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MICROBIAL OPTIMIZED PRODUCTION OF INDOLE ACETIC ACID AND ASSESSMENT OF OTHER PLANT GROWTH PROMOTING ACTIVITIES

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ABSTRACT
Till date many different plant growth promoting bacteria are isolated which enhance the plant growth by different mechanisms. IAA is one of most important hormone which is secreted by plant as well as bacteria. In the present study six IAA producers were isolated from the agricultural area of the Ahmednagar city. The isolates were studied for optimum values of tryptophan concentration, pH and temperature for production of IAA. These isolates were also studied for other plant growth promoting abilities such as phosphate solubilisation, nitrogen fixation, ammonia production, HCN production, siderophore production and antifungal activity against the *Alternaria alternate.*

KEYWORDS: Microbial, IAA, plant growth, nitrogen fixation, *Alternaria alternate.*

INTRODUCTION

As plant roots grow through soil, they release water-soluble compounds such as amino acids, sugars and organic acids that supply food for the microorganisms. In return the microorganisms provide nutrients for the plants. All this activity makes the rhizosphere, the most dynamic environment in soil. (Sahasrabudhe Madhuri M., 2011). 80% of the bacteria isolated from plant rhizosphere are to produce Indole 3-Acetic Acid (Zakharova *et al.,* 1999). IAA plays a key role in regulation of plant growth and development. (Moore, 1989). IAA performs optimally at low concentration compared to the nutrients and vitamins that normally affect plant processes. The production and concentration of IAA could also influenced by other factors besides species or strain of rhizobacteria such as culture and medium conditions, growth stage and availability of substrates (Frankenberger and Arshad, 1995). Kumar Ajay *et al.,* (2012) worked on the isolating, screening and characterization of bacteria from rhizospheric soils for different plant growth promoting activities, IAA production, phosphate solubilization, HCN production, siderophore production, ACC deaminase activity and catalase. Screening of *Rhizobium* species for IAA production and effect of tryptophan concentration, carbon sources and pH on IAA production was assessed by Sahasrabudhe Madhuri M. (2011). Mahalakshmi S. and D. Reetha (2009), worked on the assessment of IAA production, phosphate solubilization, HCN production, siderophore production, ACC deaminase and antifungal activity of bacterial isolates from the rhizosphere of tomato. The assessment of plant growth promotion activities of six bacterial isolates using *Zea mays* was carried out by Ana Marques P. G. C. *et al*. (2010). They showed the ability of IAA production, HCN production, ammonia production and siderophore production by these isolates. They concluded that by using these organisms crop yield in Maize plant increased.

MATERIALS AND METHODS

The soil sample was collected from the agricultural field near the New Arts, commerce & Science College, Ahmednagar. The soil 5cm below form surface was collected in sterile container.

Isolation of IAA producing bacteria For enrichment of IAA producing bacteria, 0.1 gm of soil sample was added in 250ml capacity conical flask containing 100ml of sterile nutrient broth (N.B) supplemented with 0.1 % (w/v) tryptophan. The flask was then incubated at 37^0C for 72 hrs on rotary shaking incubator at 200 rpm. After incubation sample from the incubated flask was further streaked on sterile nutrient agar (N.A.) plates containing 0.1 % (w/v) Tryptophan. These plates were incubated at 37^0 C for 24 hrs.The isolated colonies were inoculated in 10 ml of sterile nutrient broth tubes supplemented with 0.1 % (w/v) of tryptophan and incubated 37^0 C for 24 hrs. 2 ml broth from each tube was centrifuged at 7000 rpm for 10 minutes at 4^0C. 1 ml of supernatant was mixed with 4 ml of Salkowskis reagent and the tubes were incubated at room temperature in dark for 30 minutes and observed for development of pink colour.

Effect of different factors on IAA production

Different concentrations of IAA were prepared in nutrient broth ranging from 1 to10 µg/ml. For estimation of IAA, 1 ml of broth was mixed with 4 ml of Salkowskis reagent and incubated at room temp in dark for 30 mins. After incubation the absorbance of the mixture was measured at 530 nm using UV/Visible spectrophotometer. Standard graph of concentration of IAA against absorbance was plotted and used for estimation of unknown concentration of IAA during subsequent studies.

Effect of Tryptophan concentration

Sterile nutrient broth tubes supplemented with range of tryptophan (mg/ml) viz., 1, 2, 3, 4 and 5 were used prepared. Each tube was inoculated with isolate and incubated at 37^0 C for 24 hrs. After incubation the

concentration of IAA in each tube was determined by Salkowskis method as mentioned above.

