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RESEARCH PAPER

Arsenic Bioremediation Potential of Arsenite Oxidizing Bacteria Isolated from Geogenic and Anthropogenically Contaminated Soil

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Abstract
The soil of many places of eastern India contains high amount of arsenic, due to several geogenic activities in this area. In the specific regions of the country where there is no such type of Geogenic activities, the soil is found to be almost free of arsenic. In such places where there are industries, the soil is being contaminated with the arsenic due to anthropogenic activities. One of such site which was selected for the study was in close vicinity to the textile industries in Jaipur, Rajasthan, India discharging their effluents having 423 µg/g arsenic. While the soil sample collected from the far eastern part of Tezpur Assam, India, contaminated by Geogenic sources contained 443µg/g arsenic. Four arsenite resistant bacterial strains were isolated from each of the samples. Strains SE^{-3} and TB^{-1} isolated from Jaipur and Tezpur, respectively showed highest minimum inhibitory concentration of 46.5mM and 38.7mM sodium arsenite. Based on 16S rDNA sequencing and nucleotide homology and Phylogenetics analysis strain, SE-3 was identified as *Pseudomonas sp*. SE^{-3} (accession no. KP730605) and TB^{-1} as Bacterium TB^{-1} (accession no KP866680). Complete oxidation of arsenite to less toxic form arsenate was observed in *Pseudomonas sp*. SE^{-3}, while 64.6% by Bacterium TB^{-1}. The arsenite oxidation was supported on the molecular level by confirming the presence of *aox* gene by PCR amplification. The enzyme activity of arsenite oxidase was also established. Arsenic hyper tolerant bacteria isolated from these soils having arsenite oxidizing ability show a promising way for the bioremediation of arsenic in contaminated soil.

Keywords: Arsenic, Arsenite resistant bacteria, Arsenite bioremediation, Characterization of isolates, aox genes.

INTRODUCTION

Arsenic is considered a heavy metal, which shows high toxicity and is incapable of natural degradation in the environment. It persists in the environment due to various natural and anthropogenic activities (Wan et al., 2019). The acute and chronic exposure of arsenic above the permissible limit leads to adverse effects on human health (Mandal and Suzuki, 2002). The global average arsenic level in the soil is 10ppm and according to the European Union, the maximum acceptable arsenic limit for agricultural soil is 20ppm (Rahaman et al., 2013). Primarily inorganic forms that are present predominantly are arsenate [As(V)] and arsenite [As(III)] where arsenite is more soluble, mobile, and 100 times more toxic than arsenate (Quéméneur et et al., 2010).

The general mechanism of toxicity in a cell as studied in different animals and human beings suggests that arsenic enters the cell at neutral pH (Kim, 1985). The cellular toxicity of trivalent arsenite involves two mechanisms: 1) by binding to the sulphydryl group of cysteine residues of protein which results in the inactivation of critical enzymes.2) by depletion of Lipoate, which

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helps in the synthesis of intermediates of kreb cycle. The formation of dihydrolipoyl-arsenite chelate complex results in Lipoate depletion results in ATP reduction, and in the inhibition of Kreb cycle and oxidative phosphorylation. (Muehe et al., 2014; Sharma et al., 2014). The toxicity of different forms of arsenic decreases from arsine being highly toxic followed by inorganic arsenite, organic arsenite, inorganic arsenate, organic arsenate being at the least level in the sequence of toxicity (Mandal and Suzuki 2002; Aksornchu et al., 2008).

Arsenic has gained the attention of researchers due to its unprecedented release and its entrance in terrestrial (soil) and aquatic environments by natural and anthropogenic activities (Pegu et al., 2019;Sher and Rehman, 2019). Geogenic sources are estimated to 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). These natural sources include natural weathering of rocks, burning vegetation, volatilization and volcanic action, mining, soil-forming, biogeochemical process, etc. While anthropogenic sources include combustion of municipal waste, arsenal pesticides (herbicides, fungicides, insecticides), desiccants, land filling of industrial waste (glass, paper, textile, semiconductors) and the land disposal of these wastes are contaminating surface and groundwater as a result indirectly effecting the whole ecosystem (Derome, 2000;Deshpande et al., 2005).

