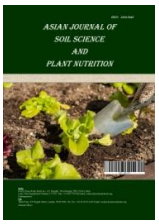

Asian Journal of Soil Science and Plant Nutrition

7(2): 41-55, 2021; Article no.AJSSPN.67025
ISSN: 2456-9682

Isolation, Morphological and Biochemical Characterization of Rhizobacteria from Arsenic Contaminated Paddy Soils in Bangladesh: An *In vitro* Study

Mohammad Mosharraf Hossain[1,2*], G. K. M. M. Rahman[2], M. A. M. Akanda[3], A. R. M. Solaiman[2], M. T. Islam[4], and M. M. Rahman[2]

[1]Department of Soil Science, Sher-e-Bangla Agricultural University, Dhaka, Bangladesh.
[2]Department of Soil Science, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur, Bangladesh.
[3]Department of Plant Pathology, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur, Bangladesh.
[4]Department of Biotechnology, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur, Bangladesh.

Authors' contributions

This work was carried out in collaboration among all authors. Author MMH designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors GKMMR, ARMS and MMR managed the analyses of the study. Authors MAMA and MTI managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/AJSSPN/2021/v7i230110
Editor(s):
(1) Dr. Paola Angelini, University of Perugia, Italy.
Reviewers:
(1) Ali Athafah Tomah, University of Misan, Iraq.
(2) Dionisio S. Bucao, Mariano Marcos State University, Philippines.
Complete Peer review History: http://www.sdiarticle4.com/review-history/67025

Original Research Article

Received 20 January 2021
Accepted 24 March 2021
Published 26 March 2021

ABSTRACT

Soil-plant–microbes relations within the plant rhizosphere are the determinants of plant and soil health, which is important for soil ecological environment for plant-microbe interactions. Plant growth-promoting rhizobacteria (PGPR) are considered to encourage plant growth and development directly or indirectly in soil. PGPR can demonstrate a diversity of characteristics responsible .for influencing plant growth and development. During this study, Twenty four different

**Corresponding author: Email: mosharraf6791sau@sau.edu.bd;*

bacterial isolates were isolated, and detailed morphological, biochemical, and physiological characterizations of those isolates were accomplished. This experiment was performed with the 24 bacterial isolates to see their gram stain test, KOH test, catalase activity, cellulose degradation capability, in dole acetic acid (IAA) production, and phosphate solubilization activities, and also tested for growth within the different arsenic and salt stress conditions and 37°C temperature. Results revealed that among the rhizobacterial isolates, fifteen bacterial isolates were negative and nine was positive in gram reaction, while some were showed high IAA production ability, phosphate solubility capability, and cellulose degradation capacity within the culture media. The isolates were isolated from paddy soils and a few were characterized by a yellow color, flat elevation, and gram-positive, while some were characterized because of the yellowish color with round colony shape, raised elevation, gram-negative, and every one the isolates were positive in catalase production capacity and phosphate solubilization activity which is able to increase the available phosphorus within the soil for plants and also produced indole acetic acid that may use as a hormone to be used in growth enhancement of plants. Hence, these isolates need to be tested further for their effect on arsenic dynamics at the plant rhizosphere, selection of suitable plant species for the bacterial association, bacterial effect on arsenic uptake by plants, and potentials for field applications for sustainable agriculture.

Keywords: Rhizobacteria; paddy soil; arsenic; Bangladesh.

1. INTRODUCTION

Worldwide arsenic (As) toxicity poses a serious health risk to a lot of people [1]. Arsenic is a toxic pollutant released into the environment either by natural phenomena or anthropogenic activities. Arsenic is a poisonous contaminant released into the environment either by natural or anthropogenic action. Arsenic was found to be abundant in rocks, soil, water, sediments, and air. Soil-plant-microbe interactions are complex and known to influence plant health and productivity. Bangladesh is a delta of high arsenic contamination in groundwater and therefore the water is widely used for irrigation. Arsenic uptake and accumulation by a plant are considerably predisposed by arsenic concentration within the soil or growth medium and significantly increased with rising As levels [2]. Plants can develop toxicity symptoms like inhibition of seed germination [3]; decrease in plant height [2,4,5] depressed tillering [6]; reduction in root growth [7,5]; decrease in shoot growth [7] lower fruit and grain yield [4] and reduction in photosynthesis rate while they are exposed to excess As either in soil or in solution culture. A wide variety of microorganisms is capable of growth within the presence of heavy metal ions and tolerates high concentrations [6]. Several bacteria belonging to the genera *Acidithiobacillus*, *Bacillus*, *Deinococcus*, *Desulfitobacterium*, and *Pseudomonas* [8] have been reported to be resistant to arsenic. Anderson and Cook [9] have depicted that the strains of *Aeromonas*, *Exiguobacterium*, *Acinetobacter*, *Bacillus*, and *Pseudomonas*, which can be resistant in elevated concentrations of arsenic species (up to 100 mM arsenate or up to 20 mM arsenite). During the past couple of decades, the employment of plant growth-promoting rhizobacteria (PGPR) for sustainable agriculture has increased tremendously in various parts of the planet. Significant increases in growth and yield of agronomically important crops in response to inoculation with PGPRs have been repeatedly reported [10]. By geomicrobiological processes, several microbes have been isolated and identified which will transform arsenic species like from arsenate to arsenite and contrariwise [11]. Growth-promoting microbes possessing arsenic-resistant genes can be used to stimulate the growth of the crop plants in arsenic-affected soils. Selection of efficient As-resistant bacteria and inoculation into the seeds and/or roots of suitable plant species will widen the perspectives of bioremediation of soils laid low with arsenic [12]. The importance of metal/metalloid-resistant bacteria possessing PG traits are often enormous in metal/metalloid-contaminated soils since these bacteria can increase the tolerance of plants against abiotic stress, stimulate plant growth, and contribute in this way to accelerated remediation of contaminated soils [13,14]. Heavy metal-resistant and plant growth-promoting bacteria (PGPB) have been reported to extend the uptake efficiency and enhancement of metal translocation, promote growth and increase heavy metal tolerance in plants [13,14,12]. The rhizobacteria secrete metabolites that help plant growth like auxins [15], cytokinins [16] and gibberellins [17] yet as from first to last the

solubilization of phosphate minerals in soils. Plant growth-promoting rhizobacteria controlling disease through the assembly of antifungal metabolites like indole-3-acetic acid (IAA), indole-3-acetamide (IAM) pathway, indole-3-pyruvate (IPyA) pathway, methionine-S-adenosylmethionine (SAM), 1-aminocyclopropane-1-carboxylate (ACC), 1-aminocyclopropane-1-carboxylate synthase (ACS), phosphatase (Ptase), ammonia (NH3), hydrogen cyanide (HCN) [13]. Additionally to the development of plant growth, PGPR is directly involved in increased uptake of nitrogen, synthesis of phytohormones, solubilization of minerals like phosphorus, and production of siderophores that chelate iron which will be available for the growth of plant root [18]. It has also been reported that PGPR can solubilize inorganic and/or organic phosphates in soil [19]. Being one among the foremost severely arsenic-affected countries within the world, Bangladesh faces the challenges of producing arsenic-safe agricultural products. Due to the scarcity of agricultural land, there is no alternative but to grow crops in the arsenic-affected soils by following appropriate techniques to attenuate the toxicity. Bioremediation of arsenic within the agricultural soils of Bangladesh has the potential to challenge this problem from the priority of sustainability. However, there is no substantial report available on the sensible application of bioremediation in Bangladesh. From the abovementioned circumstances, the following experiment was undertaken to isolate, morphological and biochemically characterize the rice rhizospheric bacteria from arsenic-contaminated paddy soils.

