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As(III) Oxidation and Electron Mass Transfer Kinetic in an Enriched Mixed Culture of *Bacillus sp*., and *Exiguobacterium sp*., Isolated from Cow Dip in South Africa

Tony Ebuka Igboamalu*, Evans M.N Chirwa

Water Utiization Division, Department of Chemical Engineering, University of Pretoria, Pretoria 002, South Africa
Tony.Igboamalu@gmail.com

Catalytic bio-oxidation of toxic Arsenite (As(III)) to Arsenate (As(V)) in a mixed culture of bacteria strains isolated from cow dip in Tzaneen (Limpopo Province South Africa) was investigated under anaerobic condition. Anaerobic bio-treatment of As(III) is thermodynamically feasible since facultative microbes perform a significant role in the biogeochemical cycling of (As) under anaerobic environment, while deriving energy for cell growth and metabolism. If this metalloid is not efficiently treated before disposal of wastewater, it could result in cancer even at very low concentrations. An experiment was conducted and conditions were enhanced by varying different parameters such as pH, carbon source, oxidation-reduction potential (ORP), and As(III) concentration levels (20, 30, 50,100, 300, 500, and 1000 mg/L), under anaerobic condition, utilizing HCO_3 as a carbon source. As(III) oxidizing activity was optimum at temperature of 32±0.3^oC, ORP in the range of -60 to -180 MV and pH of 7±0.3, while incomplete oxidation was observed at pH of 1, 4 and 10. Average of 50 % As(III) oxidation efficiency was observed at pH 1, followed by 30 %, 16 % and 99 % at pH 4, 10 and 7.3. At different initial As(III) concentrations, results showed that at concentrations ≤ 500 mg/L, near complete As(III) oxidation was achieved with corresponding increases in As(V) concentrations. Inhibition effect was observed when As(III) concentration was set at ≥ 1000 mg/L. This study suggests that the isolated bacteria strains developed a mechanism to resist and detoxify As(III) in the arsenic contaminated site under anaerobic condition. Initial evaluation of the bacteria using 16S rRNA partial sequence method showed that cells in the mixed culture comprised predominantly of the Gram-positive species: *Bacillus sp*., and *Exiguobacterium sp*.

1. Introduction

The environmental pollution associated with toxic metallic compounds such as arsenite (As(III)) is usually from anthropogenic activities; mining and pesticides usage, wood processing industries, and pesticides manufacturing (Jain and Ali, 2000). Arsenite poses a serious threat to human health and the environment even at very low concentrations (Federal Register, 2004). However, treatment of arsenic containing waste before final disposal of effluent becomes very important because of its carcinogenic and mutagenic effect. No matter how small As(III) concentration is, it could cause skin lesions, and cancer of the brain, liver, kidney and stomach (Tochounwuo etal., 2004). Recently, it was reported that about 14.6 million people in Northwest China are exposed to drinking water containing arsenic with a concentration of ≥ 0.03 mg/l (Zhang etal., 2002). However, lowering drinking water standard to ≥ 0.01 mg/l, will demand an advanced level of treatment that might not be cost effective or user friendly.

Arsenic exists in the environment as {(Arsenite) As(+3) and (Arsenate) As(+5)} while the rest of its species (elemental) As(0) and (arsine) As(-3), are unstable (Suttigarn and Wang 2005). The toxicity of this metal depends on their speciation, which is by control by environmental redox potential (Eh), and pH. On this factor, As(III) is known to be more toxic and mobile than As(V) (Smedley and Kinniburg 2002), however, detoxification or treatment strategy could involve transforming toxic As(III) to less toxic As(V) which could precipitate as hydroxyl complexes.

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Various absorption or membrane technologies have been considered effective for arsenic removal from wastewater. Compared aluminium coagulants with iron(III) salts, the later have been found to be more effective for arsenic removal (Jiang, 2001). The problems normally with these technologies include: the need for pH adjustment thereby making it less cost effective, energy and labour intensive, and generate a secondary stream of harmful sludge that might require pre-treatment before disposal (Igboamalu and Chirwa, 2016). Biodetoxification may be considered as a cleaner alternative to physical and chemical processes, as it can be achieved under natural pH and redox conditions (pH 6.8-7.5, and Redox 0), and therefore it is less environmental, energy and labour intensive, and cost effective.

