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Research Article

ISOLATION AND CHARACTERIZATION OF A MINERAL SOLUBILIZING *AZOTOBACTER* SP. OBTAINED FROM SOIL SAMPLE COLLECTED FROM RANGWASA JAIVIK FARM

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Abstract- A bacterial strain with the ability to grow on nitrogen free media under aerobic condition was isolated from the soil collected from Rangwasa Jaivik farm which has been practicing traditional and organic farming practices for the last twenty-five years. The strain was identified as *Azotobacter* sp. and was found to have the ability to solubilize minerals such as phosphate, potassium and zinc. The isolate also produced Indole Acetic Acid and was found to improve germination of seeds in Fenugreek when used as bio-inoculant. The plants from the seeds inoculated with the isolated strain showed significant reduction in yellowing of the leaves.

Keywords- *Azotobacter, Nitrogen Fixing Bacteria, Phosphate solubilizing bacteria, Potassium solubilizing bacteria, Zinc solubilizing bacteria*

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Introduction

Nitrogen is one of the essential macronutrients required for plant growth and development. It is the second most abundant element found in the plant cell, and is required for synthesis of proteins, nucleic acids and some vitamins. Despite an abundant supply of nitrogen in the atmosphere, it is unusable by the biological systems without combining with the element hydrogen; the process is called nitrogen fixation [1]. Several soil microbes can carry out what is called biological nitrogen fixation (BNF) that converts the atmospheric nitrogen into a plant usable form. The bacteria and archaea that can carry out nitrogen fixation are termed as "diazotrophs". BNF is mainly carried out by symbiotic microorganisms that partner with the host plant, where the plants provide sugars that is required as a source of energy for nitrogen fixation and in exchange, they provide fixed nitrogen to the plant. Examples of such bacteria are *Rhizobium* and, the *cyanobacterium Anabaena azollae* which consists of specialized cell called heterocyst for nitrogen fixation. The associative nitrogen fixation is carried out by species such as *Azospirillum* that can form close association with several members of the grass family including paddy, wheat, barley *etc.* Free-living soil bacteria such as *Azotobacter, Clostridium, Klebsiella* and certain *Bacilli* however, do not require any direct interaction with other organisms and are able to obtain the energy required for nitrogen fixation by oxidizing organic molecules released by other organisms or from decomposition. Nitrogen fixation by legumes was considered as the major source of available nitrogen in plants for long, until it was demonstrated that associative and free living rhizospheric bacteria also contribute agronomically significant amount of Nitrogen particularly to cereals [2]. Especially for crops like Paddy, non-symbiotic N_2 fixers are very important [3] and presence of such microbes in soil brings down the need for additional Nitrogen fertilizers significantly. The diazotrophs consist of a nitrogen enzyme system consisting of the iron protein (Fe-protein) and the molybdenum iron protein (MoFe-protein), the two component metalloproteins that catalyse the ATP-dependent reduction of dinitrogen to ammonia [4]. Phosphorus and potassium are the other vital macronutrients, besides nitrogen, that are indispensable for the proper growth and development of plants.

Their presence in the soil is mostly in the form of insoluble rocks, minerals and other deposits [5]. However, plants are unable to utilize these macronutrients in these forms as they can uptake these nutrients in solubilized forms. This important task is carried out by several beneficial microbes present in the soil. Several species belonging to the genera *Bacillus, Rhizobium, Psuedomonas, Agrobacterium etc.* can solubilize inorganic compounds present in the soil to convert them into soluble forms. A generally accepted mechanism for such solubiliztion is through secretion of organic acids by these microbes. Phosphate solubilization is also carried out by certain rhizospheric bacteria by secretion of acid phophatase enzymes that catalyze the hydrolysis of organic phosphates at acidic pH [6]. The micronutrients such as zinc, copper magnesium *etc.* are also indispensible for optimum growth and development of plants. Zinc plays an important role in metabolism of carbohydrates, synthesis of auxin, and is a component of several metalloproteins [7]. The zinc solubilising bacteria present in the rhizosphere of the roots do so by producing organic acids that sequester the zinc cations and decrease the pH of surrounding soil. Other processes such as siderophore formation [8] or oxidoreduction [9], could also be involved in zinc solubilization. In this study, we report an *Azotobacter* sp. isolated from soil sample collected from Rangwasa Jaivik Farm. *Azotobacter* is a free-living diazotroph that has demonstrated benefits as a bio-inoculant [10], not only because of increase in fixed nitrogen in the soil [11], but also because of the secretion of beneficial growth stimulating hormones such as IAA, gibberlins and cytokinins[12-14], that help in better growth and higher yield. *Azotobacter* has been demonstrated to fix 10mg of atmospheric nitrogen per gram of carbohydrate consumed, under certain conditions [15]. Several strains of *Azotobacter* have found use in industry due to the production of exopolysaccharides with high potential for commercial application [16]. The bacterial strain reported here was isolated on selective media for nitrogen fixing bacteria and was also found to efficiently carry out phosphate, zinc and potassium solubilization. The bacterial strain was subjected to morphological and biochemical characterization and identified as *Azotobacter* sp. based on Bergey's manual of determinative bacteriology.