Effect of pH

Sterile nutrient broth tubes supplemented with 0.1 % (w/v) tryptophan were prepared individually with pH 4, 5, 6, 7 and 8. The tubes were inoculated with culture and incubated at 37^0 C for 72 hrs. After incubation concentration of IAA in each tube was determined by using Salkowskis method as mentioned above.

Effect of Temperature

Sterile nutrient broth tubes supplemented with 0.1 % (w/v) tryptophan were prepared. The tubes were inoculated with culture and incubated at varying temperature viz., 30^0C, 35^0C and 45^0C for 72 hrs. After incubation concentration of IAA in each tube was determined by using Salkowskis method as mentioned above.

Effect of Pesticide concentration

Sterile nutrient broth tubes supplemented with 0.1 % (w/v) tryptophan were prepared and pesticide was added to the individual tubes at varying concentration viz., 10, 50, 100, 150, 200, 250, 300, 350 and 400 µl/ 100ml. The tubes were inoculated with culture and incubated at 37^0C for 4 days. After incubation the concentration of IAA was determined using Salkowskis method as mentioned above.

Assessment of other plant growth promoting activities of the isolates

Phosphate solubilization

For assessment of phosphate solubilizing ability of the isolates, individual isolates were spot inoculated on sterile Pikovaskaya agar plate and incubated at 37^0 C for 48 hrs. After incubation the plates were observed for zone of clearance around the colony.

Nitrogen fixation

Nitrogen fixing ability of the isolates was assessed by streaking the individual culture on Norris agar plates. The plates were then incubated at 37^0C for 24 hrs. and after incubation the plates were observed for growth.

Siderophore production

Individual isolates were separately inoculated in sterile succinate broth and incubated for 3 days at 37^0C. The production of siderophore was detected by addition of CAS reagent in 1:1 proportion of succinate broth. The mixture was then observed for colour change from blue to yellow.

Determination of Type of Siderophore

- **Determination of Cathecol group** Presence of cathecol group in the siderophore was determined by Arnow's Assay. The assay was performed by mixing the following components: 1 ml culture supernatant, 1 ml 0.5 M HCL, 1 ml Nitrite-Molybdate reagent (10 % sodium nitrate (W/V) and 10 % sodium molybdate (W/V) in double distilled water), and 1ml of 1 M NaOH. The mixture was then incubated for 5 minutes. Formation of yellow colour indicates presence of catechol type of siderophores. A negative control was kept which includes all the components except culture sample.

- **Determination of Hydroxamate group** Presence of Hydroxamate group in the siderophore was determined by Gibson and Magrath Assay. For this assay, 1ml culture supernatant was hydrolysed with 1 ml of 6 N H_2SO_4 in a boiling water bath for 6 hrs or at 130^0C for 30 min. The mixture was then buffered by adding 3 ml of Sodium Acetate Solution. 1 ml of 1% sulphanilic acid solution was then added to it followed by addition of 0.5 ml iodine solution. After 3-5 min excess iodine was destroyed by adding 1 ml of 2% aq. solution of sodium arsenate solution. 1 ml of α-napthylamine solution was then added to the mixture. Finally 10 ml of distilled water was added to it and mixture was then allowed to stand for 20-30 min. After incubation of 30 min mixture was then observed formation of faint pink colour. (Mahmoud A-L.E., *et al*., 2001).

Hydrogen Cyanide production

Hydrogen cyanide production was assessed by streaking the individual culture on sterile nutrient agar plate amended with 0.44 % (w/v) of glycine. A circular disk (10 cm diameter) of Whatmann filter paper was soaked in 2% sodium carbonate in 0.5 % picric acid solution and placed in the lid of the plates. Plates were sealed with para film and incubated at 37^0 C for 96 hrs. After incubation the plates were observed for development of brown colour on filter paper.

Ammonia Production

Individual isolates were inoculated in test tubes containing 10 ml of sterile peptone broth. The tubes were then incubated at 37^0 C for 48 hrs in shaking incubator at 120 rpm. After incubation 0.5 ml Nessler's reagent was added to each tube and observed for the development of faint yellow to dark brown colour.

Antifungal activity

The antifungal activity of individual isolates was studied using agar well diffusion method. 0.1 ml of 24 hrs old culture of fungi (*Alternaria alternata*) was spread on sterile *Mueller-Hinton agar*. Wells of 8 mm diameter were prepared into the agar. 200µl of cell free broth obtained by centrifuging 24hr old culture of each individual isolate was added in the well. Sterile nutrient broth was used instead of cell free broth for keeping negative control. The plates were kept in the refrigerator for 20 mins. The Plates were incubated for 48 hrs at 37^0 C. After incubation the plates were observed for zone of inhibition around the wells.

