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Isolation and Characterization of *Azotobacter* and Phosphate Solubilizing Bacterial Isolates

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Isolation from 20 soil samples collected from rhizosphere of watermelon was done on Ashby's medium and Pikovskaya's agar media.8 *Azotobacter* and 6 Phosphate solubilizing bacteria PSB isolates were obtained from all samples. All the obtained isolates of *Azotobacter sp* and PSB were gram negative. On morphological study it was observed that, all *Azotobacter* isolates were motile and positive for KOH test. In case of PSB, the colony shapes of 4 obtained isolates were circular and irregular in 2. All the isolates showed smooth appearance on the media with white colony colour. The biochemical results for both *Azotobacter spp* and PSB revealed that, most of them were positive for methyl red test, catalase test, starch hydrolysis, gelatine hydrolase test, oxidase test

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and indol test. Out of eight *Azotobacter* isolates, Isolate 4 (*Azob*-1) showed highest (14.25 mg/ml) N fixing ability whereas, from of six PSB isolates, Isolate1 (PSB-1) showed highest solubilising index (4.13) therefore these two isolates were found most efficient in their roles and therefore recommended for use for field experiments.

Keywords: Watermelon; azotobacter; PSB; yield.

1. INTRODUCTION

The rhizosphere of plants is a complex zone where the soil, plant, and microbial interactions take place. Plant roots host a wide range of microorganisms, some beneficial and others detrimental. *Azotobacter* is a genus of bacteria that are aerobic, free living, gram negative, motile, and either oval or spherical in shape and form thick walled cysts that play an important role in nitrogen fixation. Genus *Azotobacter* is a non-symbiotic heterotroph capable of fixing an average of 20 kg of nitrogen per hectare annually. Genus *Azotobacter* also produces auxin which is phytohormone that stimulates plant growth [1,2]. They also make it easier for heavy metals to move about in the soil, which speeds up the bioremediation of lead, cadmium, and other heavy metals from the soil. Phosphate solubilizing bacteria is Gram-negative bacteria. They are characterized by their cell envelopes, which are composed of a thin peptidoglycan cell wall sandwiched between an inner and outer membrane. Phosphate solubilizing bacteria are beneficial bacteria capable of solubilizing inorganic phosphorus from insoluble compound. Solubilization ability of rhizosphere microorganisms is considered to be one of the most important traits associated with plant phosphate nutrition. A considerably higher concentration of phosphate solubilizing bacteria is commonly found in the rhizosphere in comparison with non rhizosphere soil. The soil bacteria belonging to the genera *Pseudomonas* and *Bacillus* and fungi are more common [3]. Thus, in this research paper the investigation has been carried Isolation and identification of isolates of *Azotobacter spp* and PSB from rhizospheric soil of watermelon growing regions of Kolhapur were carried out then the morphological and biochemical character of obtained isolates were studied

2. MATERIALS AND METHODS

Soil samples were collected from different Watermelon growing villages in Kolhapur district of Maharashtra and brought to the laboratory for isolation of *Azotobacter spp* and PSB bacteria. The rhizospheric soil was kept in fresh plastic bags after labelling and tagging. These samples were preserved in refrigerator at 4°C temperature for further use. The isolation was carried out by serial dilution and pour plate technique using Ashby's medium and Pikovskaya's agar medium (PKV) for *Azotobacter spp* and PSB bacteria respectively. Isolation was done by using Serial dilution and pour plate technique One gram of well mixed soil sample was added in 9 ml of distilled water blank. Tenfold serial dilution was prepared up to 10^7 dilutions. One ml aliquot was transferred from 10^4 to 10^6in sterilized petriplates under aseptic conditions. After that each petriplates with aliquot was filled with sterilized Ashby's and Pikovskaya's agar medium (15-20 ml) and mixed gently. After solidification of the medium, plates were incubated at 28+2 °C for 4 to 5 days. Later on, the morphological and biochemical characteristics of obtained colonies on both the medium (Ashby's and Pikovskaya's agar medium) were compared with those defined in Bergey's manual [4] to confirm them as *Azotobacter spp* and PSB strains. The strains with similar characters of *Azotobacter spp* and PSB were streaked onto another medium plates and were purified by subsequent streaking after each growth till all the colonies in petriplates appeared similar in morphology and characters. Morphological characteristicssuch as gram staining, cell shape, stain colour, cell morphology, motility test, 3% KOH test were studied for both for both *Azotobacter spp* and PSB isolates.

Selection of efficient strain of *Azotobacter spp* was done on the basis of nitrogen fixing ability (mg/ml) of obtained isolates of *Azotobacter spp* by using Kjeldhal method. Whereas, efficient strain of Phosphate solubilizing bacteria by screening of the isolates for phosphate solubilizing activity on the basis of development of zone of phosphate solubilisation on Pikovskaya's agar medium.