2. MATERIALS AND METHODS

The experiment was conducted at the Soil Microbiology Laboratory of the Department of Soil Science at Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur, Bangladesh during the period from January 2016 to June 2016 to fulfill the abovementioned objective. The materials used and method logies adopted for the research works are elaborately described below.

2.1 Geographical Location of the Soil Sampling Sites

Soil samples were collected from different sites of arsenic-contaminated paddy fields of Shahrasti Upazila of Chandpur district of Bangladesh located in between 23°08' and 23°17' North latitudes and in between 90°51' and 91°02' East longitudes within the 23°008´ to 23°308´ North latitude and from 90°328´ to 91°028´ East longitude [20] of Chandpur District in Bangladesh (Fig. 1). The latitude and longitudes (GPS reading) of the sampling sites were recorded (Table 1).

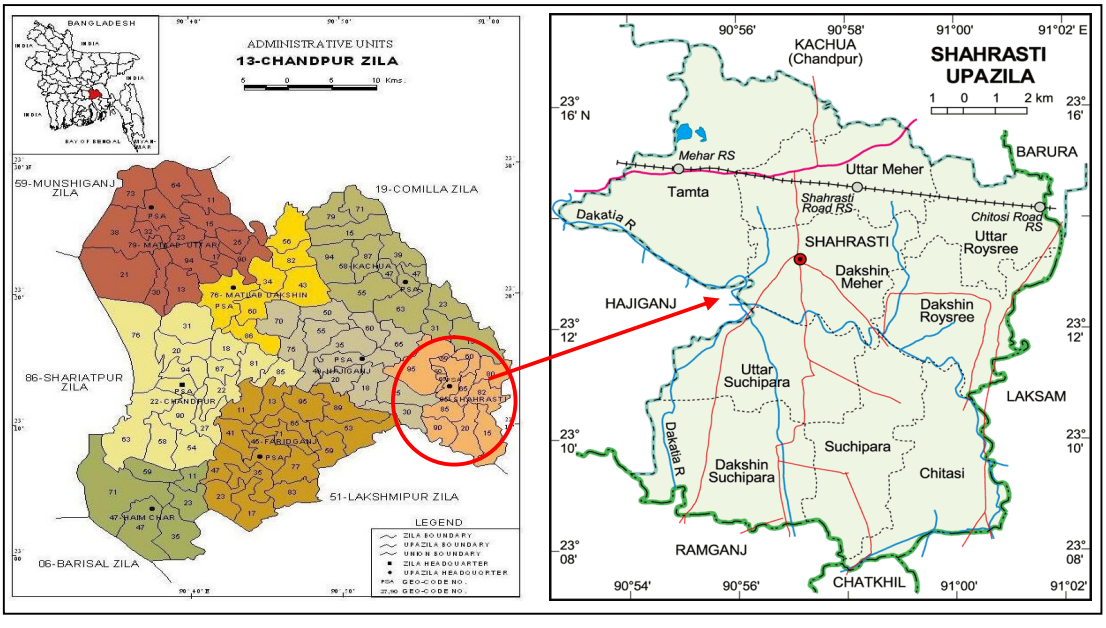

Fig. 1. Geographical location map of soil sampling sites

Table 1. Biochemical properties of collected soil samples

Soil Samples ID	GPS Reading		Soil pH	Organic matter (%)	Total N (%)	Available P (µg /g)	Total As (ppm)	Bacterial population ($\times 10^{-7}$ cfu/gm soil)
	Latitude	Longitude						
Sample 1	23°14′255″	090^{0}55′405″	5.66	1.81	0.12	9.69	16.87	3.3
Sample 2	23^{0}14′240″	090^{0}55′406″	5.51	1.81	0.12	9.34	16.98	2.9
Sample 2	23^{0}14′229″	090^{0}55′398″	6.10	1.85	0.11	11.09	22.23	3.9
Sample 3	23^{0}14′215″	090^{0}55′403″	5.10	1.79	0.11	12.23	23.91	4.5
Sample 4	23^{0}14′210″	090^{0}55′415″	5.66	1.82	0.11	8.34	12.45	2.9
Sample 5	23^{0}14′254″	090^{0}55′350″	5.76	1.81	0.13	7.99	12.76	4.1
Sample 6	23^{0}14′292″	090^{0}55′355″	6.33	1.76	0.12	10.39	18.89	2.3
Sample 7	23^{0}14′308″	090^{0}55′317″	5.58	1.73	0.13	13.54	22.43	2.9
Sample 8	23^{0}14′303″	090^{0}55′298″	5.82	1.67	0.10	14.65	19.45	4.8
Sample 9	23^{0}14′317″	090^{0}55′266″	5.56	1.36	0.09	18.48	31.87	4.8
Sample 10	23^{0}14′304″	090^{0}55′254″	5.50	1.75	0.08	17.97	24.23	2.5
Sample 11	23^{0}14′294″	090^{0}55′243″	5.70	1.77	0.11	17.87	28.34	2.1
Sample 12	23^{0}14′337″	090^{0}55′231″	5.99	1.57	0.10	13.45	17.98	3.6
Sample 13	23^{0}14′343″	090^{0}55′206″	5.52	1.57	0.14	11.21	12.56	3.4
Sample 14	23^{0}14′348″	090^{0}55′171″	5.67	1.68	0.13	11.02	18.99	2.2
Sample 15	23^{0}14′380″	090^{0}55′166″	6.29	1.77	0.12	11.12	17.45	3.3
Sample 16	23^{0}14′383″	090^{0}55′188″	5.82	1.67	0.13	17.65	23.78	3.1
Sample 17	23^{0}14′378″	090^{0}55′202″	5.91	1.78	0.08	21.32	32.76	3.3
Sample 18	23^{0}14′390″	090^{0}55′225″	6.48	1.70	0.09	18.97	27.09	3.9
Sample 19	23^{0}14′387″	090^{0}55′291″	5.51	1.70	0.11	19.49	25.34	2.4
Sample 20	23^{0}14′363″	090^{0}55′247″	5.60	1.69	0.13	16.79	21.34	3.9

2.1.1 Collection of soil samples

Soil samples were collected from the rice rhizosphere of different sites of arsenic contaminated paddy fields randomly and carefully labeled and put in zip lock bags, wrapped in foil paper, and transported to the laboratory, and stored in a refrigerator at 40°C temperature for culturing the bacteria. All the soil sampling points were geo-referenced with the GPS tool. A portion of each sample was mixed, transferred to the laboratory, sun-dried and passed through a 2 mm sieve, and used for subsequent physical-chemical and microbial analysis.

