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Original Research Article

Isolation and Characterization of Arsenic Resistant Bacteria Isolated from Arsenic Polluted Area

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A B S T R A C T

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An experiment was conducted to isolate and characterize arsenic resistant bacterial isolates. In this study, soil and water samples were collected from arsenic contaminated areas of Raichur and Yadagir district. Twenty nine arsenic resistant bacterial isolates were isolated from soil and water sample also screened for the efficiency to resist arsenic. From them, twelve isolates were finally selected and studied for their morphological, biochemical and beneficial characteristics in detail. The isolates were identified as *Pseudomonas* sp. and *Bacillus* sp. tentatively.

Introduction

Arsenic (As) is a toxic metalloid particle, available ubiquitously in earth's crust. In addition, natural processes such as weathering of rocks and volcanic emissions as well as human activities like mining, combustion of fossil fuels, smelting of ores or the application of arsenical herbicides, pesticides and wood preservatives are the sole sources leading to As contamination in the environment (Smedley and Kinniburgh, 2002).

Arsenic mainly occurs in two inorganic forms, arsenate As (V) and arsenite As (III) (Neff, 1997). Contamination of terrestrial and aquatic ecosystems by arsenic (As) is a very sensitive environmental issue due to its adverse impact on human health. The level of arsenic accumulation in environment dramatically increased over the years that cause increasing soil contamination and thereby enter the food chain through biomaginification. It is the only known human carcinogen for which there is an adequate evidence of carcinogenic risk by both inhalation and ingestion, IARC, Monographs (2004). Acute exposure to high levels of inorganic arsenic by humans can be fatal while acute exposure to lower levels can result in vomiting, decreased production of red and white blood cells, abnormal heart rhythm, and damage to blood vessels. Skin lesions are a common sign of arsenic poisoning (Smedly *et al*., 2002). Chronic exposure especially damages the liver and leads to cirrhosis. It is also a neurotoxin, damaging peripheral and central nervous systems (Mazumder *et al*., 1999). At cellular level, arsenic has been shown to induce chromosomal aberrations. However, the results of genotoxicity studies have indicated that it is not mutagenic. It has been suggested that arsenic might interfere with the DNA repair system or DNA methylation state, inhibition of p53 and telomerase activities (Chou *et al*., 2001). Oxidative stress,

promotion of cell proliferation and signal transduction pathways leading to the activation of various transcription factors (Wang *et al*., 2001). Therefore, removal of arsenic from environment is of great significance to local agriculture and the population.

The conventional techniques such as chemical precipitation, chemical oxidation and reduction, ion exchange, filtration, reverse osmosis have been employed for the removal of arsenic (Malik, 2004). The disadvantage of these methods is that they are not accurate, particularly when these toxic heavy metals are present in very low concentration (Chaalal *et al*., 2005). The greater public awareness of arsenic poisoning in animal and human nutrition has been a growing interest in developing regulatory guidelines and remediation technologies for mitigating arsenic-contaminated ecosystems (Mahimairaja *et al*., 2005). The other drawbacks associated with the present techniques include secondary environmental pollution due to the chemicals used in the remediation process and the cost of the prevailing techniques.

Bioremediation is an option that offers the possibility to destroy contaminants or render them innocuous using natural biological activity. microorganisms play a major role in the biochemical cycle of arsenic and can convert to different oxidation states with different solubility, mobility and toxicity (Silver and Phung 2005).

Certain microorganisms in nature have evolved the needed genetic mechanism, which enable them to survive and grow in an environment containing toxic levels of arsenic. These microorganisms can be potentially used for the bioremediation of arsenic contaminated area.

Materials and Methods

Isolation of arsenic resistant bacteria from soil and water sample

Arsenic resistant bacteria were isolated from the collected soil and water samples using enrichment culture technique. The soil samples were serially diluted and plated on minimal salt agar plates amended with 10 mg L^{-1} of sodium arsenite. The plates were incubated at room temperature (30±1 °C) for 2 days. The colonies exhibiting proper growth on the plates were selected and purified by four-way streak plate method. After careful selection of single colony further purified by streak plate method on fresh minimal agar plates. These purified cultures of bacteria were maintained on agar slants of minimal medium for further studies.

Morphological characterization

The selected bacterial isolates were morphologically characterized by observing cell shape, motility and gram reactions were carried out as per the standard procedures given by Bartholomew and Mittewer (1950).