Microbial detoxification of As(III) has been known as far back as 1918. The first heterotrophic As(III) oxidizing bacteria was isolated in cattle dip in South Africa by Green (Igboamalu and Chirwa, 2017). Subsequently, various studies have reported the microbial interaction with As (III), of which could be either be by As resistance structure as ars genes (Rosen 2002) or respiratory/non-respiratory oxidation structure (Muller etal., 2003). A previous study has shown that bacteria have developed various mechanisms for oxidizing As(III), resulting to energy generation while utilizing CO2 as carbon source (Santini etal., 2000). This is evident by demonstrating that some facultative bacteria may survive by utilising As(III) as an electron donor (Sun et al., 2010). As much as 256 KJ/mol of energy can be released during the oxidation of As(III) to As(V) which can be trapped for microbial growth (Aniruddha and Wang, 2010). The proposed microbial catalytic cell oxidation of As(III) to As(V) follows equation 1.1 to 1.3. The viability of the overall equation 1.3 is because of the arsenite oxidase structure which constitutes two subunits, namely; a larger 88-kDa polypeptide containing the Mo-pterin with a HiPIP 3Fe^4S centre, and a smaller 14-kDa subunit with the Rieske 2Fe^2S center (Anderson etal., 2001). Arsenite cell interaction was proposed to bond closely to the Mo(VI) of the oxidized cofactor, allowing a direct nucleophilic attack and transfer of two electrons (Mukhopadhyay etal.,2002). Under equilibrium condition, Mo(IV) is oxidized back to Mo(VI) with electron transfer from the 3Fe^4S HiPIP center.

$$As^{3+} \rightarrow 2e^{-} + As^{5+} \quad (1.1)$$

$$Mo^{6+} + 2e^{-} \leftrightarrow Mo^{4+} \quad (1.2)$$

Overall red-ox reaction; from combining Equation 1.1 and 1.2, gives
Simultaneous red-ox reactor occurs in the larger and small subunits of Arsenite oxidase structures

$$As^{3+} + Mo^{6+} \rightarrow As^{5+} + Mo^{4+} (\Delta G = -256\ kJ/mol) \quad (1.3)$$

Considering bio-catalytic redox reaction described equation 1.1-1.3, As(III) oxidation removal seems to be feasible in the presence of Mo(VI). It could be seen that from the cell protoplasm under normal favourable environmental conditions, As(III) tends to donates 2e-, and oxidizes to As(V), while Mo(VI) accepts 2e- and reduced to Mo(IV). A reduced form of molybdenum Mo(VI) is continuously regenerated through oxidation of Mo(IV) by accepting 2e- from 3Fe^4S HiPIP centre. However, based on bioenergetics consideration, the reaction is feasible as indicated, and it is potentially exothermic process, which generates sufficient energy to drive metabolic processes, and cell growth if the toxic effects of both compounds are mitigated (Igboamalu and Chirwa, 2014). The present study, evaluates the feasibility of utilizing the isolated bacteria strains for As(III) oxidation under anaerobic condition and neutral pH.

2. Material and Method

2.1 Enriched culture isolation and media

2 g of soil and 5 ml of water samples used as inoculum were collected from the old cow dip in Tzaneen Limpopo South Africa, Figure 1a. Sample collection was evenly distributed at different locations within the cow farm. The isolation site was proposed because it has been reported that various cow dips in Limpopo South Africa have utilized arsenic-based compounds for cattle dipping for about half a century to combat East Coast fever in cattle. High As(III) concentration up to 46.6 mg/kg was reported in one of the cow dips in this province (Ramudzuli and Horn, 2014). In this regard, it was assumed that the water and soil samples from cow dip consist of arsenic-insecticide content, in which microbes at such contaminated site has developed mechanisms to resist or detoxify arsenic.

As(III) and As(V) were determined using metrohm ion chromatography with UV/VIS and conductivity (Metrohm, South Africa) (Figure 1b). Arsenic resistant strains were grown in a basal mineral medium (BMM) containing a mixture of the following solution: 10 mM NH4Cl, 30 mM Na2HPO4, 20 mM KH_2PO_4, 0.8 mM Na_2SO_4, 0.2 mM $MgSO_4$, 50 µM $CaCl_2$, 25 µM $FeSO_4$, 0.1 µM $ZnCl_2$, 0.2 µM $CuCl_2$, 0.1 µM NaBr, 0.05µM Na_2MoO_2, 0.1µM $MnCl_2$, 0.1µM KI, 0.2 µM H_3BO_3, 0.1 µM $CoCl_2$, and 0.1 µM $NiCl_2$ into 1 L of distilled water. The medium was sterilized before use by autoclaving at 121°C and 115 kg/cm^2 for 15 minutes.