Among bioremediation strategy a wide range of arsenic-resistant bacteria species has been reported which promise successful degradation of this pollutant from the environment by various metabolic processes. Microbes tolerate the toxicity of arsenic either by withstanding the toxicity by intrinsic properties or by surviving in a high concentration of arsenic in the surrounding by detoxification mechanism. The growth of microbes in low to high concentrations of arsenic suggests that the bacterial strains possess some stress resistance within them (Dave, 2020; Pratush et al., 2018). Therefore, microbes either tolerate the arsenic i.e. the ability to withstand the toxicity by intrinsic properties or resist the toxicant by surviving in high concentrations of arsenic by adapting its detoxification mechanism. Arsenite oxidation is a widely distributed detoxification mechanism found among bacteria (Green et al., 1918). Arsenite oxidation is the capability of the bacteria to transform Arsenite [As(III)], which is the 100 times more toxic form to less toxic Arsenate[As(V)].

Arsenite oxidation has been reported in bacteria that possess genes encoding enzymes, responsible for the catalysis of arsenic transformation reaction. Investigation on arsenite oxidation reveals the presence of *aox* (arsenite oxidizing) operon bearing structural (*aox* A, *aox* B), and regulatory genes (*aox* R, *aox* S) constituting *aox* operon responsible for detoxification process (Chang et al., 2010).Arsenite oxidase is a periplasmic soluble enzyme that catalyzes arsenite oxidation. Arsenite oxidase transfers the electrons obtained from the oxidation of arsenite towards the soluble periplasmic electron carriers which are proven by in vitro assays (Anderson et al., 1992). The bacterial arsenic oxidation is an inducible system as it requires arsenite for activation of the process. The presence of the enzyme can be determined by the reduction 2,4-dichlorophenolindophenol (DCIP) which can be monitored on UV-Vis spectrophotometer at 600nm (Anderson et al., 1992; Prasad et al., 2009; Bachate et al., 2012). The aim of this study is to isolate the arsenic resistant bacteria from both the sites exposed to arsenic by Geogenic and Anthropogenic activities and explore the potential of isolates in bioremediation of toxic arsenic to less toxic form by oxidation.

MATERIAL AND METHOD

Soil sample (S) was taken from open field in Sanganer area of Jaipur City, Rajasthan (26°49'37.8"N 75°45'46.3"E) where Textile Industries discharge their effluents. The second soil sample (T) was collected from the nearby area of a tube well in Tezpur , Sonitpur district, Assam (26°38'52.2"N 92°47'21.9"E) based on reports of arsenic-contaminated tube wells and rings

Fig. 1. Showing Location of Jaipur (Rajasthan) and Tezpur (Assam) in India, (Source: Google maps)

wells by Geogenic sources (Sabhapandit et al., 2010) (Figure 1). Fresh Samples (S and T) were processed for bacterial isolation. All the soil samples were collected in sterile polypropylene zip lock bags, the samples were passed through a 2 mm sieve to remove plant debris and stored at 4°C for Physicochemical analysis, metal estimation.

The Physical and chemical parameters of fresh soil samples such as pH, Electrical conductivity(EC), water holding capacity (Gravimetric method), texture, calcium, magnesium, sodium, potassium ions were analyzed using standard protocols given by APHA 2005 and Maiti, 2003. The presence of arsenic and its concentration was estimated by Atomic Absorption Spectroscopy (AAS, model Chemito AA2013).

Arsenic resistant bacteria were isolated by the serial dilution method (up to 10^{-5}). From each dilution, 100µl was spreaded on nutrient agar (HiMedia) and incubated for 48 hours at 37±2°C in an incubator. On the basis of this preliminary screening, bacteria showing resistance against supplemented arsenite were selected.

For determining the lowest concentration that completely inhibits bacterial growth after overnight incubation, isolates were inoculated in nutrient broth supplemented with the gradient doses of sodium arsenite starting from 1g/L (7.7mM) to 6g/l (46.5mM) in triplicates. The growth after 48 hours was judged based on optical density at 600nm using UV-Vis Spectrophotometer. The strains SE-3 and TB-1(Lab nomenclature) exhibited the highest Minimum Inhibitory Concentration (MIC) of arsenite among the isolates i.e.5g/l (38.7mM) and 6g/l (46.5mM) respectively. Thereby SE-3 and TB-1 were selected for further analysis.

To understand the growth curve and nature of pH change the strains TB-1 and SE-3 were allowed to grow in Nutrient Broth (HIMEDIA) in the absence of arsenic. To obtain a dose curve the growth of bacteria at different doses of sodium arsenite (1, 2, 3, 4, 5, 6g/l) with respect to time (0, 24, 48, 72, 96 hours) was determined in nutrient broth in triplicates. The cultures were incubated at 37±2°C in a shaker incubator at 120rpm. The growth of isolates was determined by the optical density at 600nm for each set using UV-Vis Spectrophotometer. The Dose curve (with Sodium Arsenite) thus obtained was compared to the growth curve (without sodium Arsenite) to understand the effect of arsenite (toxicant) on the growth of bacteria.