2.1.2 Isolation of rhizobacteria from collected soil samples

From each soil sample, 1 g of soil was suspended in 9 ml of double-distilled water and vortex for 3 minutes. The resulting suspensions were serially diluted to 10-10. Each dilution (0.1 ml) was spread over a pre-solidified Nutrient Agar (NA) media plates (Beef extract- 10.0 g, NaCl 5.0 g, Peptone 10.0 g, Distilled water 1.0 L, Agar 20.0 g, Adjust pH to 7.0-7.5). The rhizobacterial isolation process a solid nutrient rich agar medium was used and each media was autoclaved at 121°C with 15 psi for 20 minutes before inoculation. After inoculation the samples were spread over with the help of a sterile spreader and then incubated in an incubator at 28°C for 2 days. Growth was monitored daily and single colonies were picked up with a sterile toothpick and sub-cultured over the pre-solidified NA media. All the subsequent *In vitro* plate assay analyses were done in triplicate until the colonies were more purified.

2.1.3 Enumeration of the bacterial population in soil samples

A serial dilution technique was used to isolate the bacterial population from the soil. From each soil sample, 1 g of soil was suspended in 9 ml of double-distilled water and vortex for 3 minutes. The resulting suspensions were serially diluted to 10-10. Each dilution (0.1 ml) was spread over a pre-solidified Nutrient Agar (NA) media plates (Beef extract- 10.0 g, NaCl 5.0 g, Peptone 10.0 g, Distilled water 1.0 L, Agar 20.0 g, adjust pH to 7.0-7.5). After 3 days of the incubation period, the CFU (colony forming unit) of the bacteria that developed on the respective agar plates was counted on an enumerator with a standard method from each dilution by the following formula.

$$\text{CFU (colony forming unit) or viable cells per gm of dry soil} = \frac{\text{Mean plate count} \times \text{Dilution factor}}{\text{Amount of dilution}}$$

Data from each replicate were averaged for a soil sample and expressed as CFU per gram of oven-dried soil.

2.2 Characterization of the Rhizobacterial Isolates

2.2.1 Colony morphology

Bacterial isolates were thus randomly selected morphologically from initial diluted agar plates before sub-culturing for purification. Recovered bacterial isolates were phenotypically (morphotypes and physiological) characterized. The colony morphology of the isolates was studied under a light microscope. This included shape, edge, elevation, surface, color, and pigmentation. Cellular morphology was based upon cell shape and gram staining. Cellular morphology and biochemical characteristics were determined based upon Bergey's Manual of Systematic Bacteriology [21].

2.2.2 Gram stain test

A little drop of bacterial suspension was taken on a clean slide. The drop was spread and allowed to dry in the air. The air-dried smear was passed once through the top of a spirit lamp and allowed to cool. Then the smear was stained with crystal violet solution for one minute and washed in tap water following the steps below. The film was immersed in iodine solution for one minute, washed in tap water, and then dried. Then ethanol was added to the film and washed after one minute in tap water and it was dried. Then a counter stain, safranine was added with the film and washed in tap water and it was dried. The color of the smear was observed under an oil immersion object in a light microscope. The color of the smear appeared violet if the bacterium was gram-positive. On the other hand, the color of the smear was turned red if the bacterium was gram-negative.

2.2.3 KOH test

This is an alternative test for Gram staining especially when strain numbers to be tested are more. A clean glass slide was taken and one drop of 3 % KOH solution was kept in the center.

Using a toothpick the bacterial strain from the agar plate is placed on the KOH drop and thoroughly mixed. The formation of a sticky and thread-like appearance within 45 seconds when the toothpick lifted indicates that the test bacterium is Gram-negative and the formation of an insoluble white precipitate indicates that it is a Gram-positive strain.

2.2.4 Qualitative estimation of phosphate solubilization by the rhizobacteria

The test of the relative efficiency of isolates for producing phosphate solubilization was carried out by selecting the microorganisms that are capable of producing a halo/clear zone on a plate owing to the production of organic acids into the surrounding medium [22,23]. The ability of all bacterial isolates to solubilize phosphate was tested by an agar assay using Pikovskaya media in 1 liter: Glucose-10g, $Ca_3(PO_4)_2$-5g, $(NH_4)_2SO_4$-0.5g, NaCl-0.2g, $MgSO_4.7H_2O$-0.1g, KCl-0.2g, Yeast extract-0.5 g, $MnSO_4.H_2O$-0.002g, $FeSO_4.7H_2O$-0.002g and Agar-15g [24] with bromophenol blue broth medium containing insoluble tricalcium phosphate as the single phosphorus source were used to detect phosphate solubilization activity of the isolates. About 40 µl bacterial isolate was spot inoculated on the agar plate and incubation at 30^0C for at least 7 days, the clear zones around grown bacteria indicated the phosphate solubilizing activity of bacterial isolates [25]. The ability of the bacteria to solubilize insoluble phosphate was described by the following formula [26].

$$\text{Phosphate solubilization index (PSI)} = \frac{(\text{Colonydiameter}+\text{halozonediameter})}{\text{colonydiameter}}$$

2.2.5 Determination of Indole acetic acid (IAA) production

Auxin, Indole-3-acetic acid (IAA), produced by the bacterial cultures was estimated by growing the isolates were inoculated in 50 ml Jenson's broth (In 1 liter distilled water: Sucrose -20.0 g, dipotassium phosphate-1.0 g, magnesium sulfate -0.50 g, sodium chloride -0.50 g, ferrous sulfate-0.10 g, sodium molybdate -0.005 g and calcium carbonate-2.0 g), and incubated at 30°C for 48 hours according to Bric et al. [27]. 1mL of the bacterial culture was transferred into a new 50 ml Jenson's broth with the addition of 5 ml L-Tryptophan as the precursor of Indole-3-acetic acid. Jenson's broth without bacterial inoculation served as the control. 1.5 ml of the bacterial culture were transferred into a sterile Eppendorf tube and centrifuged at 7000 rpm for 7 min. The supernatant (1ml) was mixed with 2ml of Salkowsky reagent {2% of 0.5 M $FeCl_3$ in 35% $HClO_4$ (Perchloric acid)} according to the method by Gordon and Weber [28]. The solution was allowed to settle for 25 min and the development of a pink color would indicate IAA production.

