International Journal of Applied and Natural Sciences (IJANS)
ISSN 2319-4014
Vol. 2, Issue 4, Sep 2013, 23-30

International Academy of Science, Engineering and Technology
Connecting Researchers; Nurturing Innovations

ARSENATE REDUCTION BY SOME BACTERIA ISOLATED FROM INDUSTRIAL EFFLUENTS OF RAJSHAHI, BANGLADESH

M. A. SALEH & A. E. EKRAM

Microbiology Laboratory, Department of Genetic Engineering and Biotechnology, University of Rajshahi, Bangladesh

ABSTRACT

Arsenic is a heavy metal whose significance in microbial ecology and as environmental toxins has recently been recognized. Arsenite, a reduced form of arsenic, is more toxic and mobile than arsenate. The aim of this study was to isolate the arsenic resistant bacteria from industrial effluents and to assess their ability to reduce arsenate to arsenite. Two different species of bacteria *i.e. Klebsiella oxytoca* and *Rahnella aquatilis* were isolated and these two could tolerate arsenic up to 260 mg/L and 220 mg/L respectively. Both the species showed maximum growth at pH 7.0.The maximum growth for *K. oxytoca* was observed at 30 º C while the maximum growth of *R aquatilis* was observed at 35º C. *K oxytoca* and *R aquatilis* showed 75% and 69% ability to reduce As(V) to As(III) respectively. So, they may potentially be used in the bioremediation of arsenic.

KEYWORDS: Arsenic, Industrial Effluents, *Klebsiella oxytoca, Rahnella aquatilis*

INTRODUCTION

Arsenic is a toxic metalloid naturally found as inorganic oxyanion arsenate As(V) and arsenite As(III) species. Arsenate is often found co-precipitated with iron oxyhydroxide, which may be immobilized under acidic and moderately reducing conditions. Under reducing conditions, arsenic is found as arsenite which can co-precipitate with metal sulphides (Niggemyer *et al.*, 2001). The sources of environmental arsenic may derived from various natural sources (*i.e*. weathered volcanic, marine sedimentary rocks, fossil fuels, minerals), water, air, living organisms and anthropogenic activities including mining, agricultural chemicals (e.g. pesticides, herbicides), wood preservatives, medicinal products, industry activities (Dowdle *et al.*,1996; Stolz and Oremland,1999; Liest *et al*.,2000; Mandal and Suzuki,2002)

The toxicity of different forms of arsenic decreases in the order: arsine>inorganic arsenite>organic arsenite>inorganic arsenate>organic arsenate>free arsenic (Mandal and Suzuki,2002).Toxicity depends on different factors such as, gas, physical state, solution or powder particle size, cell adsorption rate, elimination rate and the nature of chemical substituents in the toxic compound (Anderson and Cook,2004). Conventional methods for removing metals from industrial effluents include chemical precipitation, chemical oxidation or reduction, ion exchange, filtration, electrochemical treatment, reverse osmosis, membrane technologies and evaporation recovery (Ahluwalia and Goyal, 2007). These process may be ineffective or extremely expensive especially when the metals in solution are in the range of 1-100 mg/l (Nourbakhsh *et al*.,1994). Therefore, it is important to develop an innovative, low cost and eco- friendly method for removal of toxic heavy metals from the wastewater. Biological remediation techniques, either using living/dead cells or biosynthesized molecules have been examined (Katsoyiannis *et al*,2002). Studies have shown that both plant and microorganisms are able to accumulate metal ions via processes such as transportation across the cell membrane, biosorption onto cell wall, entrapment in extracellular capsule, precipitation, oxidation- reduction reaction and biosorption to extracellular polysaccharide (Malik,2004). Hyperaccumulating plant species, such as *Pityrogramma calomelanos* and *Pteris vitta*, were shown to accumulate arsenic in the form of arsenate at the leaf section (Visoottiviseth *et al.*,2002).

