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SCREENING AND OPTIMIZATION OF ARSENIC DEGRADING BACTERIA AND THEIR POTENTIAL ROLE IN HEAVY METAL BIOREMEDIATION

TRIAGEM E OTIMIZAÇÃO DE BACTÉRIAS QUE DEGRADAM ARSÊNICOS E SEU PAPEL POTENCIAL NA BIORREMEDIAÇÃO DE METAIS PESADOS

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ABSTRACT: Industrialization has added extremely toxic metalloid arsenic into the environment which at high concentration severely threatens the biota. Naturally, some microbes possess the ability to bio-accumulate metals and also to transform arsenite (As III) a toxic form to a non-toxic arsenate As V. The present study aimed to isolate arsenic resistant bacterias from the arsenic contaminated soil and water. Among eleven bacterial isolates, three FAs 1, 4 and 9 exhibited tolerance against sodium arsenite at 100mM concentration by achieving growth of 7.48×10^9,1.57×10^9 and 2.23×10^9 C.F.U./ml, respectively. Optimization at different conditions such as temperature, pH and arsenic concentration revealed high arsenic tolerance from isolate FAs 4 (5.33×10^8) at 37°C and FAs 1 (4.43×10^8 C.F.U./ml) at pH 7. Arsenic resistance at optimum conditions for the bacterial strains FAs 1, FAs 4 and FAs 9 showed maximum growth at 80mM concentration of arsenite. These bacterial isolates did not show redox ability to oxidize arsenite As III to arsenate As V. However bacterial isolates FAs 1, FAs 4 and FAs 9 were able to accumulate arsenic 39.16, 148 and 125 µg/L on the 4th, 3rd and 5th day of incubation, respectively. The isolates FAs 1, FAs 4 and FAs 9 were identified as Gram negative non endospore forming rods. In future, these novel isolates possess a great potential in biotechnology field, as bioremediation of arsenic contaminated soil and water can be done by employing arsenic accumulating bacteria which is an eco-friendly and cost effective method.

KEYWORDS: Arsenic contamination. Arsenic bioremediation. Arsenic biodegradation. Bio-accumulation.

INTRODUCTION

The advent of industrialization and human activities in some parts of the world has polluted the soil and water bodies with heavy metalloids. One of the alarming pollutant is arsenic a lethal metal contaminant, resulting in major health related issues. Naturally, arsenic is present in the environment in very minute amount but its concentrations intensifies due to use of certain other products having arsenic in it such as wood preservatives, pesticides, and in processes of making coal and smelting operations (WANG; MULLIGAN, 2006). World Health Organization (WHO) has suggested that the maximum limit of arsenic concentration in drinking water is 10µg per liter and above the limits the metalloid, pose serious health effects (DOPP et al., 2004). For providing safe drinking water, it is essential that it should be free from any harmful component (WANG; MULLIGAN, 2006).

Numbers of physicochemical methods are known to remove arsenic from the contaminated site, but these methods have limitations including high expenditure of the material, sludge formation, need of high energy and the dumping site problems. For the arsenic contaminated soil and ground water, bioremediation show great potential due to its environment friendliness and cost effective approach as compared to other physicochemical methods. Microbes rely on number of methods such as absorption, methylation, redox reaction and complexation to remove arsenic from contaminated soil and ground water (STOLZ; OREMLAND, 1999; SANTINI et al., 2000; ZOBRIST et al., 2000; NIGGEMYER et al., 2001; SILVER; PHUNG, 2005; RHINE et al., 2006; FRANCHI et al., 2019). Gram negative and Gram positive bacteria contain an enzyme oxidase that converts arsenite to arsenate (SANTINI; VANDEN HOVEN, 2004). Bioconversion of arsenite to arsenate has been reported from other bacteria such as *Sulfurosprillium barnesii* (ZOBRIST et al., 2000), *Pseudomonas arsentioxidans* NT-26 (SANTINI et al., 2000), *Thermus thermophilus* and *Thermus aquaticus* (GIHRING et al., 2001), *Herminiimonas arsenicoxydans* (MULLER et al., 2007), *Sphingomonas, Caulobactor, Rhizobium and Pseudomonas* (MACUR et al., 2001). From Asia