3. RESULTS AND DISCUSSION

Total of 20 soil samples were collected from rhizospheric zones of watermelon from different fields of Kolhapur district in year 2022-23. From

20 soil samples 8 isolates of *Azotobacter spp* and 6 isolates of PSB were obtained.

Identification of *Azotobacter spp*: Identification of obtained isolates was done on the basis of Morphological characteristics. Data presented in Table 1, revealed that, all the isolates of *Azotobacter spp* were gram negative, motile and positive for KOH test. Most of the *Azotobacter spp* isolates, namely Isolate 1, Isolate 2, Isolate 3, Isolate 4, Isolate 6, and Isolate 8, were cocci-shaped, while Isolate 5 and Isolate 7 was bacillus shaped. The cell arrangement of these isolates was mostly scattered in chain, except for Isolate 3 and Isolate 7, which were scattered in single. Similar results are accordance with the findings of Raut et al*.,* [5] and Andhare et al. [6].

Biochemical characterization of isolated *Azotobacter*: Numerous biochemical tests were used to examine the biochemical properties of the isolated *Azotobacter spp*. The obtained biochemical test results are presented in Table 2. The results revealed that, Isolates 1, 2, 3, 4, 5, and 7 were tested positive for the methyl red test, catalyse test and Indol test while isolates 6 and 8 were negative. Isolates 1, 2, 4, 5, 7, and 8 were positive for starch hydrolysis, while isolates 3 and 6 were negative. The gelatine hydrolase test was positive for all the Isolates. All isolates, except for Isolate 4 were positive for the oxidase test. The current investigation of biochemical test results revealed similarities with Patil et al*.* [7] and Roychowdhury et al*.* [8].

Selection of efficient strain of *Azotobacter spp*.: On the basis of morphological and biochemical characterization of obtained *Azotobacter spp* isolates the most efficient strain was confirmed by determining the nitrogen fixing capacity on Ashby's medium, an effective strain of *Azotobacter* was chosen. From data presented in Table 3 it was found that, the range of N_2 fixed by various obtained *Azotobacter* strains was varied between 9.24 mg $ml^{-1}$to 14.25 mg ml^{-1}. Among these Isolate 4 was found most efficient which fixes 14.25 mg ml^{-1} nitrogen as compare to rest of the isolates, while Isolate 2 to the tune of 9.24 mg/ml nitrogen found less efficient in nitrogen fixation (Table 3). Based on above conclusions Isolate 4 (Azob-1) was proved most effective strain and therefore it was chosen for further studies.The results obtained are consistent with those of Kizilkaya [9] and Kaviyarasan et al*.* [10] who also estimated the N fixing capacity of *Azotobacter spp* isolates with values ranging from 6.58 to 14.86 mg ml^{-1}.

Table 1. Morphological characteristics of *Azotobacter spp* isolates

Sr. No.	*Azotobacter*	Cell morphology	Cell arrangement	Gram reaction	Stain colour	Motility	KOH test
1	Isolate 1	Cocci	Scattered in chain	- Ve	Pink	+ Ve	+ Ve
2	Isolate 2	Cocci	Scattered in chain	- Ve	Pink	+ Ve	+ Ve
3	Isolate 3	Cocci	Scattered single	- Ve	Pink	+ Ve	+ Ve
4	Isolate 4	Cocci	Scattered in chain	- Ve	Pink	+ Ve	+ Ve
5	Isolate 5	Bacillus	Scattered single	- Ve	Pink	+ Ve	+ Ve
6	Isolate 6	Cocci	Scattered in chain	- Ve	Pink	+ Ve	+ Ve
7	Isolate 7	Bacillus	Scattered single	- Ve	Pink	+ Ve	+ Ve
8	Isolate 8	Cocci	Scattered in chain	- Ve	Pink	+ Ve	+ Ve

Legends: (+) Positive test, (-) Negative test

Table 2. Biochemical characterization of *Azotobacter spp* isolates

Sr. no.	*Azotobacter*	Methyl red test	Catalase test	Starch hydrolase test	Gelatine hydrolase test	Oxidase Test	Indol test
1	Isolate 1	+	+	+	+	+	+
2	Isolate 2	+	+	+	+	+	+
3	Isolate 3	+	+	-	+	+	+
4	Isolate 4	+	+	+	+	-	+
5	Isolate 5	+	+	+	+	+	+
6	Isolate 6	-	-	-	+	+	-
7	Isolate 7	+	+	+	+	+	+
8	Isolate 8	-	-	+	+	+	-

Legends: (+) Positive test, (-) Negative test

Table 3. Nitrogen fixing ability of *Azotobacter spp* isolates in Ashby's broth (mg ml^{-1})

Sr. No.	Isolates of Azotobacter	N fixation in broth (mg ml^{-1})
1.	Isolate 1	11.74
2.	Isolate 2	9.24
3.	Isolate 3	12.38
4.	Isolate 4	14.25
5.	Isolate 5	10.78
6.	Isolate 6	13.24
7.	Isolate 7	10.26
8.	Isolate 8	11.10