The water and sludge samples were cultivated for 24 h in 100 mL of sterile nutrient broth amended with 70 mg/L of As(III) under shaking at 120 rpm in a rotary Environmental Shaker (Labotech, Gauteng, South Africa).

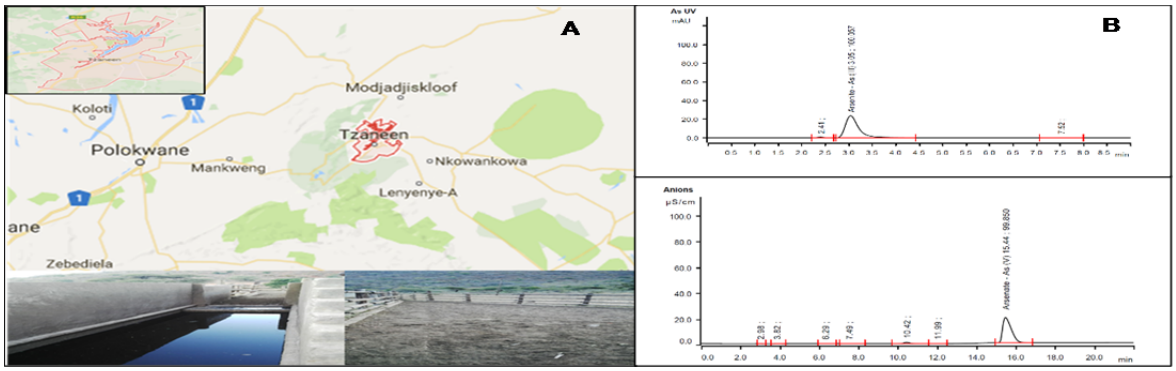

Figure 1: (a) The location off the soil sample and water samples from cow dip farm in Tzaneen Limpopo province South Africa (b) As(III) and As(V) chromatogram for standard calibration analysis

2.2 Batch investigation

Isolated cultures amended with As(III) concentrations ranging from (20 -1000) mg/L were grown anaerobically for 264 hours in a 100 mL bottle containing nutrient broth. This was covered with aluminium foil to prevent light penetration. Anaerobic condition was achieved by purging 99 % N_2 gas for 10 minutes. Prior to inoculating the bottles with harvested cells in the temperature control room, 5 mL of the sample was withdrawn for As(III) and As(V) determination. The experiment was amended with 0.15 gram of sodium hydrogen bi-carbonate as carbon source. In the temperature controlled room, the bottles were kept at optimum temperature of 32±0.2 ^{o}C on the orbital shaker at 120 rpm (Labotec, Gauteng, South Africa). 5mL sample was withdrawn periodically, and centrifuged at 6,000 rpm for 10 min in a Minispin® Microcentrifuge (Eppendorf, Hambury, Germany). Similarly, in a different batch experiment at As(III) concentration of (50 and 70) mg/L, different parameters such as pH, carbon source, oxidation-reduction potential (ORP) was also evaluated.
The strain identification was based on the ± 700 bp partial sequence of the 16S rRNA gene of the organisms. The sequences were compared against the GenBank of the National Centre for Biotechnology in the United States of America using a basic BLAST search.

3. Result and Discussion

3.1 Microbial analysis

Resistant and Oxidation of As(III) by microorganism signifies a potential bio detoxication mechanism that allows microorganisms to tolerate higher levels of poisonous metabolites like As(III) (Mukhopadhyay etal.,2002). Isolated anaerobic cultures show the ability to grow on the nutrient solution containing Arsenite concentration ≥ 70 mg/L and utilization of bicarbonate as a carbon source, Table (1). Among 6 strains isolated from six different locations (As_1-As_6), only 4 shows 100% similarity with blast search. As(III) oxidation efficiency ranging from 70 – 87 % was achieved at 120 hr of incubation, at optimum pH of 7.2 and temperature of 32±0.2. In addition, oxidation-reduction potential (ORP) for all the strains show a negative value ranging from (-46 – -61) MV, however indicating the oxidation strength of the solution. The phylogenetic analysis using 16S rRNA and blast search shows the presence of gram negative bacteria of 99-100% similarity to *Bacillus sp*., and *Exiquobacterium* sp. These strains were combined as mixed culture for further studies. The strain *Exiquobacterium profundum* showed comparable similarity with previous reported moderately thermophilic As(III) oxidizing bacteria (Crapart etal., 2007). Quite number of researchers have reported bacillus sp., as As(III) bacteria (Bachate etal., 2013).