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different countries such as Bangladesh, India, Nepal, Vietnam, China and Myanmar are facing great threat to public health because of arsenic contamination in the environment. Following arsenic crisis in most of the developing countries there is need for monitoring presence and limiting arsenic contamination in drinking water. According to WHO, about 20% of the population of Punjab and 36% population of Sindh is exposed to arsenic contamination above limits (ARAIN et al., 2009; ARSHAD et al., 2015; ARSHAD; IMRAN, 2017).

The present study aimed to isolate arsenic resistant bacterias from the arsenic contaminated soil and water.

MATERIAL AND METHODS

Sampling

Arsenic detoxifying bacteria were isolated from arsenic contaminated water and soil samples of Lahore and Kasur. Sites for sample collection were Canal water, sewage water and drinking water.

Isolation of Arsenic Resistant Bacteria

Specific media was prepared to isolate arsenic resistant bacteria. For that sodium arsenite stock (100mM) was mixed with Luria Britani agar (LB) to obtain 10mM concentration (SALAM et al., 2009). Media plates were inoculated with the 0.1ml of the sample dilutions (10^{-2}-10^{-8}) and were incubated at 37˚C for 24h. Colony morphology of the bacteria was observed and pure cultures were preserved as 80% glycerol stocks at -20°C.

Screening for Arsenic Resistant Bacteria

Bacterial isolates were assessed for their arsenic tolerance at different arsenic (10mM, 50mM, 100mM and 200mM) concentrations. For that the inoculum for each strain was made in the lowest concentration of sodium arsenite i.e., 10mM. LB broth of varying arsenic concentrations were inoculated and incubated at 37˚C for 24h. After the incubation period growth was measured at 600 nm absorbance using a UV/Vis spectrophotometer (Hitachi, US 2800 spectrophotometer) (ESCALANTE et al., 2009).

Optimization of Growth Physicochemical Conditions

Effect of physicochemical conditions on bacterial resistance to arsenic was assessed by observing the growth at different pH and temperature. Different temperature studied were room temperature RT (30°C), 37°C and 45°C and pH range used were 6.0, 7.0 and 8.0. Bacterial culture in LB broth was provided with different physico-chemical conditions and incubated for 24hr. Effect of different parameters was observed by measuring the absorbance of culture at 600 nm (CHANG et al., 2007).

Estimation of Arsenic Resistance for the Selected Bacterial Isolates

For determination of arsenic concentration under optimum conditions, the medium containing 80mM, 100mM and 120mM of sodium arsenite with pH 7 was inoculated with 0.1ml of freshly prepared culture of bacterial isolates 10^6 cfu/ml at 37°C for 24h and absorbance was measured at 600 nm using a UV/Vis spectrophotometer (Hitachi, US 2800 spectrophotometer). The experiment was performed in triplicates.

Determination of Arsenic Accumulation by the Isolates

Arsenic bio-accumulation for the bacterial isolates was performed according to the method (KOSTLE et al., 2004). About 50ml of the media having 80mM sodium arsenite was inoculated 10^6 cfu/ml in 250ml flask under optimum conditions (37˚C, pH 7 for 5 days). About 5mL the culture was withdrawn daily to determine the growth and arsenic bio-accumulation. For analyzing the bio-accumulation of arsenic $KMnO_4$ test was performed where oxidation determines the arsenic speciation. The culture was centrifuged at 14,800×g for 5min, supernatant was removed and cell pellets were re-suspended in distilled water and again centrifuged at 14,800×g for five minutes. The supernatant was discarded and cell pellets were dried at room temperature for 2 days. Pellets were dissolved in HNO_3 and volume was made 10ml by adding distilled water then filtered and arsenic analysis was done through atomic absorption Perkin Elmer, Analyst 800 (HATCH; OTT, 1968).