2.3 Catalase Test

A loop full of culture was taken and placed on a clean glass slide. A drop of 3% hydrogen peroxide (H_2O_2) solution was placed on the bacterial culture and mixed well using a sterile loop. The effervescence indicated catalase activity that is the formation of air bubbles within 10 seconds indicates a positive result for the catalase test. A negative result is no bubbles or only a few scattered bubbles.

2.3.1 NaOH test

To perform NaOH test for bacterial isolates, 3% NaOH solution was taken into a slide and then with a sterile inoculating loop, a small number of pure culture isolates was sunk into it. Smeared the sample using a loop and observed either it is sticky or not. If sticky, it is considered as negative and if not, it is considered as positive.

2.3.2 Cellulose degradation test

For cellulose degradation ability of the bacterial isolates, Carboxymethyl Cellulose (CMC) ($MgSO_4.7H_2O$-0.005 g, K_2HPO_4- 0.005 g, Carboxymethyl cellulose-0.02 g, Congo red-0.002 g and adjusted in pH 7.0) agar plates were prepared by screening for cellulase enzyme production according to the method by Kasana et al. [29]. A sterile paper disc was dipped into the microbial culture and transferred onto the CMC agar plates. The plates were incubated overnight at 33°C. After incubation, the plates were flooded with Congo red solution (Congo red 0.2%, in SD 3A alcohol, 80% saturated with sodium chloride) (Sigma-Aldrich, Germany) for 15 min, followed by de-staining with the salt solution for 15 min. Unstained areas indicate where the CMC has been degraded due to the production of cellulose by the bacterial strain.

2.3.3 Growth attributes test of rhizobacteria in different arsenic level

The pure sub-culture bacterial isolates were streaking over the nutrient agar plate by sterile copper loop and incubated at 30^0C for 3 days to maintain the varying arsenic doses (0, 10, 20, 30,

40, and 50 ppm). After 3 days of incubation, the agar plates were tested by visual observation and record the growth appearance.

2.4 Analyses of Soil Chemical Properties

All the physical and chemical properties of collected soil samples were analyzed by standard analytical protocol. Soil pH was measured with the help of a glass electrode pH meter as described by Jackson [30]. Organic carbon was determined following the wet oxidation method as described by Page et al. [31] and the organic matter content was calculated by multiplying the % organic carbon with the Van Bemmelen factor 1.73 [32]. Total N of soil was estimated following the micro-Kjeldahl method. The phosphorus in the extract was then determined colorimetrically [33]. The absorbance was measured by a double beam spectrophotometer (Model no. 170-30, Hitachi, Japan) at 710 nm wavelength. Exchangeable potassium of soil was determined from ammonium acetate (1N NH_4OAC) extract as described by Jackson [34] and was measured by using a flame-photometer (Atomic absorption spectrophotometer, model No. Hitachi, Japan). Then total As was determined by using the method as described by Loeppert and Biswas [35] using hydride generation atomic absorption spectrophotometer (HG-AAS).

3. RESULTS AND DISCUSSION

The present investigation included the collection of soil samples, isolation of bacterial population, phenological and biochemical characterization of rhizobacteria *in vitro*. The results presented in Tables and Figures are discussed under the following heads:

3.1 Isolation of the Bacterial Isolates

A total of 24 bacterial isolates were isolated from the arsenic-contaminated rice rhizosphere and all the bacterial isolates were designated as SS 01 to SS 24.

3.1.1 Morphological characterization of the bacterial isolates

The rhizobacterial isolates were characterized based on their morphological features such as shape, margin, elevation, surface, color, and odor (Fig. 2). All the rhizobacterial isolates were fast-growing having different morphological characters in the nutrient agar medium. On nutrient agar (NA), 4 (SS 05, SS 09, SS 11and SS 18) isolates out of 24 isolates produced round-shaped and raised colonies, having smooth margin, odorless with a smooth shiny surface (Table 2). The 4 isolates also produced creamy, brownish, and yolk yellowish color in nutrient agar plates. Another 14 rhizobacterial isolates (SS 01, SS 02, SS 03, SS 04, SS 06, SS 08, SS 10, SS 13, SS 14, SS 16, SS 17, SS 21, SS 22, and SS 24) produced rod-shaped and raised margin colonies having a smooth shiny surface, smooth margin and creamy, yellowish and off white odorless color in the nutrient agar plate media (Table 2). On the other hand, SS 07, SS 12, SS 15, SS 19, SS 20, and SS 23 of the rhizobacterial isolates produced short rod shape and raised margin, smooth shiny surface, smooth margin with yellowish, brownish, and off white odorless color in the nutrient agar medium (Table 2).

3.2 Gram Staining and KOH Test

Microscopic observations were performed to investigate some characteristics of rhizobacterial isolates such as Gram staining and KOH test. Among 24 isolated isolates, fifteen of them were found as Gram-negative in reaction and nine were Gram-positive in reaction (Figs. 3,4). The KOH test also showed similar results (Table 3). KOH test was positive means that the bacterial isolates were Gram-negative.

3.3 Catalase Activity Test

A catalase test was performed with all the bacterial isolates. It was observed that all the bacterial isolates were positive in catalase activity reaction (Table 3 & Fig. 5). Catalase activity in the bacterial isolates may potentially be very advantageous. Bacterial isolates with catalase activity positive are highly resistant to environmental, mechanical, and chemical stress [36]. The catalase test detects the catalase enzyme present in most cytochrome-containing aerobic bacteria which form hydrogen peroxide as an oxidative end product of the aerobic breakdown of sugars. Catalase decomposes hydrogen peroxide (H_2O_2) to water and oxygen. This study showed that all the 24 isolates were catalase-positive as implied by the formation of bubbles upon addition of hydrogen peroxide to the cultures. Catalase decomposes hydrogen peroxide to water and oxygen (Figure 5). A positive reaction was indicated by the formation of bubbles on the addition of hydrogen peroxide to the cultures. It was observed that all the isolates were catalase-positive (Table 3).