3.2 As(III) oxidation at varying initial concentration ranging from 20-500 ppm

As(III) oxidation with the mixed isolates (AS_1-AS_6) with bicarbonate (HCO_3^-) as carbon source Table 1, was investigated. Under anaerobic condition, the media amended with 20-500 mg/L of As(III) concentrations was checked. For an initial As(III) concentration ranging from 20-500 mg/L, near complete As(III) oxidation was achieved, Figure 2. At different hours of incubation, it was seen that As(III) oxidation is a function of time for example at 20 mg/L, 25% was achieved at 12 hrs., and at 72 hrs., 41% was achieved. There is evidence of As(III) oxidation under anaerobic condition occurring slowly since the microbes are in the process of

acclimatizing to its environment. Within 240 hrs., the mixed cultures achieved 96% As(III) oxidizing efficiency with formation of As(V), but this was not applicable to the control experiment, Table 2. The appearance of As(V) shows the stoichiometric nature of the cultures in oxidizing As(III) to As(V). The evidence of microbial growth and As(III) oxidation within these concentrations utilizing bicarbonate indicates that these cultures do not depend on an external carbon source for growth and metabolism, rather, it depends on energy derived from the electron mass transfer pathway. As(III) oxidation was indeed inhibited as As(III) concentration increases up to 1000 mg/L, Figure 2. Only 4% As(III) oxidizing efficiency was observed with 240 hrs. of incubation with formation of 21 mg/L As(V), Table 2. This suggests that the maximum threshold value for these strains lies within ≥ 1000mg/L.

Table 1: Microbial analysis and identification

Site No	Isolates	Similarity (%)	Incubation time(hr)	pH	Optimum temperature °C	ORP (mv)	As(III) Concentration (mg/L)	Oxidizing Efficiency (%)
As_1	*Exiquobacterium profundum*	100	120	7.25	32±0.2	-45.9	70	85
As_2	*Bacillus Licheniformis*	100	120	7.25	32±0.2	-58.0	40	80
As_3	*Bacillus Sonorensis*	100	120	7.25	32±0.2	-60.8	40	70
As_4	*Bacillus bacterium*	100	120	7.25	32±0.2	-55.4	40	85
As_5	*Bacillus Cytotoxicus*	99		7.25	32±0.2	-65.3	40	74
As_6	*Bacillus cereus*	99	120	7.25	32±0.2	-57.1	40	87