Biochemical characterization

The selected bacterial isolates were biochemically characterized for the following tests such as Gelatin liquefaction, Catalase test, Voges Prausker's test, Starch hydrolysis, Urease test, Indole acid test, Methyl red test, Citrate utilization test and Nitrate reduction test using standard procedure.

In vitro screening of efficient arsenic resistant bacterial isolates for their other beneficial attributes

Test for IAA production

Indole acetic acid production was quantitatively measured according to Gordon

and Weber (1951). Bacterial test cultures were grown in a minimal broth amended with tryptophan (5 mM) for 3-4 days. Cultures were centrifuged at 10,000 rpm for 20 min. Two ml of supernatant was mixed with two drops of orthophosphoric acid and 4 ml of Salkowaskis reagent. Tubes were incubated at room temperature for 25 minutes. The intensity of pink colour was read at 530 nm spectrophotometrically and the amount of IAA produced was extrapolated from the standard curve. Standard curve was prepared by plotting absorbance at 530 nm against concentration of IAA solution (Gordon and Weber, 1951).

Siderophore production

Siderophores assay will be carried out based on the CAS shuttle assay of Payne (1994). The culture extract (0.5 ml) will be mixed with 0.5 ml of CAS reagent. The color obtained will be measured using the spectrophotometer at 630 nm after 20 min of incubation. The blank will be prepared using uninoculated broth medium.

Oxidation and reduction of arsenic by the isolates

The ability of bacterial isolates to oxidize As (III) or reduce As (V) was tested by the usage of silver nitrate ($AgNO_3$). Separate YEM agar containing 1mM sodium arsenite and 5 mM sodium arsenite was used for the determination of As (III) oxidation or As (V) reduction respectively. 0.1 ml of aliquot of efficient arsenic resistant bacterial isolates were transferred on these plates and kept for incubation for 5 days at 30 ^{0}C. After incubation period, the agar plates were flooded with 0.1 M $AgNO_3$ solution. The reaction between $AgNO_3$ and As (III) or As (V) resulted in the formation of a colored precipitate. If the media turned brown, it confirms the presence of silver arsenate and if it turns yellow, the presence of silver arsenite was confirmed.

Results and Discussion

Isolation and purification of arsenic resistant bacteria

Twenty-nine arsenic resistant bacteria were isolated from different soils and water of arsenic contaminated areas. After 48 hr of incubation at 30 ^{0} C, observed a profused growth of the bacterial colonies on MSM agar plates supplemented with 10 mg/kg of sodium arsenite as sole source of nutrition. The bacterial isolates which showed prolific growth and distinct colony morphology were picked up and purified by repeated streaking on minimal agar plates. The purified isolates thus obtained were maintained on agar slants prepared with nutrient medium and codes given for the isolates were ASR-1 to ASR-29.

Morphological characterization

The selected isolates were studied for cell shape, motility and gram reactions under microscope and the results were shown in table 1. From the observation Among 12 rod shaped isolates 7 found to be gram positive and 5 found to be gram negative.

Biochemical characterization

The selected isolates were studied for biochemical tests *viz.*, Gelatin liquefaction, Catalase test, Voges Prausker's test, Starch hydrolysis, Urease test, Indole acid test, Methyl red test, Citrate utilization test and Nitrate reduction test were carried out for all the 12 isolates and results obtained for these biochemical tests have been presented in Table 2.

Table.1 Morphological characteristics of arsenic resistant bacteria isolated from soil and water samples of arsenic contaminated area

Sample	Isolate code	Colony morphology on minimal medium	Gram reaction	Cell shape	Fast/Slow growers	Motility
1	ASR-1	White, Creamy, Smooth	G -ve	Rod	Fast grower	Motile
2	ASR-2	Dull white, large, irregular	G +ve	Rod	Fast grower	Motile
3	ASR-3	Dull white, large, irregular	G +ve	Rod	Fast grower	Motile
4	ASR-5	Dull white, large, irregular	G +ve	Rod	Fast grower	Motile
5	ASR-7	White, Creamy, Smooth	G -ve	Rod	Fast grower	Motile
6	ASR-9	Dull white, large, irregular	G +ve	Rod	Fast grower	Motile
7	ASR-12	Dull white, large, irregular	G +ve	Rod	Fast grower	Motile
8	ASR-14	White, Creamy, Smooth	G -ve	Rod	Fast grower	Motile
9	ASR-17	White, Creamy, Smooth	G -ve	Rod	Fast grower	Motile
10	ASR-23	Dull white, large, irregular	G +ve	Rod	Fast grower	Motile
11	ASR-25	White, Creamy, Smooth	G -ve	Rod	Fast grower	Motile
12	ASR-26	Dull white, large, irregular	G +ve	Rod	Fast grower	Motile