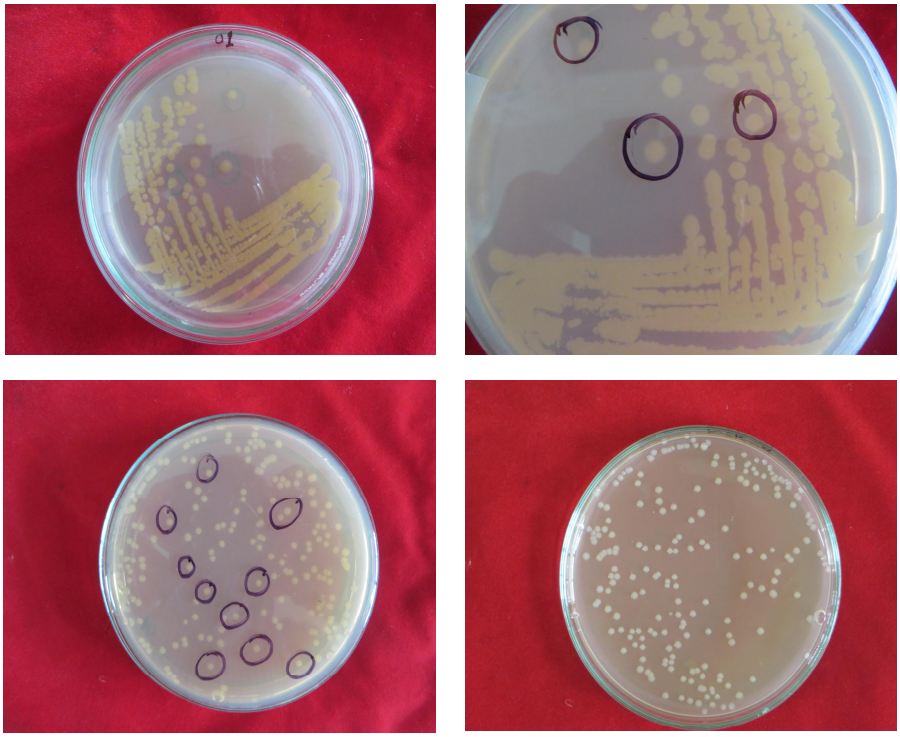

Fig. 2. Agar plate identification of bacterial isolates

Table 2. Morphological characteristics of rhizobacterial isolates isolated from arsenic contaminated soils

Isolates	Shape	Elevation	Surface	Margin	Colour	Odor
SS 01	Rod	Raised	Smooth shiny	Smooth	White	Odorless
SS 02	Rod	Raised	Smooth shiny	Smooth	Yellowish	Odorless
SS 03	Rod	Raised	Smooth shiny	Smooth	Off white	Odorless
SS 04	Rod	Raised	Smooth shiny	Smooth	Off white	Odorless
SS 05	Round	Raised	Smooth shiny	Smooth	Creamy	Odorless
SS 06	Rod	Raised	Smooth shiny	Smooth	Creamy	Odorless
SS 07	Short rod	Raised	Smooth shiny	Smooth	Brownish	Odorless
SS 08	Rod	Raised	Smooth shiny	Smooth	Yellowish	Odorless
SS 09	Round	Raised	Smooth shiny	Smooth	Yellowish	Odorless
SS 10	Rod	Raised	Smooth shiny	Smooth	Yellowish	Odorless
SS 11	Round	Raised	Smooth shiny	Smooth	Yellowish	Odorless
SS 12	Short rod	Raised	Smooth shiny	Smooth	Yellowish	Odorless
SS 13	Rod	Raised	Smooth shiny	Smooth	Creamy	Odorless
SS 14	Rod	Raised	Smooth shiny	Smooth	Creamy	Odorless
SS 15	Short rod	Raised	Smooth shiny	Smooth	Grayish	Odorless
SS 16	Rod	Raised	Smooth shiny	Smooth	Yellowish	Odorless
SS 17	Rod	Raised	Smooth shiny	Smooth	Creamy	Odorless
SS 18	Round	Raised	Smooth shiny	Smooth	Yolk yellowish	Odorless
SS 19	Short rod	Raised	Smooth shiny	Smooth	Off white	Odorless
SS 20	Short rod	Raised	Smooth shiny	Smooth	Off white	Odorless
SS 21	Rod	Raised	Smooth shiny	Smooth	Off white	Odorless
SS 22	Rod	Raised	Smooth shiny	Smooth	Off white	Odorless
SS 23	Short rod	Raised	Smooth shiny	Smooth	Brownish	Odorless
SS 24	Rod	Raised	Smooth shiny	Smooth	Brownish	Odorless

Table 3. Biochemical characteristics of rhizobacterial isolates isolated from arsenic contaminated soils

Isolates	Gram stain Test	KOH Test	Catalase Test	Phosphate Solubilization Test
SS 01	-	+	+	+
SS 02	-	+	+	+
SS 03	+	-	+	+
SS 04	+	-	+	+
SS 05	-	+	+	+
SS 06	+	-	+	+
SS 07	-	+	+	+
SS 08	-	+	+	+
SS 09	-	+	+	+
SS 10	-	+	+	+
SS 11	-	+	+	+
SS 12	-	+	+	+
SS 13	-	+	+	+
SS 14	-	+	+	+
SS 15	-	+	+	+
SS 16	+	-	+	+
SS 17	+	-	+	+
SS 18	+	-	+	+
SS 19	-	+	+	+
SS 20	+	-	+	+
SS 21	+	-	+	+
SS 22	-	+	+	+
SS 23	-	+	+	+
SS 24	+	-	+	+

(+ = Gram positive, - = Gram Negative for Gram stain test and KOH test) & + = positive growth for catalase and phosphate solubilization test)

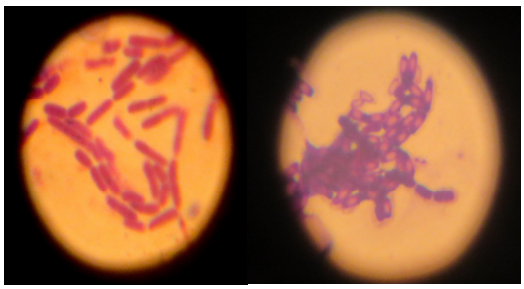

Fig. 3. Gram staining test of the bacterial isolates

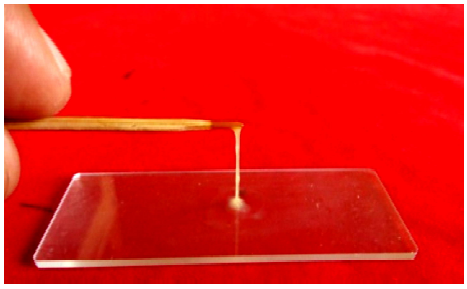

Fig. 4. KOH test of the bacterial isolates

Fig. 5. Catalase test of the bacterial isolates

3.4 Phosphate Solubilization Test

3.4.1 Qualitative phosphate solubilization test

Many soil bacteria are known to release phosphate (P) from the bound or absorbed (nutritionally unavailable) forms present in the soil thus improving the availability of this highly important mineral nutrient to the plants [37] in both acidic and alkaline soils [38]. Besides improving plant growth through the stipulation of optimal P concentrations, phosphate solubilizing bacteria (PSB) also exhibit antagonistic activities against deleterious organisms in the rhizosphere [39,40]. Out of 24 bacterial isolates, all bacterial isolates were tested positive for P-solubilization (Table 3 & Fig. 6).