Materials and Methods

Sample collection

The soil sample was collected from Rangwasa Jaivik farm.

Isolation and culturing of nitrogen-fixing bacteria

Burk's N-free medium was used for the isolation of aerobic nitrogen-fixing bacteria *Azotobacter* sp. Serial dilutions were made using 1gm of soil sample and plated on Burk's agar plates and incubated for 2-3 days at 28 ± 2°C.The fastest growing colony was selected for further characterization. Pure cultures were kept on agar slant at 4°C.

Identification and characterization of the *Azotobacter* sp.

Pure isolate of the selected *Azotobacter* sp. was characterized using the criteria of Bergey's Manual of Systematic Bacteriology [17]. The following morphological, physiological and biochemical tests were used: Colony morphology, Gram staining. The biochemical tests included methyl red test, VP test, indole test, oxidase test, starch hydrolysis test, citrate utilization and Triple sugar Iron test were carried out. An in-house commercial strain of *Azotobacter chroococcum* which has earlier been characterized was used as control. Optimum temperature and pH required for growth were determined by incubating at a range of various temperatures and pH.

Characterization of mineral solubilizing activity of the isolated strain

Phosphate solubilization assay was carried out using sterilized PVK-BPB (Pikovskaya's-Bromophenol Blue), where 0.1% Bromophenol blue was added to Pikovskaya agar medium (Himedia). The experiment was carried out in triplicate. A known Phosphate solubilizing bacterial reference strain (*Bacillus megatarium*, MTCC1684) was used as positive control. Commercial strain of *Azotobacter chroococcum* was also included for comparison. 5ml of Burk's broth media (Himedia) was inoculated with single colony of *IBAc3* / *Bacillus megatarium* / *Azotobacter chroococcum* and incubated at 30ºC for 24hrs at 100 rpm. Negative control tube consisted of uninoculated Burk's broth medium. One loopful of pure culture of each tube was dispensed onto sterilized filter paper disc placed on PVK-BPB agar medium petri-plate separately, allowed to solidify and incubated at a temperature of 30ºC for a maximum period of 5 days. Negative control consisted of uninoculated PVK-BPB agar medium. Based on the appearance of yellow zone, strains were visually assayed [18]. Potassium and Zinc solubilization assays were carried out in similar manner except that for potassium solubilization, modified Aleksandrov medium (Himedia) (ALK-BTB) with 0.01% bromothymol blue (BTB),was used [19]; and for zinc solubilization, media consisting (g/l); Glucose, 10.0g; K_2HPO_4, 0.1g; $(NH_4)_2SO_4$, 1g; KCl, 0.2g; $MgSO_4$, 0.2g, ZnO, 0.1%, bromothymol blue (BTB),0.1%, Agar, 2%, pH 7.1 ± 2 (Zn-BTB). A known Potassium and Zinc solubilizing bacterial reference strain (*Paenibacillus polymyxa*, MTCC122) was used as positive control.

Production of Indol Acetic Acid (IAA)

Culture containing the isolated strain was grown at 30°C in Burk's media containing tryptophan (100 mg/l) and was centrifuged. IAA production was determined in the supernatant by Salkowski's method [20] at 530 nm. The negative control was un-inoculated and positive control consisted of known culture of *Psuedomonas* fluorescence that was earlier characterized for IAA production.

Inoculation of fenugreekseeds (*Trigonella foenum*-graecum L.) with the isolated bacterial strain

The isolated *Azotobacter* strain *IBAc3* was used as bioinoculant on Fenugreek. Seeds of local variety of Fenugreek were collected from Rangwasa Jaivik farm. Fresh bacterial culture (24 h) was used to inoculate 100 ml Nutrient Broth (NB) broth, kept on a shaker for 48 h and centrifuged for 10 min at 3000 rpm. The supernatant was discarded, and the pellet was diluted with distilled water to 100 ml. 150 Seeds were surface sterilized with 1% Na hypochlorite for 2 min followed by six times washing with sterile water. Out of the sterilized seeds, 25 were soaked in the inoculum suspension for 2-4 h (the experiment aws set up in triplicates). Control seeds were soaked in DI water. Thereafter, the seeds were sown in pots containing autoclaved soil and sand in 3:1 ratio and growth characteristics were monitored.