Studies have reported the ability of algae, fungi, and bacteria to transform arsenite to arsenate and vice versa during their growth (Macy *et al*.,2000; Hasegawa *et al*.,2001; Visoottiviseth and Panviroj,2001). *Desulfomicrobium* sp. BenR-B has been shown to reduce arsenate to arsenite via enzyme arsenate reductase (Macy *et al*.,2000). The mechanisms involved in the microbial transformation and removal of arsenic from the environment included adsorption via reduction reaction by *Desulfomicrobium* sp. BenR-B,oxidation/reduction reaction by *Tricoderma harzianum* AsII and *Trichosporum mucoides* SBUG801, and methylation reaction by *Paenibacillus* sp. and *Pseudomonas* sp.(Macy *et al*.,2000). The endeavor of the present work is to study the isolation and characterization of arsenic resistant bacteria from industrial effluents and their ability to reduce arsenate to arsenite.

MATERIALS AND METHODS

Sample Collection

Industrial effluents were collected in a screw capped sterilize bottle from sopura silk industrial area of Rajshahi.

As Resistant Bacteria Isolation

100 µl of the sample was spread out on Luria Bertani (LB) agar plates that contain 100 µg As(III)/ml. The plates were prepared by combining 1 g NaCl, 1 g tryptone and 0.5 g yeast extract in 100 ml distilled water. The pH was adjusted to 7, and 1.5 g agar was added in the 250 ml flasks. The medium was then autoclaved. The bacterial colonies were observed after 24 hours of incubation. Effect of As(III) on the growth of bacterial isolates was determined in acetate minimal medium. To determine the minimum inhibitory concentration (MIC) of the bacterial isolates, the As (III) concentrations were increased successively until it was obtained and experiments were carried out in triplate.

Identification of the Bacterial Isolates

Morphological Characteristics

Bacterial isolates were grown in BSMY 1 with the addition of 50mM sodium arsenate and incubated at 30^0C and gram stain and cell morphology were investigated under the microscope.

Biochemical Characteristics

Biochemical chatacteristics of the two isolates were tested according to the Berger's manual of systematic bacteriology. Motility test, Glucose fermentation test, Lactose fermentation test, MR VP test, Citrate test, H_2S production test, Oxidase test, Indole test and Nitrate reduction test had been done.

16S rRNA Gene Amplification

Genomic DNA was isolated and 16S rRNA gene was amplified by using the universal Primers. The PCR reaction mixture (25 µL) contained bacterial genomic DNA (50 ng), 1X Taq buffer, 1.0 U Taq polymerase, 1.5 mM MgCl2, 200 µM dNTPs, and 0.5 µM of each primer. PCR conditions were as 94° C for 5 min (initial denaturation), followed by 40 cycles of 94° C for 1 min (denaturation), 36° C for 1 min (annealing), 72°C for 1 min (extension) and final extension 72°C for 10 mins. Amplified products were checked on agarose gel electrophoresis and sequenced. Sequence analysis was performed using the sequence alignment software BLASTn with the NCBI database (National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

Determination of Optimum Growth

Two parameters i.e., temperature and pH were considered for optimum growth of the bacterial isolates, For determination of optimum temperature, the samples were incubated at 25^0C,30^0C,35^0C,40^0C and 45^0C and for

determination of optimum pH, the pH was adjusted at 5.0, 6.0, 7.0, 8.0,9.0 and 10.0. Then after an incubation period of 12 h, O.D. was taken at 600 nm.

Effect of Arsenic on Bacterial Growth

To determine the effect of As on bacterial growth acetate minimal medium containing 100µg/ml of As was used. 50µl freshly culture was added in 100 ml medium and incubated at their respective temperature and O.D. was taken at regular interval (0, 5, 10, 15, 20, 25, 30 and 35 hours) in 600 nm.

Antibiotic Sensitivity Test

To check the resistance or sensitivity of the arsenic resistant bacterial isolates, different types of antibiotic discs were used. The discs were placed on agar plate with bacterial culture. The plates were then incubated at their respective temperature for overnight. After 16 hours of incubation, the diameter of clear zone around the discs were measured with the help of millimeter scale.