Investigation of Redox Properties

Redox properties were investigated according to the method of SALMASSI et al. (2002). The selected strains were grown in minimal media with As III for 48h at 25˚C. To 5ml of the culture 60µl of $KMnO_4$ (0.01M) was added. Development of pink color indicates the arsenate whereas yellow or orange color indicates arsenite.

Statistical Analysis

All the experiments were performed in triplicates. The analysis of data was done through one way analysis of variance (ANOVA) by applying

Duncan´s multiple comparison to determine the significant difference between parameters.

RESULTS AND DISCUSSION

The present study was undertaken to isolate bacteria having ability to bio-accumulate arsenic or to convert arsenite to nontoxic arsenate. For this isolates having arsenic resistance were isolated from water and soil samples of arsenic contaminated areas of Lahore and Kasur. Eleven different bacterial isolates i.e., FAs 1-11 were isolated on medium comprising of sodium arsenite (10mM) (Fig. 1A-D). These bacterial isolates were arsenic resistant because of their ability to grow and tolerate arsenic in the medium. Previous studies have also reported isolation of arsenic resistant bacteria from arsenic contaminated area (CHANG et al., 2007; VALENZUELA et al., 2009; TURPEINEN et al., 2004).

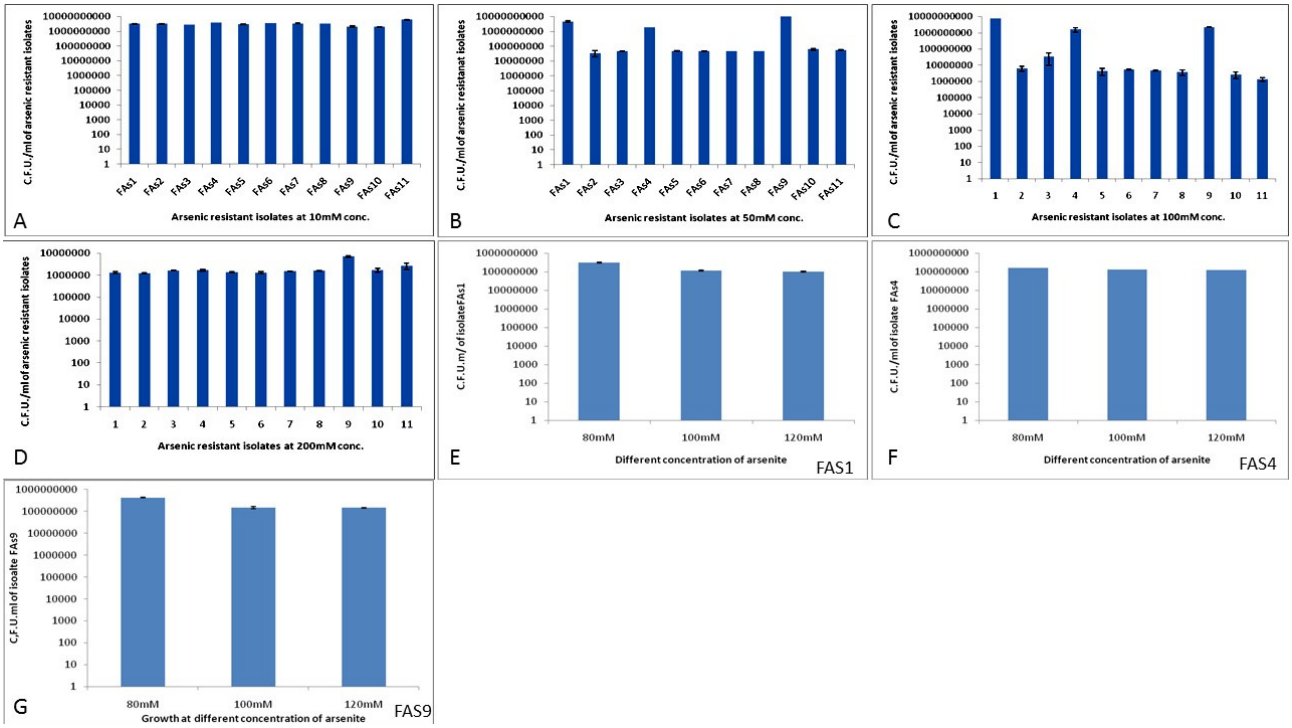

Figure 1. Screening of arsenic resistant isolates in media containing different concentrations of sodium arsenite (A) Growth measured in C.F.U./ml of the isolates at 10mM sodium arsenite concentration (B) Growth measured in C.F.U./ml of the isolates at 50 mM sodium arsenite concentration (C) Growth measured in C.F.U./ml of the isolates at 100 mM sodium arsenite concentration (D) Growth measured in C.F.U./ml of the isolates at 200 mM sodium arsenite concentration (E) Growth measured in C.F.U./ml of the isolate FAs1 at different concentrations of sodium arsenite (F) Growth measured in C.F.U./ml of the isolate FAs4 at different concentrations of sodium arsenite (G) Growth measured in C.F.U./ml of the isolate FAs9 at different concentrations of sodium arsenite

