is carried out to differentiate the family of Enterobacteriaceae. All the three strains show negative results for these three strains (Table 3). Starch hydrolysis test is done to detect whether a bacteria is able to use starch as a carbon source and an energy source. Starch hydrolysis is positive for species like Bacillus subtilis, it shows negative result for E.coli, Staphylococcus aureus. Here only S<sub>1</sub> shows positive result for this test (Table 3). Carbohydrate fermentation test is performed to detect that whether the bacterial strain can ferment carbohydrate or not. E.coli shows positive result in this test. Here only N<sub>1</sub> shows positive result in this test (Table 3). Hydrogen Sulphide test is used to check for enteric gram negative bacilli by checking

### Table 1: Chemical character of soil Sample

<table>
<thead>
<tr>
<th>Sampling sites</th>
<th>pH</th>
<th>Organic carbon (%)</th>
<th>Chloride (mg kg&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Nitrate (mg kg&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Phosphate (mg kg&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Sodium and Potassium (mg kg&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Total nitrogen (%)</th>
<th>Arsenic content in soil (mg kg&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saontalpara</td>
<td>8.24</td>
<td>1.2</td>
<td>7.1</td>
<td>0.59</td>
<td>0.38</td>
<td>Na-9.4</td>
<td>0.15</td>
<td>7.3</td>
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<td>K- 4.6</td>
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<tr>
<td>Nischindipur</td>
<td>7.49</td>
<td>3.54</td>
<td>5.68</td>
<td>0.82</td>
<td>0.36</td>
<td>Na- 9.8</td>
<td>0.24</td>
<td>16.2</td>
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<td>K- 5.8</td>
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<tr>
<td>Dogachinagarghata</td>
<td>8.40</td>
<td>5.08</td>
<td>5.68</td>
<td>0.72</td>
<td>0.39</td>
<td>Na- 7.4</td>
<td>0.07</td>
<td>4.2</td>
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<td>Panchpota</td>
<td>7.76</td>
<td>5.24</td>
<td>8.52</td>
<td>2.6</td>
<td>0.190</td>
<td>Na-6.8</td>
<td>0.13</td>
<td>9.7</td>
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<td>K- 8.1</td>
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<td>Johura kalibari</td>
<td>7.75</td>
<td>5.49</td>
<td>5.68</td>
<td>1.2</td>
<td>0.322</td>
<td>Na-7.8</td>
<td>0.231</td>
<td>6.8</td>
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<td>K- 2.4</td>
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<tr>
<td>Dhakapara- I</td>
<td>7.71</td>
<td>7.8</td>
<td>11.36</td>
<td>1.23</td>
<td>0.223</td>
<td>Na-2.7</td>
<td>0.45</td>
<td>9.7</td>
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<td>Dhakapara- II</td>
<td>7.87</td>
<td>6.0</td>
<td>7.1</td>
<td>0.59</td>
<td>0.174</td>
<td>Na-8.7</td>
<td>0.17</td>
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<td>Dhakapara-III</td>
<td>7.69</td>
<td>7.52</td>
<td>2.84</td>
<td>0.58</td>
<td>0.07</td>
<td>Na-9.1</td>
<td>0.133</td>
<td>8.06</td>
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<td>K- 5.0</td>
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<tr>
<td>Methopara</td>
<td>7.74</td>
<td>7.30</td>
<td>4.26</td>
<td>0.63</td>
<td>0.216</td>
<td>Na-9.5</td>
<td>0.231</td>
<td>7.6</td>
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<td>K- 4.0</td>
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</table>
for the enzyme thiosulfate reductase which is produced by various intestinal microorganisms. Here all the strains show negative result for this test (Table 3). The above tests suggest that all three species are separate from each other.

**Arsenic tolerance test**

All the three strains can tolerate higher concentration of arsenate and arsenite concentration. S<sub>16</sub> can tolerate both arsenate and arsenite up to 1000 mg/L<sup>-1</sup>. Where as, S<sub>1</sub> and N<sub>1</sub> can tolerate arsenate up to 1000 mg/L<sup>-1</sup> but arsenite up to 500 mg/L<sup>-1</sup>. S<sub>16</sub> strain can tolerate higher concentration of arsenate up to 9<sup>th</sup> day after that their growth decreases (Fig. 1). The strain exhibit increased growth rate under arsenite media than arsenate media. The strain can grow up to 7<sup>th</sup> day in arsenite containing media but after that their growth decreases (Fig. 2). S<sub>16</sub> strain showed highest growth rate in case of 500 mg/L<sup>-1</sup> of arsenite containing media. S<sub>1</sub> strain can grow in arsenate containing media up to 9<sup>th</sup> day but after that their growth decreases (Fig. 3). The strain exhibit increased growth rate in arsenite up to 7<sup>th</sup> day. Only in case of 50 mg/L<sup>-1</sup> concentration the strain can grow up to 9<sup>th</sup> day (Fig. 4). Where in case of 20 mg/L<sup>-1</sup> concentration after 5<sup>th</sup> day growth rate decreases. N<sub>1</sub> exhibit a decreased growth rate than the other two strains in arsenate containing media. After 7<sup>th</sup> day their growth decreases (Fig. 5). The strain exhibit increased growth rate in case of arsenate containing media than arsenate containing media (Fig 6). Thus these studies referred that all these isolates can tolerate arsenite more than arsenate and their tolerable limit extend upto 7<sup>th</sup> to 9<sup>th</sup> day. So their growth decreases after that period of time.

This result suggested that all the three strains have developed metal resistant systems in an attempt to protect sensitive cellular components. In general, microbial ability to grow at high metal concentration is
found coupled with a variety of specific mechanisms of resistance and environmental factors. Mechanisms of resistance by microorganism include microbial surface sorption, enzymatic transformation, precipitation by oxidation/reduction reaction and biosynthesis of metal binding proteins or extracellular polymers, whereas environmental factors may include the surrounding pH and redox potential, metal speciation, soil particulates and soluble organic matters (Srinath et al., 2002). In this study all the three strains showed decreased growth rate after 7th day this suggests that the resistance mechanism failed after a certain period. This may occurred due to lack of nutrients.

**Arsenic transformation test**

In the present work, all the three isolates were tested with AgNO₃ test. The silver nitrate test is based on the quality reaction between AgNO₃ and arsenite or arsenate ions. The interaction of AgNO₃ with As [III] generate bright yellow precipitate and As [V] generate brownish precipitate. All the three isolates revealed as arsenite oxidizing bacteria.

It was found that all soil samples collected from the study sites, which were previously reported as arsenic contaminated site, contain high amount of arsenic. From the soil samples three iron bacteria were isolated. All the iron bacteria were aerobic, two of them were Gram positive coccus and one was gram negative coccus. Biochemical test revealed that these three strains are different species. All the three strains can tolerate high amount of arsenic. S₁, N₁, can survive in arsenic containing broth up to 7th day. One strain (S₁₆) can survive up to 9th day, after that there growth decreases. In case of transformation test all the three bacterial strains revealed as arsenite oxidizing bacteria. Arsenite-oxidizing bacteria inhabiting natural systems require high-level arsenic detoxification systems that are not yet understood (Colin et al., 2001). Oxidation of arsenite can also be important as a detoxification process or can serve as an energy source for chemolithoautotrophic metabolism. As arsenite is more mobile, highly soluble and more toxic than arsenate, the best approach to remove arsenite is to oxidize it into arsenate; this last form is less soluble and much more easily to removed.

In order to fully appreciate the arsenic remediation potential of these three selected isolates, further transformation mechanism and molecular characterization is required.

**REFERENCES**