The strains TB-1 and SE-3 were further identified by their morphological features and biochemical properties. The biochemical properties of the strains were tested according to Bergey's

Manual of Systematic Bacteriology. The bacterial colonies were subjected to morphological and biochemical tests such as the utilization of various sugars, H_2S production, IMViC test, Oxidase, Catalase activity, Starch hydrolysis, nitrate reduction, urease activity, Gram staining. Based on the biochemical properties of both the strains, the genus of each was identified. The determined genus was confirmed with 16s rDNA sequencing. The sequences obtained were submitted in Genbank as *Pseudomonas sp.* SE-3(accession no. KP730605) and *Bacterium* TB-1 (accession no KP866680).The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 500 replicates was taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Kimura 2-parameter method. Phylogenetics analyses were conducted in MEGA4.

The isolates SE-3 and TB-1 were tested for the potential to oxidize Arsenic (III) by using Silver Nitrate test (Simenova et al., 2004). Sodium arsenite (1g/l) supplemented nutrient agar plates were streaked with the strains and flooded with a solution of 0.1M $AgNO_3$ along with the control plates and were incubated for next 24 hours at 37±2°C. The presence of Brown precipitate on the plates indicates the presence of silver arsenate which is the result of oxidation by arsenite oxidizing bacteria while the presence of bright yellow color confirms the presence of silver arsenite.

Another Qualitative method for observing arsenic species transformation was conducted using Microplate screening assay using 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 $AgNO_3$ (150µl). b) Supernatant of the culture (150µl) + 0.1M $AgNO_3$. c) Pellet suspension (150µl) + 0.1M $AgNO_3$. d) Culture without Centrifugation (150µl) + 0.1M $AgNO_3$. 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).

Quantitative estimation of arsenite oxidation was performed by Molybdene blue method, which quantitate the arsenate-molybdenum complex formed. In this method the arsenite converted (oxidized) form arsenate shows its presence by binding with molybdenum in the reaction mix (deionized water, 50% H_2SO_4, 3%Na_3MoO_4, 2% Ascorbic acid and culture) 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 arsenic species can be differentiated and can be measured (Cai et al., 2009a; Hu et al., 2012). Both the isolates 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. These pellets were then suspended in water containing 0.1g arsenic (800µM) and incubated for 0,24,48,72 hours respectively with the controls for each hour at 120rpm, 37°C in shaker incubator. The reaction mixture for each drawn sample was prepared and the volume was made up using deionized water and reading was taken at 838nm on UV-Vis spectrophotometer to quantify arsenite oxidation.

Complete oxidation of arsenite was attained by SE-3 therefore molecular analysis was performed on this strain. 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.

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 (Anderson et al., 1992; Prasad et al., 2009). 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. 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 and pellet. 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). It 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 (Morpholino Ethane 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; Prasad et al., 2009).

RESULTS AND DISCUSSION

Soil samples from Sanganer (S) Jaipur, Rajasthan, and Tezpur, Assam (T) respectively were analyzed for their physical and chemical parameters which are summarized in Table 1.

Arsenic impacted soil from both the regions (Rajasthan and Assam) showed a pH of 8.1±0.003 and 7.8±0.01 respectively. The soil pH below 5.0 and higher than 8.4 are not favorable for optimum growth of crops, but the pH range from 6.0-8.2 is suitable for bacterial growth (Sahajrao et al., 2014). Hence both the soils were bacterial predominant.

Both the soil samples from Jaipur and Tezpur were contaminated with 423μg/g and 443μg/g of arsenic respectively which exceeds the permissible limit (20μg/g) by manifolds. The high concentration of arsenic in the soil of Tezpur can be understood to be of Geogenic origin (Bora et al., 2013) and its prevalence in groundwater and the potential threats in this region have also been reported by Ghosh and Sar 2013. The high content of Arsenic exhibited by the soil of Sanganer was due to the textile effluents discharge in it, which contains mordants, dyes, caustic

Table1. Physicochemical interpretation of the arsenic contaminated soil from Jaipur and Tezpur.