Bacterial morphological analysis revealed that the isolates FAs 1, FAs 4 and FAs9 were rod shaped, Gram negative and non endospore former. Arsenic resistance in Gram positive and Gram negative bacteria has been known, is due to the arsenic resistance operon (CERVANTES et al., 1994). According to AKSORNCHU et al. (2008) Gram negative bacteria are able to resist toxic metals more due to the presence of two layers of cell membrane. Three isolates FAs 1, FAs 4 and FAs9 were able to grow at different concentrations of sodium arsenite (Figure 1E-G). In some previous studies the bacterial isolates were able to resists concentration upto10 to 400mM of arsenate and arsenite (JACKSON et al., 2001; ANDERSON; COOK, 2004). According to AKSORNCHU et al. (2008) bacteria showed resistance to heavy metals in order to protect the component of their cell. The effect of different temperature and pH on tolerance to arsenic showed that the isolates FAs 1, FAs 4 and FAs9 gave maximum growth at 37˚C at 50mM concentration of sodium arsenite (Figure 2A-G).

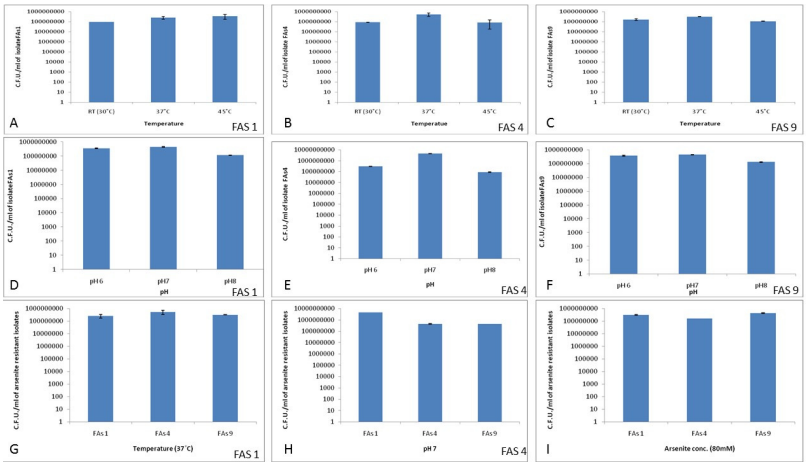

Figure 2. Optimization of physicochemical parameters for the potential arsenic resistant isolates (A) Arsenic tolerance measured as growth for isolate FAs1 at different temperatures (B)Arsenic tolerance measured as growth for isolate FAs4 at different temperatures (C) Arsenic tolerance measured as growth for isolate FAs9 at different temperatures (D) Arsenic tolerance measured as growth for isolate FAs1 at different pH (E) Arsenic tolerance measured as growth for isolate FAs4 at different pH (F) Arsenic tolerance measured as growth for isolate FAs9 at different pH (G) Comparison of potential isolates having arsenic tolerance by measuring growth as C.F.U./ml at 37˚C (H) Comparison of potential isolates having arsenic tolerance by measuring growth as C.F.U./ml at pH 7 (I) Comparison of potential isolates having arsenic (80mM) tolerance by measuring growth as C.F.U./ml under optimum conditions.