Arsenate Reduction by Bacteria

In this case the NADPH oxidation method was used (Anderson and Cook, 2004). The culture were grown to log phase (overnight grown culture) in 250 ml of acetate minimal medium supplemented with 100 µg/ml of arsenate, spun down at 14000 (6500 x g) for 5 min and pellet was washed twice in 50 ml of reaction buffer (10mM Tris, pH 7.5, with 1mM Na2EDTA and 1 mM $mgCl_2$), and finally resuspended in 15 ml of reaction buffer. Cells were lysed by sonication, and centrifuged at 14000 (6500 x g) for 5 min and supernatant was used for arsC enzyme assay.

The NADPH oxidation was initiated at 37^{o}C by mixing 50 µl of crude extract in 820 µl of reaction buffer, 30 µl of 10mm DTT, 50 µl of 2 mM arsenate, and 50 µl of 3 mM NADPH (final concentratuion 0.15mM). Arsenate concentration of 100 µg/ml and control (no arsenic) were assayed. Measurements were taken at 340 nm, where 0.15 mM NADPH has an absorbance of approximately 1.0. Absorbance decreases as NADPH is oxidized coupled to arsenate reduction to arsenite. The percentage reduction of arsenate with reference to NADPH oxidation was calculated.

RESULTS

Identification of the Bacterial Isolates

The results of the biochemical tests are presented in table 1. The sequenced 16 SrRNA gene of the isolates were uploaded to the NCBI website to confirm the species of the isolates. The nucleotide sequences coding for 16S rRNA gene after BLAST query confirmed the isolates as *K oxytoca* and *R aquatilis.*

Table 1: Biochemical Characteristics of the Isolates

Biochemical Test	*K oxytoca*	*R aquatilis*
Gram staining	-ve rods	-ve rods
Motility test	-ve	+ve
Glucose fermentation test	-ve	+ve
Lactose fermentation test	-ve	+ve
MRVP test	+ve	+ve
Citrate test	+ve	+ve
H_2S production test	-ve	-ve
Oxidase test	+ve	-ve
Indole test	-ve	-ve
Nitrate reduction test	+ve	+ve

+ve = Positive; -ve = Negative

Determination of Optimum Growth Condition

In order to large scale biomass production of the isolates for further application, the optimum growth condition was determined. Both the isolates showed maximum growth at pH 7 (figure1). In acidic pH the growth of both the bacteria were lower than basic pH. Effect of temperature was observed by incubating the bacteria at different temperatures. The maximum growth for *R aquatilis* was observed at 35º C and for *K oxytoca* was observed at 30 º C. It can be explained from Figure2 that both the bacteria showed better growth at high temperature(45 º C) than low temperature(20ºC). Growth studies will help when these bacteria are used for bioremediation purpose.

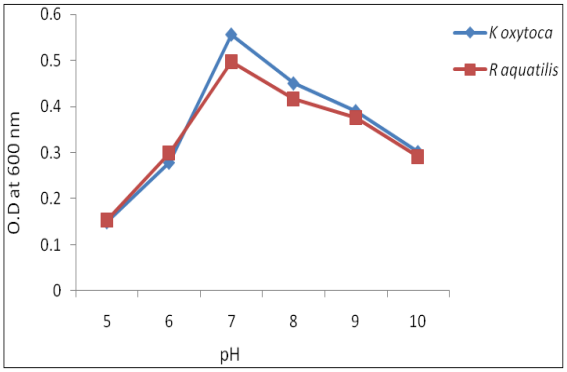

Figure 1: Effect of pH on the Growth of Bacterial Isolates Growing in LB Medium

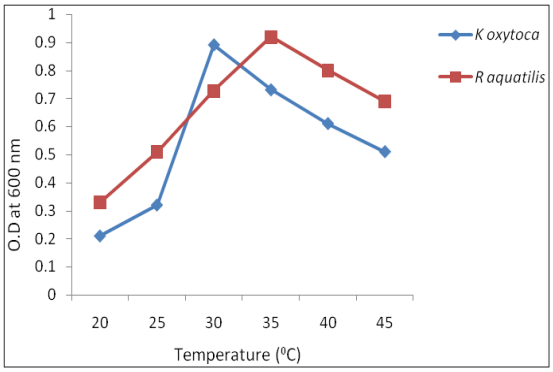

Figure 2: Effect of Temperature on the Growth of Bacterial Isolates Growing in LB Medium