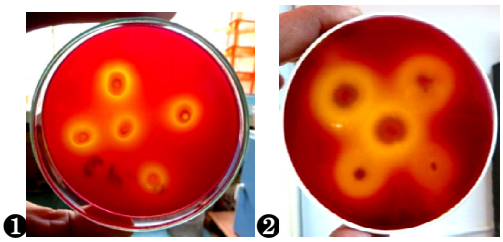

Fig. 6. Phosphate solubilization assay by the bacterial isolates

Pikovskaya plate assayed to determine phosphate solubilization activity. The cultures bacterial isolates were spot inoculated on the plates and incubated in an incubator at 28°C for 3-5 days. Formation of clear zone around the microbial colonies indicated phosphate solubilization. All 24 of the bacterial isolates were found as positive for phosphate solubilisation activity.

3.4.2 Phosphate solubilization index (PSI)

Phosphate solubilization index (PSI) based on colony diameter and halo zone diameter from bacterial isolates (Table 4). The isolated bacterial isolates were capable of solubilizing tricalcium phosphate present in Pikovskaya medium and they formed large halos with varied intensity and also found the rhizobacteria isolates were observed statistically significantly varied to produce halo zones in Pikovakaya's medium with varying intensity. All the rhizobacterial isolates were capable to solubilize the tricalcium phosphates in the Pikovskaya medium. Among all the 24 rhizobacterial isolates, the highest solubilization index was recorded from SS07 (4.4) and SS05 (4.3) isolates and lowest PSI was recorded from SS17 (1.2) isolates. Other rhizobacteria isolate SS09 and SS19 also produced higher phosphate solubilization index in Pikovskaya medium. Similarly, the lowest phosphate solubilization index was recorded from the rhizobacterial isolates SS16, SS17, and SS24. Studies on agar plates revealed that the clear halo zones formed by phosphate solubilizing microorganisms are due to the production of organic acids (Gluconic acid, Oxalic acid, and Citric acid) into the surrounding medium.

3.4.3 Indole Acetic Acid (IAA) production

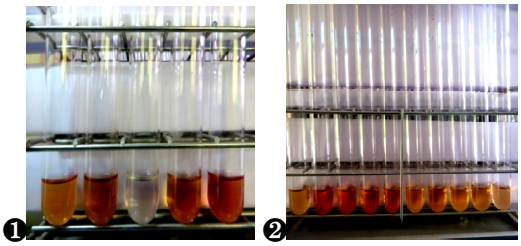

Fig. 7-8. Indole-3-acetic (IAA) production by bacterial isolates

In vitro screening for characteristics commonly associated with plant growth promotion revealed that all the 24 bacterial isolates were capable to produce indole-acetic-acid (IAA), indicating a substantial variability among rhizosphere bacterial isolates for IAA production. The isolates were also investigated for the ability to produce plant growth hormone indol-3-acetic acid (IAA) in pure culture and were found statistically significant variation (Table 4 & Fig. 7). In the presence of tryptophan, the bacterial isolates produced higher IAA which varied from 2.31-115.48 μgmL^{-1} (Table 4). The highest IAA was recorded in strain SS 08 (114.58 μgmL^{-1}) and the lowest amount of IAA (2.31 μgmL^{-1}) produced by the bacterial isolates SS18 (Table 4). On the other hand, bacterial isolates SS02, SS05, SS09, SS10, SS11, SS14, SS15, SS19, and SS22 also produced a higher amount of IAA *in vitro* (Table 4).

3.4.4 Cellulose degradation activity test

Jensen-CMC plate assayed to observe cellulose activity. The plates spotted with bacterial broth showed a transparent halo zone after stained with 0.1% Congo red indicating cellulose activity (Fig. 9). All the 24 bacterial isolates isolated from the rice rhizosphere were positive for cellulase activity (Table 5).

Table 4. Phosphate solubilizing index (PSI) and Indole acetic acid (IAA) production by rhizobacterial Isolates

Rhizobacterial Isolates	Phosphate solubilization index (PSI)	Indole acetic acid (IAA) production (µg/ml)
SS 01	3.1	34.75
SS 02	3.5	46.75
SS 03	2.4	34.5
SS 04	2.6	37.56
SS 05	4.3	75.35
SS 06	1.5	33.45
SS 07	4.4	41.69
SS 08	3.7	115.48
SS 09	4.1	100.25
SS 10	3.4	92.5
SS 11	2.6	70.36
SS 12	2.3	37.89
SS 13	2.1	36.29
SS 14	2.2	83.56
SS 15	3.3	59.78
SS 16	1.3	10.36
SS 17	1.2	6.35
SS 18	1.6	2.31
SS 19	4.2	56.98
SS 20	2.6	14.27
SS 21	1.7	16.32
SS 22	3.9	98.25
SS 23	2.5	5.68
SS 24	1.4	13.52

Table 5. Biochemical characteristics of rhizobacterial isolates isolated from arsenic contaminated soils

Isolates	Indole acetic acid test	Cellulose degradation test	NaOH test	Growth at 37°C
SS 01	+	+	+	+
SS 02	+	+	+	+
SS 03	+	+	+	+
SS 04	+	+	+	+
SS 05	+	+	+	+
SS 06	+	+	+	+
SS 07	+	+	+	+
SS 08	+	+	+	+
SS 09	+	+	+	+
SS 10	+	+	+	+
SS 11	+	+	+	+
SS 12	+	+	+	+
SS 13	+	+	+	+
SS 14	+	+	+	+
SS 15	+	+	+	+
SS 16	+	+	+	+
SS 17	+	+	+	+
SS 18	+	+	+	+
SS 19	+	+	+	+
SS 20	+	+	+	+
SS 21	+	+	+	+
SS 22	+	+	+	+
SS 23	+	+	+	+
SS 24	+	+	+	+

(+ = positive growth)

Table 6. Growth performance of rhizobacterial isolates in different arsenic (As) concentrations

Isolates	Arsenic concentrations (ppm)				
	10	20	30	40	50
SS 01	++	++	++	++	++
SS 02	++	++	++	++	++
SS 03	++	++	++	++	++
SS 04	++	++	++	++	++
SS 05	++	++	++	++	++
SS 06	++	++	++	++	++
SS 07	++	++	++	++	++
SS 08	++	++	++	++	++
SS 09	++	++	++	++	++
SS 10	++	++	++	++	++
SS 11	++	++	++	++	++
SS 12	++	++	++	++	++
SS 13	++	++	++	++	++
SS 14	++	++	++	++	++
SS 15	++	++	++	++	++
SS 16	++	++	++	++	++
SS 17	++	++	++	++	++
SS 18	++	++	++	++	++
SS 19	++	++	++	++	++
SS 20	++	++	++	++	++
SS 21	++	++	++	++	++
SS 22	++	++	++	++	++
SS 23	++	++	++	++	++
SS 24	++	++	++	++	++

(++ = Good growth)