However most of the bacteria reported in previous studies showed tolerance to arsenic at 30˚C (PRITHVIRAJ et al., 2001; SALTIKOV; OLSEN, 2002; ORDONEZ et al., 2005). Whereas in the present study, culturing at different pH the isolates FAs 1,FAs 4 and FAs9 showed maximum tolerance to arsenic (50mM) at pH 7 by presenting maximum growth. Relation of pH to arsenic tolerance can be attributed to arsenic resistance gene expression at pH ranging from 5 to 7 for the bacteria (CHANG et al., 2007). Whereas in another study by Mondal et al. (2008), optimal removal of arsenic employing bacteria was at pH 6 to 7 from the contaminated water. Arsenic resistance under optimum conditions was estimated for the isolates FAs 1, FAs 4 and FAs9 by growing them on different concentrations of arsenite (Figure 2I, Figure 3A, and B). Increased growth was observed for the isolates FAs 1, FAs 4 and FAs9 at 80mM concentration of arsenite (Figure 2I).

Although bacteria showed maximum growth at 80mM concentration but the isolates also showed significantly less growth at higher concentration indicating their tolerance level. But the ability to resist the arsenite concentration varied between these isolates. According to a study conducted by Srinath et al. (2002) and Zouboulis et al. (2004), the resistance conferred by the microorganisms against the toxic metal was due to the environmental factors i.e., pH, soil and the organic matter present in the soil as well as through a number of resistance mechanisms such as adsorption at the surface of microorganism, conversion into other form through microbial redox reactions. Anderson; Cook (2004) isolated bacteria that were able to tolerate arsenite concentration from 0 to 20mM and arsenate concentration from 0 to 100mM. Ordonez et al. (2005) found that the bacterial isolate *Corynebacterium glutamicum* was able to resist 12mM concentration of arsenite and 500mM concentration of arsenate through its resistance system of arsenic and was found to be the most arsenic tolerant bacterium. Jareonmit et al. (2010) found that the isolated bacterial isolate from the different arsenic contaminated area were able to show resistance against arsenite up to 1000mg/L.

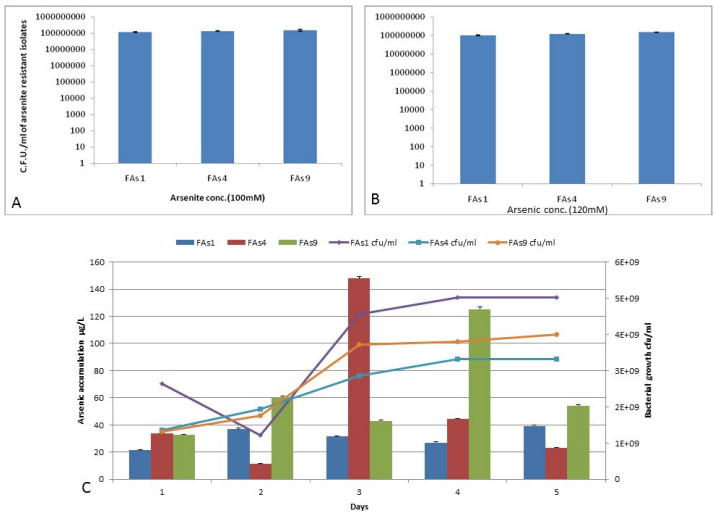

Figure 3. Comparison of arsenic tolerance among potential isolates at different arsenic concentrations (A)Comparison of potential isolates having arsenic (100mM) tolerance by measuring growth as C.F.U./ml under optimum conditions (B) Comparison of potential isolates having arsenic (120mM) tolerance by measuring growth as C.F.U./ml under optimum conditions (C) Arsenic bio-accumulation observed for up to 5 days under optimum conditions.