S.No	Physical parameters	Sanganer Soil (S)	Tezpur Soil (T)
1.	**pH**	8.1±0.00	7.8±0.01
2.	**Electrical Conductivity(mS/cm)**	0.60±0.01	0.24±0.00
3.	**Water holding capacity (%)**	60.37±0.923	46.76±0.05
4.	**Texture Analysis**	Very fine sandy	Clay loam
	Chemical Parameters		
5.	**Organic Carbon (%)**	1.11±0.10	0.27±0.01
6.	**Organic matter (%)**	1.92±0.17	0.46±0.16
7.	**Calcium (ppm)**	355±0.33	12.02±0.00
8.	**Magnesium (ppm)**	84.30±0.66	7.2±0.024
9.	**Total Hardness (ppm)**	493.6±0.33	19.22±0.02
10.	**Ca^{2+}/Mg^{2+}**	4.2	1.6
11.	**Sodium(meq/100g of soil)**	0.056±0.003	0.028±0.0003
12.	**Potassium(meq/100g of soil)**	0.021±0.001	0.008±0.0
13.	**Sodium adsorption ratio (meq/l)**	0.161±0.009	0.371±0.004
14.	**Cation Exchange Capacity (meq/100g)**	2.54±0.003	0.148±0.010
15	**Arsenic (As)**	423μg/g	443 μg/g

The maximum allowable limits of heavy metals in soils have been established by standard regulatory bodies such as World Health Organization (WHO), Food and Agricultural Organization (FAO) and Ewers U, Standard Guidelines in Europe (Chiroma *et al* 2014).

Table 2. Morphology of arsenite resistant Bacteria:

Sample	Bacterial Strain	Colony Morphology		Cell morphology
		Color	Margin	
Sanganer(S)	SE-1	Cream	Entire	Gram negative Cocci
	SE-2	Cream	Entire	Gram negative Bacilli
	SE-3	Yellow	Circular, entire	Gram negative Bacilli
	SE-4	Cream	Entire	Gram positive, Bacilli
Tezpur(T)	TB-1	Cream	Circular, entire	Gram negative Cocci
	TB-2	Yellow opaque	Entire	Gram negative, Bacilli
	TB-3	Translucent	Undulate	Gram negative Bacilli
	TB-4	Cloudy	Lobate	Gram positive, Cocci

Note: The lab nomenclature assigned to the respective isolates was in accordance with the sampling site. Where, S stand for Sanganer area in Jaipur, Rajasthan, while T is for Tezpur, Assam. Among the 5 samples (designated A to E) from Jaipur, isolation was conducted on sample E, thus "SE" was used for the isolates. In case of two samples (A and B) from Tezpur arsenite resistant bacteria were isolated from sample B; thereby "TB" was denoted for the isolates. While 1, 2, 3, 4 are the number of isolates from both the sites.

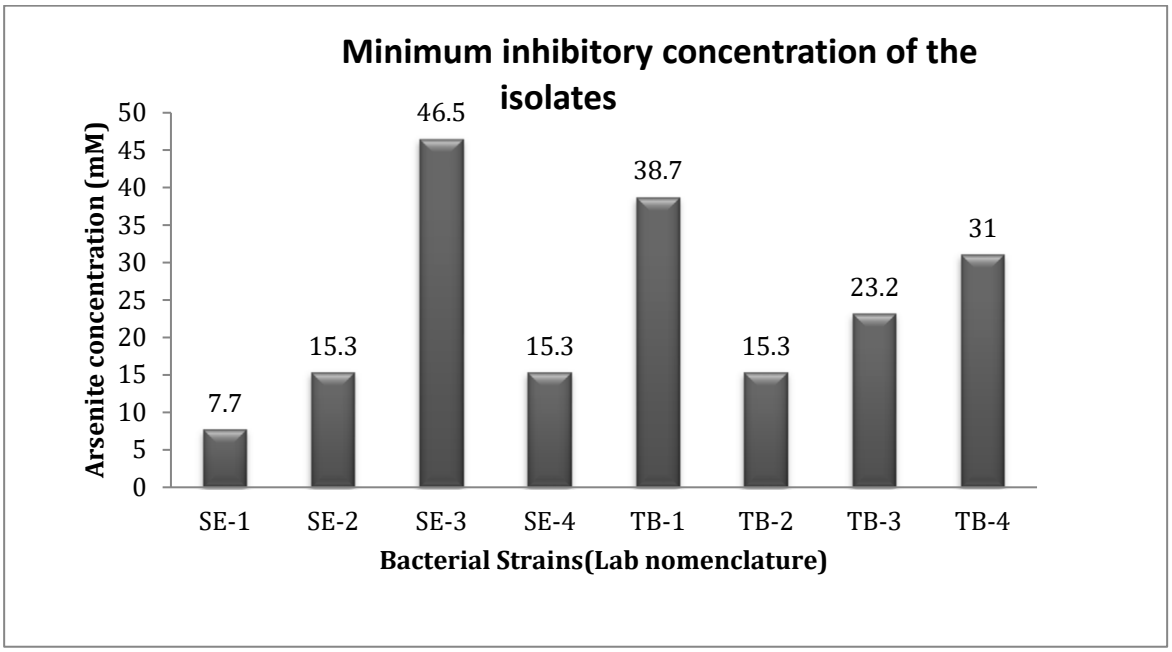

Fig. 2. Depicting Minimum inhibitory concentrations of the isolates

soda, bleaching powder which contains metals and is toxic in nature (Mahawar et al., 2015; Mobar et al., 2015).