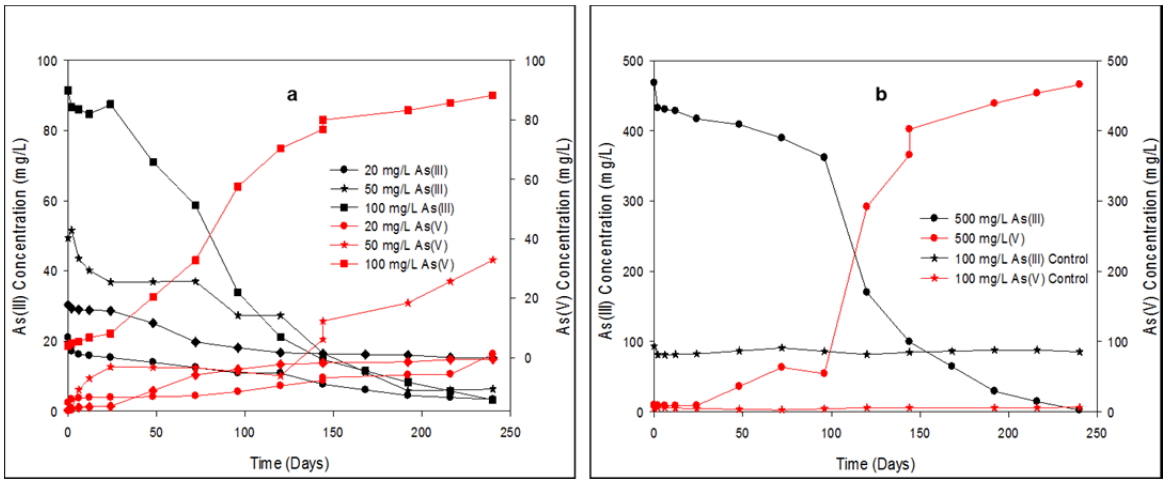

Figure 2: (a) As(III) oxidation ranging from 20-100 mg/L with As(V) formation (b) As(III) oxidation ranging from 300-500 mg/L with As(V) formation.

Table 2: As(III) oxidation at varying concentration ranging from 20-500 mg/L

As(III) Concentration @ 0 hr (mg/L)	As(III) Concentration Measured (mg/L)	Oxidizing Efficiency (%) @12 hr	Oxidizing Efficiency (%) @72 hr	Oxidizing Efficiency (%) @240 hr	As(V) Concentration formed (mg/L)	Optimum pH
20	20	25	41	90	18	7.3
50	49	19	25	96	47	7.3
100	91	7	36	98	89	7.3
300	265	17	32	93	245	7.3
500	468	9	17	99	466	7.3

Table 3: As(III) oxidation control at 100 mg/L and threshold toxicity level at 1000 mg/L

As(III) Concentration @ 0 hr (mg/L)	As(III) Concentration Measured (mg/L)	Oxidizing Efficiency (%) @12 hr	Oxidizing Efficiency (%) @72 hr	Oxidizing Efficiency (%) @240 hr	As(V) Concentration formed (mg/L)	Optimum pH
100	94	2.6	2.9	4.9	6	7.3
1000	915	2.1	2.5	4	21	7.3

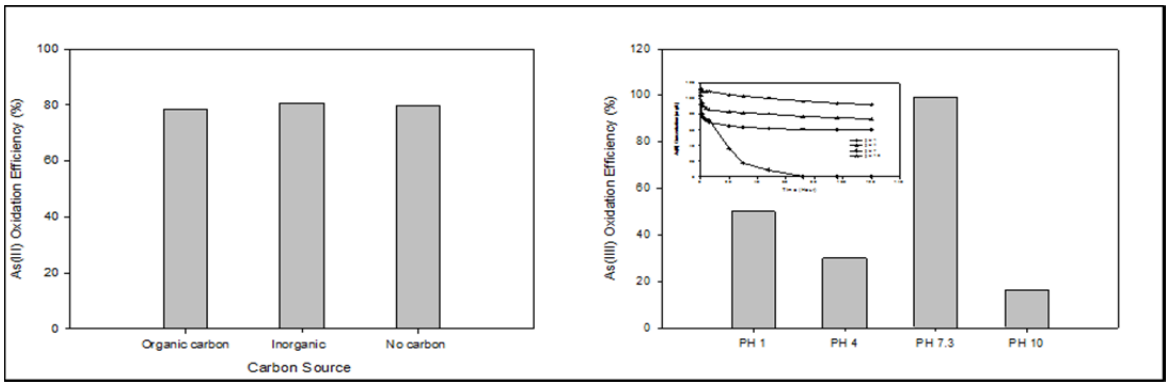

Figure 3: (a)As(III) with organic (glucose) and inorganic source (bicarbonate) (b) As(III) oxidation efficiency at different pH.