The bioconversion ability of isolates revealed that the FAs 1, FAs 4 and FAs9 were unable to oxidize arsenite to arsenate. According to a study conducted by Escalante et al. (2009) that out of 49 isolates, 16 bacterial isolates were unable to oxidize the arsenite to arsenate. According to Jackson; Dugas (2003) *arc C* genes were responsible for arsenate reduction which was encoded by enzyme reductases. Bio-accumulation of arsenic was analyzed for up to 5 days for the isolates FAs 1, FAs 4 and FAs9 in medium containing 80mM of arsenite. Bacterial isolates FAs 1 showed maximum growth at day 4 while isolate FAs 4 and FAs 5 displayed maximum growth on day 5 (Figure 3C). These isolates (FAs 1, FAs 4 and FAs9) were found to accumulate arsenite up to 39.16, 148 and 125µg/L in their cells, respectively. The FAs 1 accumulated maximum arsenite at day 5thwhereas FAs 4 and 9 were able to accumulate maximum on day 3rd and 4th, respectively. According to the research conducted by Takeuchi et al. (2007), bacterial isolates were able to accumulate 2290µg arsenic/gram from the medium containing 5mg of arsenite. The results showed the potential of these bacteria to have application in bio-accumulation of arsenic from arsenic contaminated environment.

CONCLUSION

Arsenic resistant bacterias were isolated from soil and water samples of the arsenic contaminated areas of Lahore and Kasur. These bacteria were able to tolerate high concentration of arsenic and were able to accumulate arsenic from their environment. In the future, these bacteria can be employed to remove arsenic from the contaminated environment which is not only environment friendly but also cost effective as compared to the other physical and chemical method and hence play an important role in the process of bioremediation.

RESUMO: A industrialização adicionou arsênico metalóide extremamente tóxico ao ambiente que, em alta concentração, ameaça severamente a biota. Naturalmente, alguns micróbios possuem a capacidade de bio-acumular metais e também transformar arsenito (As III) uma forma tóxica a um arsenato não-tóxico Como V. O presente estudo visa o isolamento de bactérias resistentes ao arsênico do solo contaminado com arsênico e água . Entre onze isolados bacterianos, três FAs 1, 4 e 9 exibiram tolerância à concentração de 100 mM de arseatura de sódio, obtendo crescimento de 7,48 × 10^9,157 × 10^9 e 2,23 × 10^9 C.F.U./ml, respectivamente. Otimização em diferentes condições como temperatura, pH e concentração de arsênio revelaram alta tolerância ao arsênico do isolado FAs 4 (5,33 × 10^8) a 37 ° C e FAs 1 (4,43 × 10^8 UFC / ml) em pH 7. Resistência ao arsênico em condições ótimas para as cepas bacterianas FAs 1, FAs 4 e FAs 9 apresentaram crescimento

máximo na concentração de 80 mM de arsenito. Estes isolados bacterianos não mostraram capacidade redox para oxidar o arsenito As III para arseniar como V. No entanto, os isolados bacterianos FAs 1, FAs 4 e FAs 9 foram capazes de acumular arsênico 39,16, 148 e 125 µg / L no 4º, 3º e 5º dia de incubação, respectivamente. Os isolados FAs 1, FAs 4 e FAs 9 foram identificados como bastonetes gram-negativos não endoscópicos. No futuro, esses novos isolados possuem um grande potencial no campo da biotecnologia, já que a biorremediação de solo e água contaminados com arsênico pode ser feita empregando-se bactérias que acumulam arsênico, o que é um método ecologicamente correto e econômico.

PALAVRAS-CHAVE: Contaminação por arsênico. Biorremediação de arsênio. Biodegradação de arsênio. Bioacumulação.

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