Four bacterial strains from each of the contaminated sites were successfully isolated which proves their resistance towards arsenic. Soil bacteria possess several mechanisms that help them to tolerate heavy metal ion stress around them. Microorganism utilizes arsenic compounds as electron acceptor (arsenite), electron donor (arsenate) and in arsenic detoxification mechanism (Laverman et al., 1995). This helps in arsenic mobilization and reducing the toxicity of the arsenic. The exploration of such resistant species of bacteria gains importance due to its application in bioremediation strategies. The maximum of the strains isolated were gram negative with Bacilli and Cocci features which are summarized in Table 2.

Arsenic resistant bacteria have also been reported by several other researchers in the soil

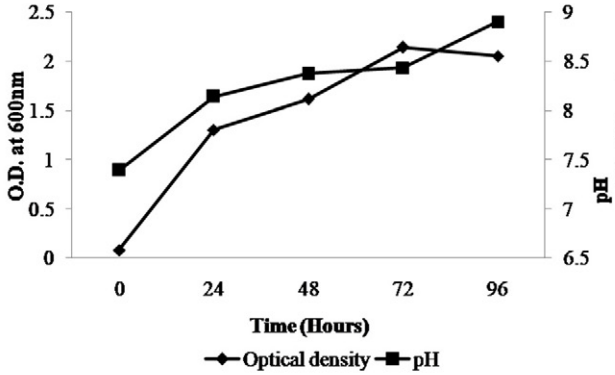

Fig. 3a. Optical density (without arsenic) and pH change during growth in SE-3.

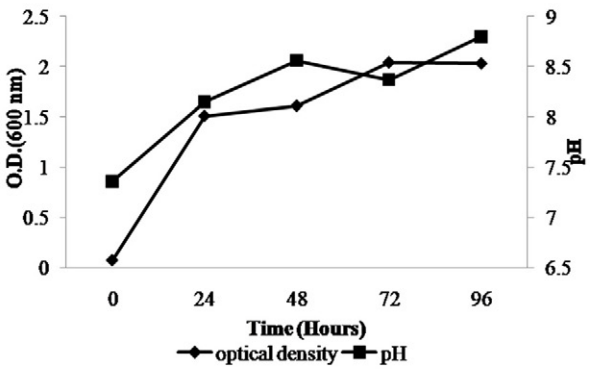

Fig. 3b. Optical density (without arsenic) and pH change during growth in TB-1.

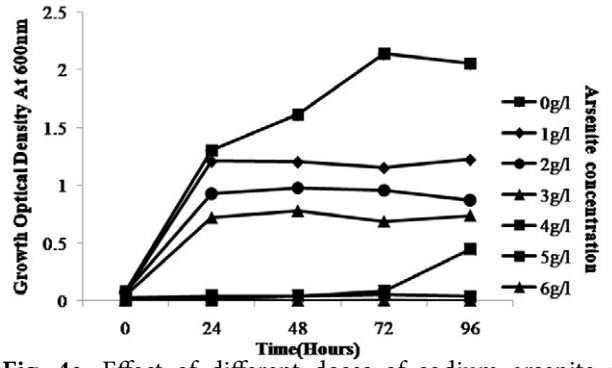

Fig. 4a. Effect of different doses of sodium arsenite on growth in SE-3, in comparison to control (0g/l)

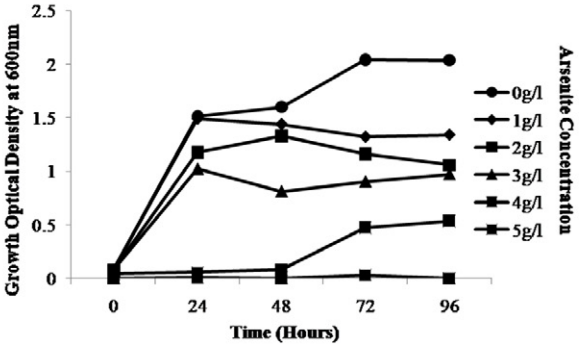

Fig. 4b. Effect of different doses of sodium arsenite on growth in TB-1, in comparison to control (0g/l)

impacted by industrial effluents (Durve, 2012; Abbas et al., 2014). The presence of arsenic tolerant species from the North-Eastern part of India, where arsenic is prevalent due to geogenic sources has also been studied and isolated (Majumder et al., 2012).