Growth Studies

The growth of the bacterial isolates were studied in the presence of arsenite (100µg/ml) and were compared with the control of culture in which no arsenite were added. The growth rate of bacterial isolates was lower in presence of arsenite than that of control (figure 3 & 4)

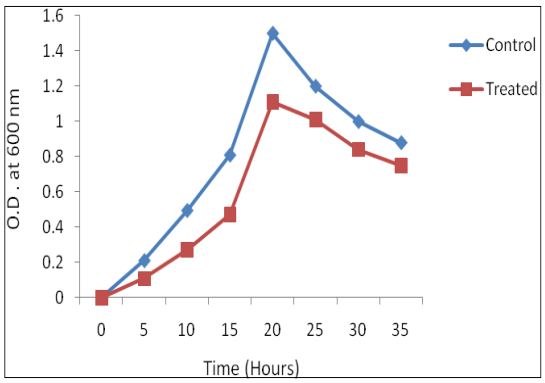

Figure 3: Effect of Arsenite (100 µg/ml) on Growth of *K oxytoca*

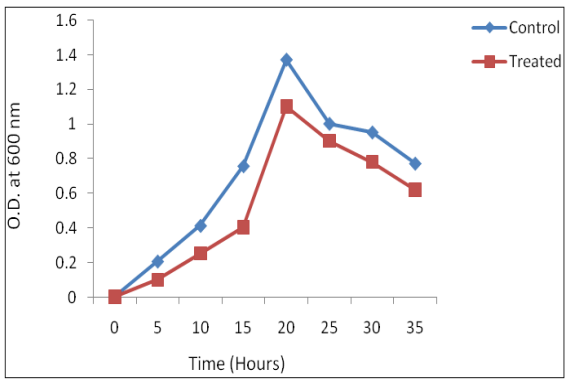

Figure 4: Effect of Arsenite (100 µg/ml) on Growth of *R aquatilis*

Antibiotic Resistance

Both the bacteria were resistant to ticarcilin, ampicilin, amoxicillin and chloramphenicol but they were sensitive to sulphamethoxazole and astreonam. In case of cefazolin, *K oxytoca*. and *R aquatilis.* were found sensitive and resistant respectively.

Arsenate Reduction

ArsC reductase crude assay showed that *K oxytoca* and *R aquatilis* could reduce As(V) into As(III) 75% and 69% respectively.

DISCUSSIONS

The reduction of As(V) to As(III) was observed within microcosms following amendment with acetate. As(V) is a toxic analogue for inorganic phosphorylation activities (Summers and Silver, 1978) ,entering cells as the toxic oxyanion, where it is capable of uncoupling oxidative phosphorylation and interfering with protein synthesis (Tamaki and Frakenberger, 1992). Bacteria with the ability to respire and reduce As(V) are ubiquitous, but as highlighted in the present study, such bacteria may exist in low numbers until provided with the opportunity to flourish by the addition of new sources of, especially, carbon and possibly the selective pressure of higher concentrations of arsenic. There exists a variety of mechanisms for the removal of heavy metals from aqueous solution by bacteria, fungi, ciliates, algae, mosses, macrophytes and higher plants (Holan and Volesky, 1994; Pattanapipitpaisal *et al*., 2002; Rehman *et al*., 2007). The cellular response to the presence of metals includes various processes such as biosorption by cell biomass, active cell transport, binding by cytosolic molecules, entrapment into cellular capsules, precipitation and oxidationreduction reactions (Gadd, 1990; Lovely and Coates, 1997) as well as protein-DNA adduct formation (Zhitkovitch and Costa, 1992) and induction of stress proteins (Ballatori, 1994).

In the present study, two different types of arsenic resistant bacteria *K oxytoca* and *R aquatilis* were isolated and they showed their ability to tolerate arsenic up to 260 mg/L and 220 mg/L. Shakoori *et al.* (2010) isolated arsenic resistant bacteria from industrial effluents and reported that *K oxytoca* can tolerate arsenic up to 240mg/L. Arsenite resistant bacteria were also isolated from industrial effluents by several researchers (de Vincente *et al*., 1990; Anderson and Cook, 2004; Aksornchu *et al* .,2008; Escalante *et al*., 2009). The ability of microbial strains to grow in the presence of arsenic would be helpful in the waste water treatment where microorganisms are directly involved in the decomposition of organic matter in biological process for waste water treatment. The growth rate of the isolated bacteria in the presence of arsenic were consistently slower than that of control. Similar result were also reported by Pal *et al.* (2004), Edward *et al*. (2006) and Shakoori *et al.* (2010).