Table 4. Morphological characterization of Phosphate solubilizing bacterial Isolate

Sr. No.	PSB isolates	Colony shape	Colony colour	Gram reaction	Stain colour	Surface
1	Isolate 1	Circular	White	- Ve	Pink	Smooth
2	Isolate 2	Irregular	White	- Ve	Pink	Smooth
3	Isolate 3	Circular	White	- Ve	Pink	Smooth
4	Isolate 4	Circular	White	- Ve	Pink	Smooth
5	Isolate 5	Irregular	White	- Ve	Pink	Smooth
6	Isolate 6	Circular	White	- Ve	Pink	Smooth

Legends: (+) Positive test, (-) Negative test

Identification of PSB: Identification of PSB was done on the basis of Morphological and biochemical characteristics of obtained isolates of PSB. Data presented in Table (4) showed that, all the obtained isolates were examined on PKV medium and identified as PSB based on the formation of halo zones surrounding the colonies and morphological characteristics such as colony shape, colony colour, surface and Gram reaction. The colony shapes of majority PSB isolates 1, 3, 4 and 6 were circular, those of isolates 2 and 5 were irregular. Every PSB isolate was smooth on the on the media with white colony colour. In case of gram reaction all the obtained isolates were gram negative in nature with pink colour staining. The present results are consistent with those of Uddin et al. [11] and Mustamu et al. [12] who also found, circular, irregular, flat elevated, smooth edged, white and yellow coloured colonies of PSB.

Dada on biochemical characterization of Phosphate solubilizing bacterial isolates presented in Table (5) revealed that,the methyl red test and starch hydrolysis test showed positive results for isolates 2, 3, 5, and 6 and negative results for isolates 1 and 4. The catalase test was positive for all isolates, except for isolate 1. The gelatine hydrolase test showed positive results for isolates 2, 4, and 5, but negative results for isolates 1, 3, and 6. The oxidase test was positive for isolates 1, 3, 4, and 6 and negative for isolates 2 and 5. In case of indol test isolates 1, 4, 5, and 6 were showed positive reaction but isolates 2 and 3 showed negative reaction with indol. The current investigation's biochemical test results revealed similarities with Bashir et al. (2019) and Damor and Goswami (2016).

Selection of efficient strain of Phosphate solubilizing bacteria by screening of the isolates for Phosphate solubilizing activity byusing Pikovskaya's medium supplemented with tricalcium phosphate, the PSB isolates were observed for the development of distinct halo zones surrounding the colonies. The solubilizing index (SI) was computed by using the colony and halo zone diameters. From data presented in Table 6 revealed that, among all the obtained strains Isolate no 1 exhibited the highest zone of solubilization, with (4.13) which was followed by isolate 3 (3.33). The Isolate 2 to the tune of 2.19 showed lowest solubilizing index as compare to rest of the treatments On the basis of data on SI of obtained isolates, Isolate-1(PSB-1) was confirmed as most efficient and it was selected for further investigations.Similar finding was reported by Nagalakshmi and Karpagan [13] who determined the phosphate solubilising index of different PSB isolates on the Pikovskaya agar medium [14].

Table 5. Biochemical characterization of Phosphate solubilizing bacterial Isolates

Sr. no.	*Azotobacter* Isolates	Methyl red test	Catalase test	Starch hydrolase test	Gelatine hydrolase test	Oxidase Test	Indol test
1	Isolate 1	-	-	-	-	+	+
2	Isolate 2	+	+	+	+	-	-
3	Isolate 3	+	+	+	-	+	-
4	Isolate 4	-	+	-	+	+	+
5	Isolate 5	+	+	+	+	-	+
6	Isolate 6	+	+	+	-	+	+

Legends: (+) Positive test, (-) Negative tests

Table 6. Phosphate Solubilizing Index (SI) showed by the phosphate solubilizing bacteria

Sr. No.	PSB Isolates	SI index of P solubilization
1	Isolate 1	4.13
2	Isolate 2	2.19
3	Isolate 3	3.33
4	Isolate 4	2.42
5	Isolate 5	3.22
6	Isolate 6	2.39

4. CONCLUSION

From present investigation it was concluded that, *Azotobacter spp* and PSB strains isolated from the rhizospheric zones of watermelon growing regions of Kolhapur district was identified on the basis of analysis of morphological and biochemical characters. All the isolated strains of PSB &*Azotobacter spp* were gram negative in reaction. Out all*Azotobacter* & PSB bacterial isolates i.e., Isolate 4 (Azob-1) of *Azotobacter*with (14.25 mg ml^{-1}) N fixing ability and PSB isolates, Isolate1 (PSB-1)with (4.13)solubilising index were found most efficient in their role.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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