The Minimum Inhibitory Concentration against Sodium arsenite in the bacterial isolates from both the sites was found to be in range of 7.7mM to 46.5mM, where bacteria SE-3 and TB-1 showed highest MIC of 46.5mM and 38.7mM respectively (Figure 2). The further study was conducted on strains with the highest MIC representing each sample.

The growth curve of the strains (SE-3 and TB-1) showed variation in pH of the medium along with the growth (Figure 3a, 3b). In the absence of arsenite colonies of both the isolates showed lag phase for up to 48 hours at 600nm. After 72 hours stationary phase was observed for both SE-3 and TB-1. The pH shift during the maximum growth in 48 hours was observed to be in range 7 to 8. The pH shift to above range in both the bacteria proves them to be of neutrophilic nature which grows in pH range between 5.5 and 8.5 (Peterson et al., 1989).

The dose response on the growth of isolates SE-3 and TB-1 against 1g/l-3g/l Sodium arsenite in Nutrient broth (HIMEDIA) shows exponential phase till 24 hours at this dose. With the increase in arsenite concentration from 4-6g/l growth of both the isolates declines which suggest the toxicity of arsenic (Figure 4a, 4b).

The bacteria require lots of energy to repair the damage on cell wall caused due to the toxic effect of arsenic present in the surrounding environmental condition Gikas et al., 2009. The same condition can be observed in the present study during the initials hour of inoculation at all the doses. The extended lag phase occurs after 24 hours of the experiment at the arsenite doses 1g/l-

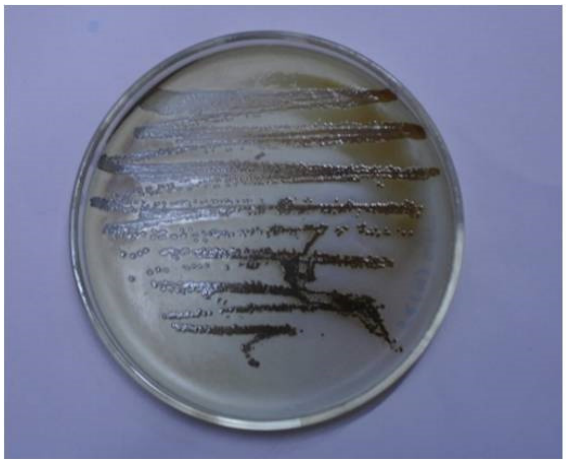

Fig. 5a. SE-3($AgNo_3$ Plate)

Fig. 5b. TB-1($AgNo_3$ Plate)

3g/l, as the bacteria now acclimatize itself with the stressful environment of arsenic (Abbas et al., 2014).On comparing the dose curves with control (without arsenic), a significant difference at high doses of both the strains proves the toxic effects of arsenic at this concentration on bacterial strains (Selvi et al., 2014).

Dey et al 2016 isolated arsenic resistant bacteria with high MIC (4500ppm) and found that microbial colonies under stress of high arsenic for long time try to combat toxicity to withstand its growth. These arsenic resistant bacteria which display high level of resistance bear capacity for bioremediation strategies (Taran et al., 2019).

On testing the bacterial culture SE-3 and TB-1 for morphological and biochemical properties they were characterized as Gram negative bacteria. Both were found to be non motile, cocci shaped and aerobic bacteria with circular and entire margins. SE-3 (Yellow color colony) and TB-1(cream color colony) were catalase positive which means they were able to degrade hydrogen peroxide by catalase production. They also possessed similarity in showing ability of nitrate reduction.SE-3 showed dextrose fermentation and was oxidase test positive where TB-1 failed to ferment any of the sugars (Dextrose, Sucrose, Arabinose, Lactose)and was found to be oxidase negative.