Results

The uniqueness of Rangwasa Jaivik farm lies in the fact that Indian traditional farming methods are practiced on this farm and no chemical fertilizers or pesticides have been used on this farm since last 25 years. In order to isolate nitrogen fixing bacteria from the sample we spread the dilutions of the sample on nitrogen free Burk's agar medium and on incubation at 28°C for 24 hours, three different morphologies were observed on the plates. The fastest growing colony was picked and isolated by streak plate method. This bacterium was further characterized morphologically and biochemically for the purpose of identification and functional characterization. The colonies formed by the isolated strain on nitrogen free Burk's medium were slightly viscous, rounded, shiny and semi-transparent [Fig-1A]. Bacteria were Gram-negative with rounded ends, after 48 h growth in nitrogen free liquid culture [Fig-1B] with an optimal growth temperature range of 15ºC to 40ºC and optimal growth pH range of 5 to 9. Based on the morphological characteristics and biochemical assay results [Table-1], the isolate was classified according to Bergey's Manual of Determinative Bacteriology as *Azotobacter* sp. and named *IBAc3*.

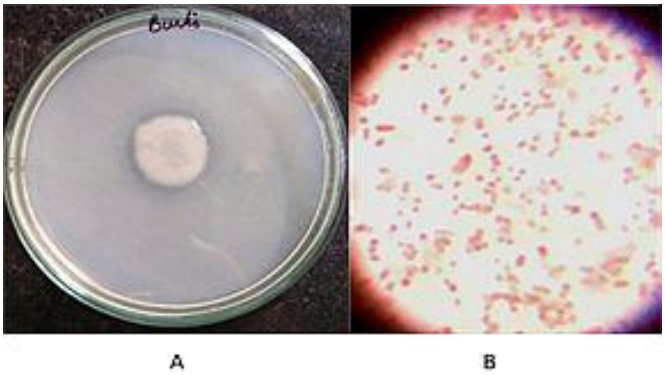

Fig-1 A. Growth of isolated strain on nitrogen-free Burk's medium, B. Microscopic view of the isolated strain after gram staining.

Table-1 *Biochemical characterization of the isolated strain*

S	Characteristics	Observation	
		Reference strain	*IBAc3*
1	Gram staining	Gram negative	Gram negative
2	Shape	Coccus	Coccus
3	Motility	Less	More
4	Oxygen requirement	Aerobic	Aerobic
5	Colony	Flat	Flat, shiny and slimy
7	Oxidase	Negative	Negative
8	Starch hydrolysis	Negative	Positive
9	Indole	Negative	Negative
10	Methyl red test	Negative	Positive
11	Vogus Proskaur	Negative	Positive
12	Citrate Utilization	Positive	Positive
13	Triple Sugar Iron	Red slant/red butt with less gas production	Red slant/red butt with more gas production
14	IAA production	positive	positive

In-vitro solubilization of Phosphate, Potassium and Zinc

In the qualitative confirmatory analysis, the strain*IBAc3* produces yellow halos by de-colorization of blue colour of PVK-BPB, ALK-BTB and Zn-BTB, agar media. Visually, the de-colorization of PVK-BPB, ALK-BTB and Zn-BTB media and formation of yellow halos by bacterial isolate *IBAc3* was like the positive control used in each experiment, while not much discoloration was observed in *Azotobacter chroococcum* [Fig-2 a-c]. It clearly defines the pH drop in the medium due to the release of organic acids by the activity of mineral solubilizing bacteria. Indeed, the yellow hallows observed on selective media for Potassium and Zinc solubilization seemed larger in diameter as compared to the positive controls [Table-2]. These results indicate that *IBAc3* is efficient in mineral solubilization.