Silver and Phung (2005) reported that Ars cytoplasmic arsenate reductase is found widely in microbes, and the arsC gene occurs in ars opetrons in most bacteria with total genomes measuring 2 Mb or larger as well as in some archaeal genomes. One potential method is microbially catalyzed reduction of As(V) to As(III), which is reported by many workers (Mukhopadhyay *et al*.,2002; Anderson and Cook, 2004; Silver and Phung, 2005; Escalante *et al*., 2009). In the present investigation arsC reductase of *K oxytoca* and *R aquatilis* showed their ability to reduce As(V) into As(III) 75%, and 69% respectively. Approximately similar results were obtained by Shakoori *et al.(*2010) where they showed that *K oxytoca* could reduce arsenate about 78% but for *R aquatilis* no references were available.

ACKNOWLEDGEMENTS

Ministry of science and technology, govt. of the people's republic of Bangladesh is gratefully acknowledged for funding this research.

REFERENCES

1. Ahluwalia, S. S. and Goyal, D. 2007. Microbial and plant derived biomass for removal of heavy metals from wastewater. Bioresour. Technol., 98: 2243-2257.
2. Aksornchu P., P.Prasertsan and Sobhon,V. 2008.Isolation of arsenic tolerant bacteria from arsenic contaminated soil.Songklanakarin J. Sci. Technol.,30(Suppl.1): 95-102

3. Anderson, C. and Cook, G. M. 2004. Isolation and characterization of arsenate-reducing bacteria from arseniccontaminated sites in New Zealand. Curr. Microbiol.,48: 341-347.

4. Anderson, C. R. and Cook, G.M. 2004. Isolation and characterization of arsenate-reducing bacteria from arsenic contaminated sites in New Zealand. Curr. Microbiol., 48:341-347.

5. Ballatori, N., 1994. Glutathionemercaptides as transport forms of metals. Adv. Pharmacol., 27:271-298.

6. De-vicente, A., Aviles, M., Codina, J. C., Borrego, J. J. and Romero, P. 1990. Resistance to antibiotics and heavy metals of *Pseudomonas aeruginosa* isolated from natural waters. J. Appl. Bacteriol., 68:625–632.

7. Dowdle, P.R., Laverman, A.M. and Oremland, R.S. 1996. Bacterial dissimilatory reduction of arsenic(V) to arsenic(III) in anoxic sediments. Appl Environ Microbiol., 62:1664–1669.

8. Edward Raja,C. Anbazhagan, K. and Selvam,G.S.2006. Isolation and characterization of a metal resistant Pseudomonas aeruginosa strain. World Journal of Microbiology and Biotechnology, 22:577-586.

9. Escalante, G., Campos, V. L., Valenzuela, C., Yanez, J., Zaror, C. and Mondaca, M.A. 2009. Arsenic resistant bacteria isolated from arsenic contaminated river in the Atacama desert (Chile). Bull. environ. Contam. Toxicol., 83:657-661.

10. Gadd, G. M., 1990. Heavy metal accumulation by bacteria and other microorganisms. Experientia, 46:834-840.

11. Hasegawa, H., Sohrin, Y., Seki, K., Sato, M., Norisuye, K.,Naito, K. and Matsui, M. 2001. Biosynthesis andrelease of methylarsenic compound during the growth of fresh water algae. Chemosphere, 43: 265-272.

12. Holan, Z. R. and Volesky, B. 1995. Accumulation of Cd, lead and nickel by fungal and wood biosorbents. Appl. Biochem. Biotechnol., 53:133-146.