On the basis of 16S rDNA sequencing and nucleotide homology and phylogenetic analysis SE-3 was found to be 99% identical to *Pseudomonas sp. BRW1* and the gene sequence was submitted in GenBank as *Pseudomonas sp. SE-3(accession no. KP730605).* While TB-1 was found to be similar to Uncultured organism clone ELU0153-T424-S-NIPCRAMgANa_000490 with 98% identity, and was submitted in GenBank as *Bacterium TB-1 (accession no KP866680).*

On the basis of BLAST search obtained by GenBank, SE-3 shows 100% similarity with *Pseudomonas stutzeri*. While the genetic sequence of Tezpur isolate TB-1 showed closest relationship (99%) with *Escherichia coli*. According to Bergeys's manual of systematic bacteriology (2012) and Bergeys's manual of Determinative bacteriology (2012), both the isolates are identified in the Phylum *Proteobacteria*, class *Gammaproteobacteria.*

Bacteria belonging to class *Gamma-proteobacteria* with the potential of arsenic resistance has previously been isolated by several researchers (Nakagawa et al., 2006; Hoeft et al., 2007; Sun et al.,2008; Campos et al.,2010).Chaturvedi et al., (2014) studied the role *Enterobacteriaecae* in combating arsenic toxicity as this family expresses the gene *ars* responsible for the arsenic resistance by expressing arsenic reductase (ArsC), which is expressed much high amount in the whole class *Gammaproteobacteria*.

Pseudomonas stutzeri strains have been isolated by various scientists (Joshi et al., 2008; LiX et al., 2012; Shakhya and Pradhan, 2013) from arsenite contaminated sources in the environment.

Table 3. Oxidation Rate by Molybdene method (SE-3)

Time Interval (Hours)	Arsenite Oxidation studied in 10ml(0.2g) SE-3 pellet [Total Arsenic supplemented(800µM)]			Arsenite Oxidation studied in 20ml(0.4g) SE-3 pellet [Total Arsenic supplemented(800µM)]		
	Concentration of Arsenate detected by Molybdene blue method(µM)	Percent of Arsenite oxidized= [Conc. As(v)/800*100]	Rate of oxidation (µmoles $min^{-1}mg^{-1}$)	Concentration of Arsenate detected by Molybdene blue method(µM)	Percent of Arsenite oxidized=[Conc. As(v)/800*100]	Rate of oxidation (µmoles $min^{-1}mg^{-1}$)
24	151.60±9.4*	18.95%	0.0005	528.20±13	66%	0.0009
48	303.24±15.7	37.9%	0.001	806±20	100%	0.001
72	390.47±9.0	48.75%	0.001	798.3±56	100%	0.001

Each Value represent mean± standard error, * =p<0.001 (Highly significant)

Table 4. Oxidation Rate by Molybdene method (TB-1)

Time Interval (Hours)	Arsenite Oxidation studied in 10ml(0.2g) TB-1 pellet [Total Arsenic supplemented(800µM)]			Arsenite Oxidation studied in 20ml(0.4g) TB-1 pellet [Total Arsenic supplemented(800µM)]		
	Concentration of Arsenate detected by Molybdene blue method(µM)	Percent of Arsenite oxidized= [concAs(v)/ 800*100]	Rate of oxidation (µmoles $min^{-1}mg^{-1}$)	Concentration of Arsenate detected by Molybdene blue method(µM)	Percent of Arsenite oxidized= [concAs(v)/800*100]	Rate of oxidation (µmoles $min^{-1}mg^{-1}$)
24	162.70±16.5*	20.33%	0.0005	266.52±9.9	33.3%	0.0004
48	242.81±19.7	30.35%	0.0008	531.27±42.6	66.4%	0.0009
72	390.47±9.0	50.66%	0.001	517.30±13.2[a]	64.6%	0.0008

Each Value represent mean± standard error, * =p<0.001 (Highly significant)

The extensive literature on the family of these bacterial cultures on arsenic removal from the environment supports the present study in unveiling the more hidden potential of these bacterial strains in bioremediation of arsenic from the environment.

The strains showed arsenite oxidizing activity by developing brown color precipitate in the culture plate after supplementation with 0.1 M silver nitrate in silver nitrate test as the oxidized product (arsenate) makes brown precipitate with silver nitrate. Arsenate generates brown colored precipitate of silver-orthoarsenate after reacting with silver nitrate (Figure 5a, 5b).

In this assay, the development of brown color precipitate, when treated with 0.1 M silver nitrate indicated the presence of arsenate in the culture pellet as well as in the supernatant. But the color in pellet was more intense concluding the presence of enzyme and more oxidation of arsenite to arsenate in pellet fraction.

On comparing the results of oxidation obtained from 10ml and 20 ml culture pellet, shows maximum oxidation of 800µM of sodium arsenite by 10 ml culture pellet of SE-3 in 72 hours was48.75%, while by 20ml pellet complete oxidation was attained in 72 hours (100%). Similarly, the oxidation of 800µM of sodium arsenite in 72 hours by 10 ml culture pellet of TB-1 was 55.79% and by 20ml pellet maximum oxidation attained was 64.6% (Table 3 and 4).