Table 7. Growth performance of rhizobacterial isolates in different NaCl concentrations

Isolates	NaCl concentration (dS/m)						
	Control	1	2	4	8	10	12
SS 01	++	++	++	++	++	++	+
SS 02	++	++	++	++	++	++	+
SS 03	++	++	++	++	++	++	+
SS 04	++	++	++	++	++	++	+
SS 05	++	++	++	++	++	++	+
SS 06	++	++	++	++	++	++	+
SS 07	++	++	++	++	++	++	+
SS 08	++	++	++	++	++	++	+
SS 09	++	++	++	++	++	++	+
SS 10	++	++	++	++	++	++	+
SS 11	++	++	++	++	++	++	+
SS 12	++	++	++	++	++	++	+
SS 13	++	++	++	++	++	++	+
SS 14	++	++	++	++	++	++	+
SS 15	++	++	++	++	++	++	+
SS 16	++	++	++	++	++	++	+
SS 17	++	++	++	++	++	++	+
SS 18	++	++	++	++	++	++	+
SS 19	++	++	++	++	++	++	+
SS 20	++	++	++	++	++	++	+
SS 21	++	++	++	++	++	++	+
SS 22	++	++	++	++	++	++	+
SS 23	++	++	++	++	++	++	+
SS 24	++	++	++	++	++	++	+

+ = Weak growth, ++ = Good growth

Cellulose is one of every of the foremost abundant polymers in plant litter, and so the breakdown of this compound may be a key step within the decomposition of the material. Cellulose-degrading microorganisms secrete cellulase enzymes that catalyze the first step of cellulose hydrolysis and release oligosaccharides that are accessible for several other lineages [41]. The primary enzymatic breakdown of cellulose naturally leads to the discharge of oligosaccharides like cellobiose.

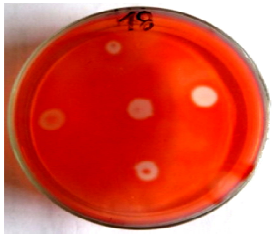

Fig. 9. Cellulose degradation activity test of the bacterial isolates

3.5 NaOH Test

All the bacterial isolates were tested in NaOH. All the bacterial isolates were showed positive results in this test (Table 5).

3.5.1 Growth at 37^0C

In growth chamber, all the isolated bacterial isolates were performed positive activity in their growth in different temperature. At 37^0C, all the bacterial isolates were able to grow in the growth medium (Table 5).

3.5.2 Bacterial growth in different arsenic content media

All the bacterial isolates were tested in numerous arsenic content media. All the isolates were able to grow in arsenic content media ranges between 10-50 ppm (Table 6). The bacterial strains isolated and studied through the present investigation have shown much higher resistance to arsenic than those isolated from soil, gold mines, and geothermal effluents in the related researches throughout world [42,43]. The bacterial strains under the present investigation were isolated from anaerobic soil environment (submerged paddy soil) which is predominated by arsenic and hence showed much higher tolerance to arsenic as compared to findings from related research established as a model microorganism for bioremediation of arsenic and one of the most arsenic-resistant microorganisms (400 mM for AsV and 60 mM for AsIII) described to date [44].

3.5.3 Bacterial growth within the different salt medium

All the bacterial isolates were tested in several salt concentrations. All the isolates were ready to grow in 1 to 12 dS/m salt concentration. The bacterial isolates were found in better growth in 1 to 10 dS/m but fair growth in over 10 dS/m salt concentration (Table 7).

4. CONCLUSION

Nowadays, soil contamination by arsenic is a major hazard in Bangladesh. The necessity to develop cost effective and eco-friendly technologies for the remediation of arsenic-contaminated soils and water has stimulated interest in arsenic-resistant organisms. For these purposes, twenty-four different bacterial isolates were isolated form arsenic contaminated soils, and detailed morphological, biochemical, and physiological characterizations were accomplished. Results revealed from the *in vitro* experiment that fifteen bacterial isolates were negative and nine were positive in gram reaction, while two were showed high IAA production ability, phosphate solubility capability, and cellulose degradation capacity within the culture media. These rhizobacterial isolates could potentially produce beneficial synergistic effects via their adaptable properties to boost soil fertility and possible plant growth stimulation through beneficial interactions and will therefore represent good candidates for the bioremediation process of arsenic-contaminated environments.

ACKNOWLEDGEMENT

The first author wishes to express gratitude to the Department of Soil Science, Department of Plant Pathology, and Department of Biotechnology, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur, Bangladesh for getting the chance to do the microbial work during the Ph.D. study.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Nordstorm DK. Worldwide occurrences of arsenic in ground water. Science.