Table.2 Biochemical characterization of arsenic resistant bacteria isolated from soil and water samples of arsenic contaminated areas

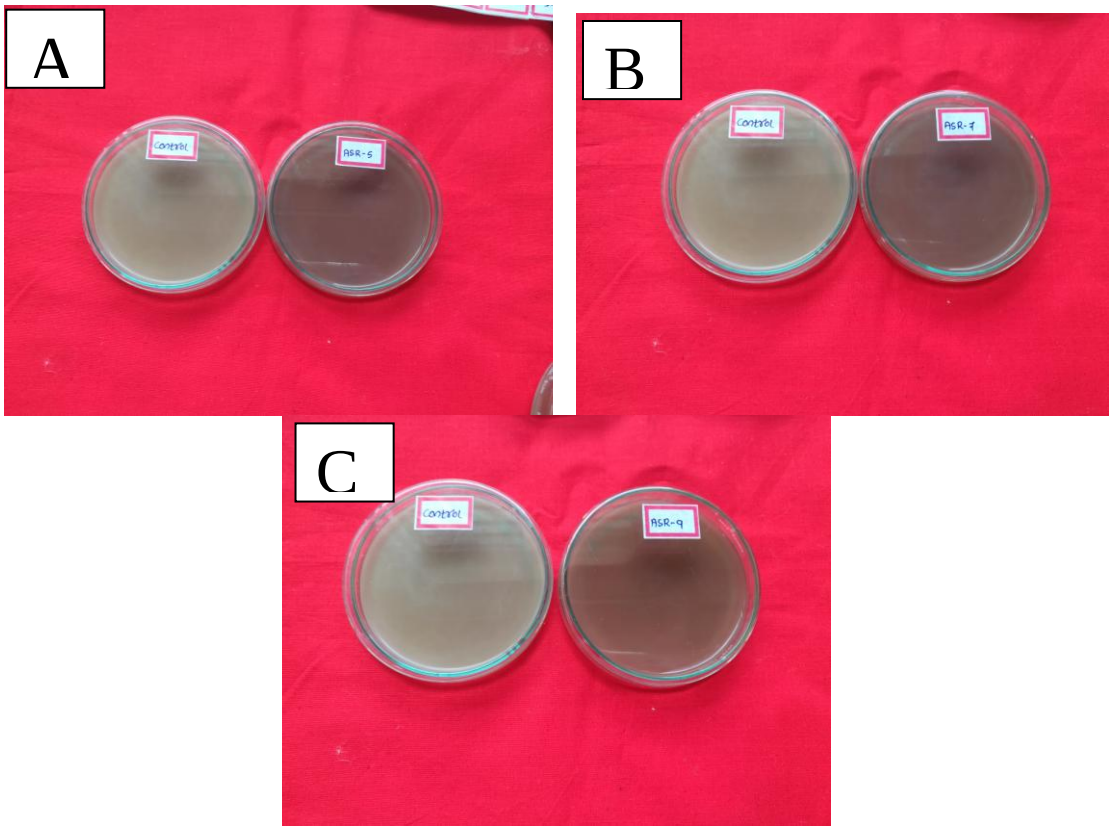
Sample	Isolate code	1	2	3	4	5	6	7	8	9	Tentative genus
1	ASR-1	-	+	-	+	-	-	+	-	+	*Pseudomonas* sp.
2	ASR-2	+	+	+	+	-	-	+	-	+	*Bacillus* sp.
3	ASR-3	+	+	+	+	-	-	+	-	+	*Bacillus* sp.
4	ASR-5	+	+	+	+	-	-	+	-	+	*Bacillus* sp.
5	ASR-7	+	+	-	+	-	-	+	-	+	*Pseudomonas* sp.
6	ASR-9	+	+	+	+	-	-	+	-	+	*Bacillus* sp.
7	ASR-12	+	+	+	+	-	-	+	-	+	*Bacillus* sp.
8	ASR-14	-	+	-	+	-	-	+	-	+	*Pseudomonas* sp.
9	ASR-17	+	+	-	+	-	-	+	-	+	*Pseudomonas* sp.
10	ASR-23	+	+	+	+	-	-	+	-	+	*Bacillus* sp.
11	ASR-25	+	+	-	+	-	-	-	-	+	*Pseudomonas* sp.
12	ASR-26	-	+	+	+	-	-	-	-	+	*Bacillus* sp.