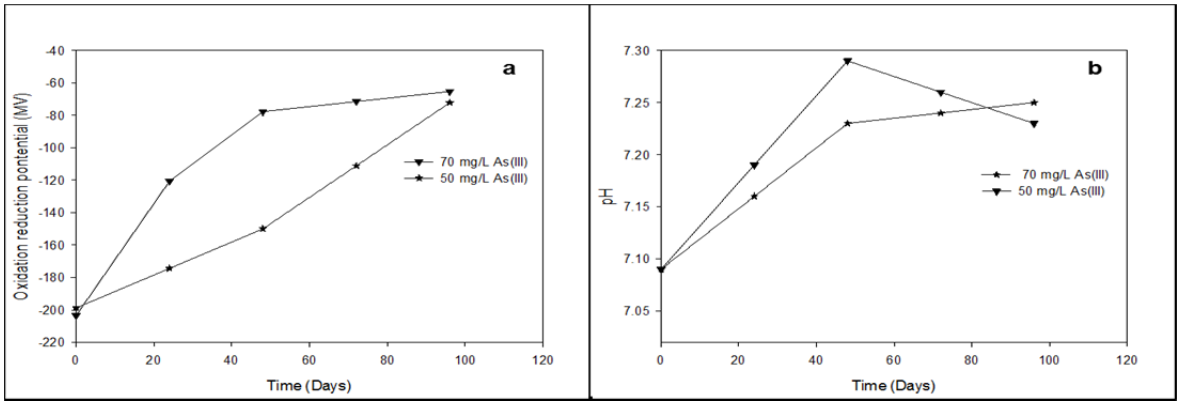

Figure 4: Oxidation-reduction potential and pH of As(III) oxidation at 50 and 70 mg/L As(III) concentration

3.3 Effect of organic or inorganic carbon on As(III) oxidation

To check whether HCO_3^- or glucose was a limiting factor for oxidation of As(III) to As(V). This was investigated with 5 g/L glucose ($C_6H_{12}0_6$), 0.5 g/L sodium bicarbonate ($NaHCO_3$) and control experiment without a carbon source. Results indicate that the redox rates for both cultures did not differ significantly, Figure 3a. As(III) oxidation rates of 2.1,1.91, and 1.90 mg As(III)/L.h and removal efficiency of 78.4, 81, and 80 % was also seen with glucose, sodium bicarbonate, and control experiment. However, this suggests that As(III) oxidation to As(V) does not depend on external carbon source rather microbial growth depends on energy derived from electron transfer process.

3.4 Optimum pH on As(III) oxidation

At different pH of 1, 4, 7 and 10, As(III) oxidation with the mixed culture was studied at 100 mg/L As(III) concentration. Within 120 hrs. of incubation, 16 % As(III) oxidation efficiency was achieved at pH of 10, followed by 30% at pH 4 and 50% at pH of 1, Figure 3 b. High As(III) oxidation efficiency (99%) was seen at pH of ≤ 7±3, suggesting the oxidation As(III) to As(V) is optimum at neutral pH. At pH of 1, it is not clearly understood why 50 % As(III) oxidation was achieved under acidic condition since As(III) dominates at pH range from 6-9. It suggests that some As(III) species may have precipitated from solution under acidic condition. However, evidence of low As(III) oxidation efficiency at pH of 1,4, and 10 recommend lack of As(III) oxidation.

3.5 Oxidation-reduction potential and optimum pH variation

Electron mass transfer was check based on the tendency of the experimental solution to gain or loss electron. At 50 and 70 mg/L As(III) concentration with mixed culture under anaerobic condition was investigated. Samples were with over interval and measure the oxidation-reduction potential in milli volts (mV). At both concentrations, initial oxidation-reduction analysis was recorded as -208 mV, pH 7.1 at 70 mg/L and -199 mV, pH 7.3 at 50 mg/L. Within 96 hr of incubation, oxidation-reduction potential was recorded as -65.3 mV, pH 7.25 at 70 mg/L and -72.2 mV, pH 7.23 at 50 mg/L as shown in figure 4. Oxidation-reduction potential of both

experimental solution tends to increase from less oxidizing condition to more oxidizing condition with corresponding increase in pH. However, this is governed by the appearance of As(V), suggesting indeed an electron transfer during As(III) oxidation, with beneficial use of energy released for cell growth-metabolism.

4. Conclusions

Mixed culture of facultative bacteria characterised as *Bacillus sp.,* and *Exiquobacterium sp*., isolated from cow dip in South Africa shows high resistance to As(III) concentration up to < 1000 mg/l. The evidence of oxidizing As(III) and formation of As(V) indicates that these cultures could be a member of chemoautotrophic As(III) oxidizing bacteria. Among different pH levels observed, As(III) oxidation was seen optimum and highly effective at neutral pH under anaerobic condition. This study indicates that the isolated mixed culture can resist and oxidize As(III) to As(V) in a contaminated, and thus afford a potential for bioremediation of As(III) contaminated sites. However, the metabolic process is less energy intensive since As(III) oxidation is achieved under anaerobic condition, no carbon source and neutral pH.

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