Table-2 *Solubilization of Phosphate, Potassium and Zinc by reference strains and IBAc3as seen by zone of clearance on the selective media plates*

S	Name of Bacteria	Average diameter of culture +halo zone (in cm) on various media		
		PVK-BPB	ALK-BTB	Zn- BTB
1	Control	-	-	-
2	*Paenibacillus polymyxa*	NA	4.00	2.14
3	*Azotobacter chroococcum*	1.06	1.06	0.80
4	*Bacillus megatarium*	2.40	NA	NA
5	IBAc3	3.47	4.27	3.47

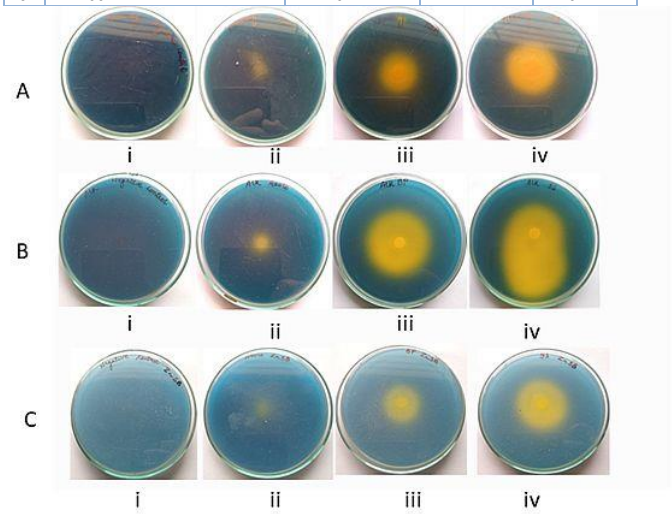

Fig-2 In-*vitro* mineral solubilization by various bacterial cultures on various selective media. A. Phosphate solubilization of PVK-BPB media (i. control, ii. *Azotobacter chroococcum,* iii. *Bacillus megatarium*, iv. *IBAc3*); B. Potassium solubilization of ALK-BTB media (i. control, ii. *Azotobacter chroococcum*, iii. *Panaebacillus polymyxa*, iv. *IBAc3*); C. Zinc solubilization of Zn-BTB media (i. control, ii. *Azotobacter chroococcum*, iii. *Panaebacillus polymyxa*, iv. *IBAc3*)

Germination and growth characteristics of Fenugreek seeds and seedlings

The seeds that were inoculated with the cells of *IBAc3* showed significant increase in germination, *i.e.*, by about 25%. We found that by day 7 after sowing, *IBAc3* inoculated seeds consisted of 90% germinated seeds while control showed only 65% seed germination. It was also found that in mature plants, control plants showed 20% higher number of yellow leaves as compared to the *IBAC3* treated seeds. Rest of the growth parameters did not show any significant difference.

Discussion

Several researches have revealed that soils that are higher in organic content and a well-maintained active biology is more fertile compared to the soil that lacks these properties. The optimal physical, chemical and biological properties of soil render the crops resistance to insect pests and diseases. Therefore, a lot of interest has been generated in the use of bio-inoculants as fertilizers. Such fertilizers also prove to be economically and ecologically sustainable. In this study we isolated an *Azotobacter* strain that exhibits multiple beneficial activities including mineral solubilization and secretion of plant growth promoting molecules. The fact that this microbe was isolated from a soil that was collected from a farm that has only been subjected to organic and or natural farming practices reinforces the fact that such soils are rich in beneficial microbial flora. The isolated strain was found to solubilize minerals even more efficiently that the commercially used microbes and was found to be efficient in improving germination in Fenugreek seeds. Fenugreek leaves are eaten as vegetable and yellowing of leaves for green leafy vegetables is undesirable. In our experiments use of *IBAc3* reduces the yellowing of leaves significantly possibly due to its multiple activities of nitrogen fixation as well as mineral solubilization hence providing better nutrition to the plants. This indicates that the isolated strain can also be used for remediation of soils that have been affected by non -judicious use of chemical fertilizers by helping in breaking down the mineral complexes that may have been formed due to inorganic fertilizers.

Conclusion

It is concluded that the isolated bacteria in this study is a multifunctional bacteria which has a number of plant beneficial activities including nitrogen fixation, mineral solubilization, production of plant growth promoting molecules and hence can be used as an excellent bio-inoculant as well as for enriching nutrient deficient soils.

Application of Research: This research can be used for isolation of *Azotobacter* and more such soil friendly bacteria and their characterization.

Research category: Agriculture microbiology

Abbreviations: sp.: species; PVK-BPB: Pikovskaya's Bromophenol blue; NB: Nutrient broth; DI: Deionized; ALK-BTB: Aleksandrov's Bromothymol blue; BNF: Biological Nitrogen Fixation; IAA: Indole Acetic Acid; Zn-BTB: Zinc containing media with Bromothymol blue

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Study area / Sample Collection: Rangwasa Jaivik farm

Species name: *Azotobacter* sp

Conflict of Interest: None declared

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.
Ethical Committee Approval Number: Nil

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