NOTE: 1.Gelatin liquefaction test 2.Catalase test 3.Voges-Prausker's test 4. Starch hydrolysis test 5.Urease test 6. Indole acid test 7. Citrate utilization test 8.methyl red test 9. Nitrate reduction test

Table.3 IAA and siderophore production by efficient arsenic resistant bacteria isolated from soil and water samples of contaminated areas

Sl. No.	Isolates	Production of IAA Conc. (µg ml^{-1})	Production of siderophore (%)
1	ASR-1	8.56	42.94
2	ASR-2	7.63	48.42
3	ASR-3	15.62	47.08
4	ASR-5	17.03	57.95
5	ASR-7	14.25	51.65
6	ASR-9	14.53	54.56
7	ASR-12	6.34	35.64
8	ASR-14	11.11	16.85
9	ASR-17	10.11	48.93
10	ASR-23	9.56	30.97
11	ASR-25	8.36	50.28
12	ASR-26	8.56	40.63
	S.Em±	**0.80**	**0.70**
	CD at 1%	**0.20**	**0.27**

Pic.1 Arsenic oxidation (A) arsenic plate inoculated with isolate ASR-5, (B) arsenic plate inoculated with isolate ASR-7 and (C) arsenic plate inoculated with isolate ASR-9



From these biochemical and morphological tests ASR-1, ASR-7, ASR-14, ASR-17 and ASR-25 were tentatively identified as *Pseudomonas* sp. and ASR-2, ASR-3, ASR-5, ASR-9, ASR-12, ASR-23 and ASR-26 shown *Bacillus* sp.

Similarly, Oller *et al.,* (2013) reported four bacterial isolates belonging to genus *Pseudomonas,* which tested positive for catalase test.

In vitro screening of efficient arsenic resistant bacterial isolates for their beneficial attributes

In vitro synthesis of IAA

The production of IAA by bacterial isolates were analyzed *in vitro* on a minimal broth medium supplemented with tryptophan as a precursor. The results obtained are presented in Table 3. The production of IAA by the isolates varied from 6.34 µg/ml to 17.03 µg/ml of broth medium. Among all the isolates verified, the isolate ASR-5 produced high amount of IAA (17.03 µg/ml of medium) followed by ASR-9 which produced 14.53 µg/ml. The lowest IAA produced by ASR-12 (6.34 µg/ml of medium). Similar findings were seen by Das and Sarkar (2018).

Siderophore production

The Chrome Azurol Sulfonate (CAS) assay was used for the detection of siderophore production. The results obtained are presented in Table 3. All the isolates were examined for their ability to produce siderophore on CAS medium. The production of siderophore by the isolates varied from 16.85 % to 57.95 % of broth medium. Among all the isolates verified, the isolate ASR-5 produced high amount of siderophore (57.95%) followed by ASR-9 which produced 54.56 %. The lowest siderophore is produced by ASR-14 (16.85%). Similar findings were observed by Lampis *et al.,* (2015).

Oxidation and reduction of arsenic by the isolates

A qualitative silver nitrate ($AgNO_3$) screening technique was used to detect the oxidation of As [III] to As [V] or the reduction of As [V] to As [III]. When silver nitrate was mixed with the culture containing arsenate, it also turned brown (Fig.2) confirming the presence of silver arsenate. Hence it is observed that none of the bacteria has the ability to reduce arsenate to arsenite, but three of them oxidized arsenite to arsenate. Similar results was obtained by Banarjee *et al.,* (2011) used AgNO3 to detect the oxidation of As[III] to As[V] or the reduction of As [V] to As [III]. The strains RJB-2, RJB-4, RJB-5 and RJB-A demonstrated oxidizing as well as reducing abilities to arsenic.

Thus from this entire study, it can be concluded that. A total number of 29 bacterial isolates were isolated from 47 soil and water samples collected from different arsenic contaminated areas. It was observed that various species of *Pseudomonas* and *Bacillus* possess a good potential to resist arsenic effectively. The isolates also produce IAA and siderophore at higher concentration and also resistant to arsenic. All the three isolates have the unique ability to oxidize arsenite to less toxic arsenate.

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