2002;296(5576):2143-2145.
2. Marin AR, Masscheleya PH, Parrick WHJ. The influence of chemicals form and concentration of arsenic on rice growth and tissue arsenic concentration. Plant Soil. 1992;139:175-183.
3. Abedin MJ, Meharg AA. Relative toxicity of arsenite and arsenate on germination and early seedling growth of rice (*Oryza sativa* L.). Plant Soil. 2002;243:57-66.
4. Carbonell-Barrachina A, Burlo-Carbonell F, Mataix-Beneyto J. Arsenic uptake, distribution and accumulation in tomato plants-human health risk. Fresenius Environ. Bull. 1995;4:395-400.
5. Abedin J, Cresser M, Meharg AA, Feldmann J, Cotter-Howells J. Arsenic accumulation and metabolism in rice (*Oryza sativa* L.). Environ. Sci. Technol. 2002;36:962-968.
6. Rehman A, Ali A, Muneer B, Shakoori AR. Resistance and biosorption of mercury by bacteria isolated from industrial effluents. Pak. J. Zool. 2007;39:137-146.
7. Carbonell-Barrachina AA, Aarabi MA, DeLaune RD, Gambrell RP, Patrick WH. The influence of arsenic chemical form and concentration on *Spartina patens* and *Spartina alterniflora* growth and tissue arsenic concentration. Plant Soil. 1998;198(1):33-43.
8. Niggemyer A, Spring S, Stackebrandt E, Rosenzweig RF. Isolation and characterization of a novel As(V) reducing bacterium: implications for arsenic mobilization and the genus *Desulfitobacterium*. Appl. Environ. Microbiol. 2001;67:5568-5580.
9. Anderson CR, Cook GM. Isolation and characterization of arsenate-reducing bacteria from arsenic contaminated sites in New Zealand. Curr. Microbiol. 2004;48: 341-347.
10. Kloepper JW, Schroth MN, Miller TD. Effects of rhizosphere colonization by plant growth promoting rhizobacteria on potato plant development and yield. Phytopathol. 1980;70:1078-1082.
11. Bachate SP, Cavalca L, Andreoni V. Arsenic-resistant bacteria isolated from agricultural soils of Bangladesh and characterization of arsenate-reducing strains. Department of Food Science and Microbiology, University of Milan, Milan, Italy. J. Appl. Microbiol. 2009;107:145-156.
12. Wenzel WW. Rhizosphere processes and management in plant-assisted bioremediation (phytoremediation) of soils. Plant Soil. 2009;321:385-408.
13. Ma Y, Prasadb MNV, Rajkumarc M, Freitasa H. Plant growth promoting rhizobacteria and endophytes accelerate phytoremediation of metalliferous soils. Biotechnology Advances. 2011;29:248-258.
14. De-Bashan LE, Hernandez JP, Bashan Y. The potential contribution of plant growth promoting bacteria to reduce environmental degradation: A comprehensive evaluation. Appl. Soil Ecol. 2012;61:171-189.
15. Asghar HN, Zahir ZA, Arshad M, Khalig A. Plant growth regulating substances in the rhizozphere: microbial productionand functions. Advances in Agronomy. 2002;62:146-151.
16. Arkhipova TN, Veselov SU, Melantiev AI, Marty NEV, Kudoyerova GR. Ability of bacterium *Bacillus* to produce cytokinins and to influence the growth and endogenous hormone content of lettuce plants. Plant Soil. 2005;272:201-209.
17. Joo GJ, Kim YM, Lee KIJ, Song S, Rhee IK. Growth promotion of red pepper seedlings and the production of gibberellins by *Bacillus cereus, Bacillus macroides, Bacillus pumilus*. Biotechnology Letters. 2004;26:487-491.
18. Lalande R, Bissonnette N, Coutlée D, Antoun H. Identification of rhizobacteria from maize and determination of their plant-growth promoting potential. Plant Soil. 1989;115:7-11.
19. Liu ST, Lee LY, Tai CY, Hung CH, Chang YS, Wolfram JH, Rogers R, Goldstein AH. Cloning of an *Erwinia herbicola* gene necessary for gluconic acid production and enhanced mineral phosphate solubilization in Escherichia coli HB101. J. Bacteriol. 1992;174:5814-5819.
20. BBS. Bangladesh Bureau of Statistics, Chandpur district cultural survey report; Cultural survey report of upazilas of Chandpur district 2001, Bangladesh; 2001.
21. Claus D, Berkeley RCW. Genus *Bacillus*. 1872. In Bergey's Mannual of Systematic Bacteriology. Edited by Sneath PHA, Mair NS, Sharpe ME, and Holt JG. Baltimore; Williums & Wilkins. 1986;2:1105-1139.
22. Mehta S, Nautiyal CS. An efficient method for qualitative screening of phosphate solubilizing bacteria. Curr. Microbiol. 2001;43:51-56.

23. Rodriguez H, Fraga R. Phosphate solubilizing bacteria and their role in plant growth promotion. Biotechnol. Adv. 1999; 17:319-339.
24. Pikovskaya RI. Mobilization of phosphorus in soil in connection with vital activity of some microbial species. Microbiol. 1948; 17:362370.
25. Frey-Klett P, Chavatte M, Clausse ML, Courrier S, Roux CL, Raaijmakers J. Ectomycorrhizal symbiosis affects functional diversity of rhizosphere Flourescent pseudomonalds. New phytol. 2005;165:317-328.
26. Edi-Premono M, Moawad MA, Vleck PLG. Effect of phosphate solubilizing *Pseudmonas putida* on the growth of maize and its survival in the rhizosphere. *Indonesian J. Crop. Sci*. 1996;11:13-23.
27. Bric JM, Bostock RM, Silverstone SE. Rapid in situ assay for indole acetic acid production by bacteria immobilized on a nitrocellulose membrane. Appl. Environ. Microbiol. 1991;57:535-538.
28. Gordon AS, Weber RP. Colorimetric estimation of indole acetic acid. Plant Physiol. 1951;26:192-195.
29. Kasana RC, Salwan R, Dhar H, Dutt S, Gulati A. A rapid and easy method for the detection of microbial cellulases on agar plates using Gram's iodine. Current Microbiol. 2008;57:503-507.
30. Jackson ML. Soil chemical analysis. Prentice Hall Inc. Engle Wood Cliffs NJ, USA. 1962;55-56.
31. Page AL, Miller RH, Keeney DR. Methods of soil analysis. Part 2. 2nd ed. Amar. Soc. Agron., Inc. Madi, Wis, USA;1982.
32. Piper GS. Soil and plant analysis. Adelaide University Press, Australia; 1950.
33. Olsen SR, Cole CV, Watanabe FS, Dean LA. Estimation of available phosphorus in soils by extraction with sodium bi carbonate. USDA Circ. 939.US Gov. Print Office, Washington, DC; 1954.
34. Jackson ML. Soil chemical analysis, Prentice Hall of India Pvt. Ltd., New Delhi. 1973;38-56.
35. Loeppert RH, Biswas BK. Methods of Analysis for Soil Arsenic. Texas A & M University, College Station, Texas, USA. 2002;2-4.
36. Glick BR, Patten CL, Holguin G, Penrose GM. Biochemical and Genetic Mechanisms Used by Plant Growth Promoting Bacteria. Imperial College Press, London;1998.
37. Richardson AE. Prospects for using soil microorganisms to improve the acquisition of phosphorus by plants. Functional Plant Boil. 2001;28(9):897-906
38. Richardson AE, Hocking PJ, Simpson RJ, George TS. Plant mechanisms to optimize access to soil phosphorus. Crop and pasture Sci. 2009;60(2):124-143.
39. Siddique Z, Akhter M. Biocontrol of a chickpea root-rot disease complex with phosphate-solubizing microorganisms. J. Plant Pathol. 2007;67-77.
40. Kukreja G, Blute SS, Mangate SA, Dhawale MN. Exploring the potential of Pseudomonas Species as phosphate solubilizer, plant growth promoter, biocontrol agent and pesticide degrader. Asian J. Exp. Biol. Sci. Spl. 2010;40-44.
41. Lynd LR, Weimer PJ, Zyl WH, Van, Isak S, Pretorius IS. Microbial cellulose utilization: fundamentals and biotechnology. Microbiol. Mol. Biol. Rev. 2002;66:506.
42. Saltikov C, Olson B. Homology of *Escherichia coli* R773 arsA, arsB, and arsC genes in arsenic-resistant bacteria isolated from raw sewage and arsenic-enriched creek waters. Appl. Environ. Microbiol. 2002;68:280–288.
43. Simeonova D, Lievremont D, Lagarde F, Muller D, Groudeva V, Lett MC. Microplate screening assay for detection of AsIIIoxidizing and AsV-reducing bacteria. FEMS Microbiol. Lett. 2004;237:249–253.
44. Mateos LM, Ordonez E, Letek M, Gil JA. *Corynebacterium glutamicum* as a model bacterium for the bioremediation of arsenic. Int Microbiol. 2006;9:207–215.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle4.com/review-history/67025