13. Katsoyiannis, I., Zouboulis, A., Althoff, H. and Bartel, H.2002. As(III) removal from ground waters using fixed bed upflow bioreactors. Chemosphere, 47: 325-332

14. Leist, M., Casey, R. J. and Caridi, D. 2000. The management of arsenic wastes: problems and prospects. J. Haz. Mat., 76:125-138.

15. Lovely, D. R. and Coates, J. D. 1997. Bioremediation of metal contamination. Curr. Opin. Biotechnol., 8:285-289.

16. Macy, J. M., Santini, J. M., Panling, B. V., O'Neil, A. H. and Sly, L. I. 2000. Two new arsenate/sulfate-reducing bacteria: Mechanisms of arsenate reduction. Arch. Microbiol., 173, 49-57.

17. Malik, A. 2004. Metal bioremediation through growing cell. Environ. Int., 30: 261-278.

18. Mandal, B. K. and Suzuki, K. T. 2002. Arsenic around the world: a review. Talanta, 58: 201-235.

19. Mukhopadhyay, R., Rosen, B. P., Phung, L. T. and Silver, S. 2002. Microbial arsenic from geocycles to genes. FEMS Microbiol. Rev., 26:311-325.

20. Niggemyer, A.,Spring,S.,Stackebrandt,E. and Rosenzweig,R.F.2001.Isolation and characterization of a novel As(V)-reducing bacterium: implications for arsenic mobilization and the genus Desulfitobacterium. Appl. Environ.Microbiol., 67(12):5568-80

21. Nourbakhsh, M., Sag, Y., Ozer, D., Aksu, Z., Kutsal, T.and Caglar, A. 1994. A comparative study of various biosorbents for removal of chromium (VI) ions from industrial waste waters. Process Biochem., 29: 1–5.

22. Pal,A. Choudhuri,P., Dutta, S., Mukherjee, P.K and Paul, A.K. 2004. Isolation and characterization of nickel resistant microflora from serpentine soils of Andaman. World Journal of Microbiology and Biotechnology, 20:881-886.

23. Pattanapipitpaisal, P., Mabbett, A. N., Finlay, J. A., Beswick, A. J., Paterson-Beedle, M., Essa, A., Wright, J., Tolley, M. R., Badar, U., Ahmed, N., Hobman, J. L., Brown, N. L. and Macaskie, L. E., 2002. Reduction of Cr (VI) and bioaccumulation of chromium by Gram-positive and Gram-negative microorganisms not previously exposed to Cr-stress. Environ. Technol., 23:731-745.

24. Rehman, A., Ali, A., Muneer, B. and Shakoori, A.R. 2007. Resistance and biosorption of mercury by bacteria isolated from industrial effluents. Pakistan J. Zool., 39:137-146.

25. Shakoori F.R., Aziz I, A. Rehman and A. R. Shakoori. 2010. Isolation and Characterization of Arsenic Reducing Bacteria from Industrial Effluents and their Potential Use in Bioremediation of Wastewater. Pakistan J. Zool., 42(3): 331-338.

26. Silver, S. and Phung, L. T. 2005. Genes and enzymes involved in bacterial oxidation and reduction of inorganic arsenic. Appl. environ. Microbiol., 71:599–608.

27. Stolz, J. F. and Oremland, R. S. 1999. Bacterial respiration of arsenic and selenium. FEMS Microbiol. Rev., 23:615-627.

28. Summers, A. O. and S. Silver. 1978. Microbial transformations of metals. Annu. Rev. Microbiol., 32**:**637-672.

29. Tamaki, S. and W. T. Frakenberger. Jr. 1992. Environmental biochemistry of arsenic. Rev. Environ. Contam. Toxicol., 124**:**79-110.

30. Visoottiviseth, P. and Panviroj, N. 2001. Selection of fungi capable of removing toxic arsenic compounds from liquid medium. Sci. Asia., 27: 83-92.

31. Visoottiviseth, P., Francesconi, K. and Sridochan, W. 2002.The potential of Thai indigenous plant species for thephytoremediation of arsenic contaminated land. Environ. Pollut., 118: 453-461.

32. Zhitkovitch, A. and Costa, M. 1992. A simple sensitivity assay to detect DNA-protein crosslinks in intact cells and in vivo. Carcinogenesis, 13:1485-1489.