The presence of *aox* genes, which is genetic determinant of arsenite oxidation proved the capability and role of *Pseudomonas sp.* SE-3 in transforming arsenite to arsenate. The presence

Table 5. Result of amplification of *aox* genes in SE-3

Marker	DNA marker	Amplification
Lane A	Negative control	No amplification
Lane B	*aox A*	No amplification
Lane C	*aox B(a)*	Amplified
Lane D	*aox B(b)*	Amplified
Lane E	*aox C*	Amplified
Lane F	*aox D*	No amplification
Lane G	*aox R*	No amplification
Lane H	*aox S*	No amplification

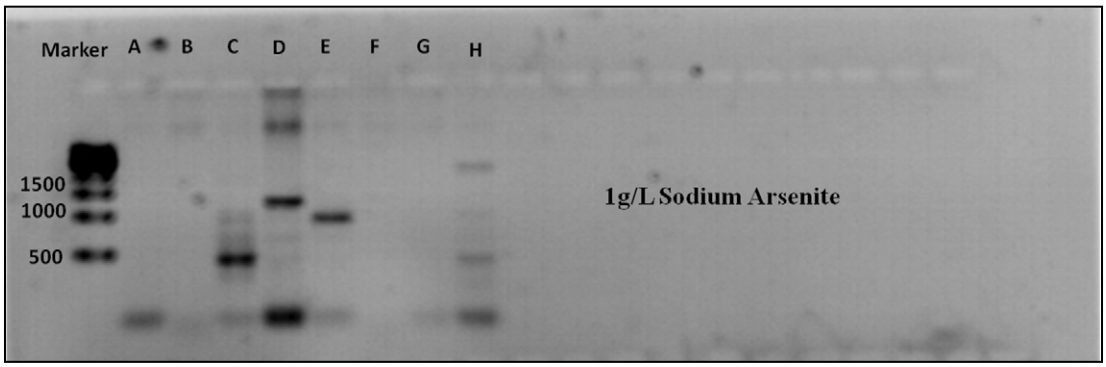

Fig. 6. PCR analysis of chromosomal DNA of Pseudomonas sp SE-3 for Arsenite oxidase Gene cluster

of the set of *aox* genes was concluded using PCR amplification and the results are summarized in Table 5, Figure 6.The Electrophoresis band pattern suggest the presence of *aoxB(a), (b)* and *aox*C genes in *Pseudomonas sp. SE-3*. These genes encode arsenite oxidase enzyme and the presence of the former confirms the expression of the latter. The *aox*B genes codes for larger subunit of the enzyme consisting of molybdopterin (Lieutaud et al., 2010; Salmeron et al., 2011; Ghosh et al., 2014) while *aox*C encodes putative nitro reductase (Koechler et al., 2010).

The specific activity of arsenite oxidase was obtained by Spheroplast preparation and its DCIP (2,4-dichlorophenolindophenol) assay. The result showed rapid color disappearance in pellet fraction by reducing DCIP to its colorless reducing form, while no change in color was observed in the supernatant. The test concludes the enzyme activity to be located in the membrane fraction of *Pseudomonas* sp. SE-3 which proves enzyme to be membrane bound.

CONCLUSION

The Geogenic activities in the environment lead to exposure of soil towards arsenic. Additional load is being added by the anthropogenic activities. This finding thus creates awareness regarding the pollution and uncontrolled arsenic liberation from textile industry in the environment. Study proves that both soils despite being from diversified area contain arsenic hyper tolerant bacteria (SE-3 and TB-1) which can play a key role in bioremediation Strategy. The biological detoxification by bacteria can prove to be more economic and efficient. The bacterial isolate SE-3 with MIC 46.5mM was able to oxidize 100% arsenite while TB-1 with MIC 38.7 mM successfully oxidized 64.6% arsenite from the medium. This successful degradation of arsenic by oxidation provides the resistance potential to bacteria. This, in turn, will help in bio remediating the toxicant from the environment. This research sends a message that we should minimize

arsenic liberation directly into the environment as we are just pilling up the toxicant which will eventually lead to Bioaccumulation of arsenic.

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CONFLICT OF INTEREST

The authors declare that there is not any conflict of interests regarding the publication of this manuscript. In addition, the ethical issues, including plagiarism, informed consent, misconduct, data fabrication and/ or falsification, double publication and/or submission, and redundancy has been completely observed by the authors.

LIFE SCIENCE REPORTING

No life science threat was practiced in this research

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