Identification and characterization of the isolates

The isolates were identified by morphological and Biochemical characteristics as per the Bergey's Mannual of Determinative Bacteriology (9^{th} edition). Colony characteristics, grams nature and motility of individual culture were observed.

Biochemical characters

Catalase: 3% solution of hydrogen peroxide was taken in test tubes and individual isolated colony was immersed into the tube and observed for effervescence.

Oxidase: Individual culture of organism was streaked on filter paper strip previously dipped in 1% aqueous solution of N, N, N, N tetra methyl para phenylene diamine dihydrochloride. and observed for formation of blue colour.

Gelatinase: Individual isolate was inoculated in sterile nutrient medium containing 12% (w/v) gelatin and incubated for 48 hrs at 37^0C. one uninoculated tube was kept as negative control. After 48 hrs, the inoculated tubes

were kept in freeze for 30 min. and observed for gelatin liquification.

Casein Hydrolysis: Individual isolates were spot inoculated on sterile Milk agar plate and incubated at 37^0C for 24 hrs. After incubation the plates were observed for zone of casein hydrolysis around the colony.

Sugar fermentation test: Individual isolates were assessed for their sugar fermentation ability. Fermentation of different sugars (Dextrose, Mannitol, Lactose, Mannose, Maltose) by the isolates was carried out by inoculating the individual culture in the sterile peptone water supplemented with 1% of the required sugar, phenol red as pH indicator and an inverted Durham's tube. After incubation at 37^0 C for 24 hrs, the tubes were observed for acid and gas production.

RESULTS AND DISCUSSION

Isolation of IAA producing bacteria

After mixing 1 ml of cell free broth with 4 ml of Salkowski's reagent and incubation at room temperature in dark for 30 mins., six isolate showed formation of pink colour indicating production of IAA. These isolates were selected for studying other plant growth promoting activities.

Effect of different factors on IAA production

Effect of Tryptophan concentration

All six isolates were assessed for IAA production under varying concentration of tryptophan viz., 1,2,3,4 and 5 mg/ml. After incubation the produced IAA was determined using Salkowski's method. The results obtained were mentioned in figure 1. The isolates were studied for optimum values of tryptophan concentration, pH and temperature for production of IAA. The optimum tryptophan concentration for PBS-2, PBS-3, PBS-5 and PBS-6 was 5mg/ml. For PBS-1 and PBS-4 the optimum concentration was 3 and 4 mg/ml respectively.

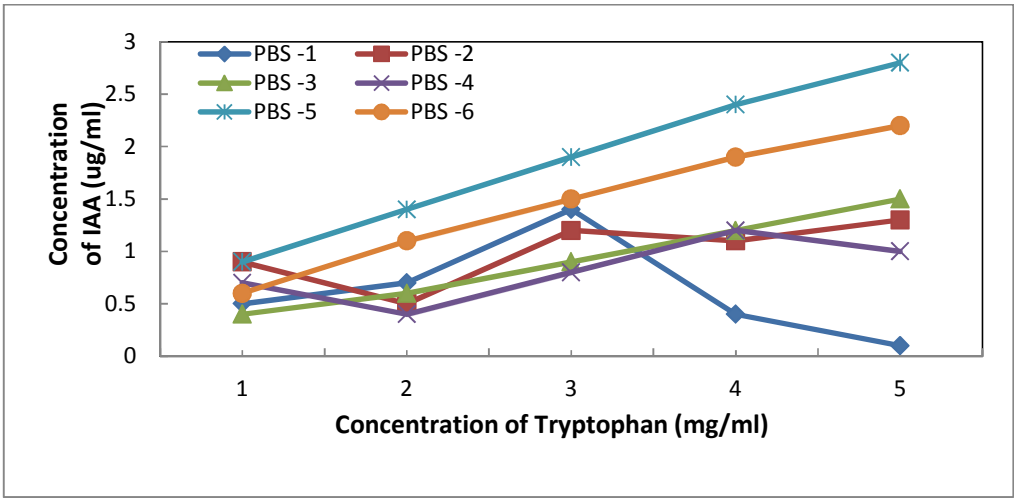


FIGURE 1. Effect of tryptophan concentration on IAA production.

Effect of pH

All six isolates were assessed for IAA production under varying pH viz., 4,5,6,7 and 8. After incubation the produced IAA was determined using Salkowski's method. The result obtained were mentioned in figure 2. PBS-1, PBS-2, PBS-3 and PBS-4 showed maximum IAA production at pH 7, whereas optimum pH for PBS-5 and PBS-6 was 5.

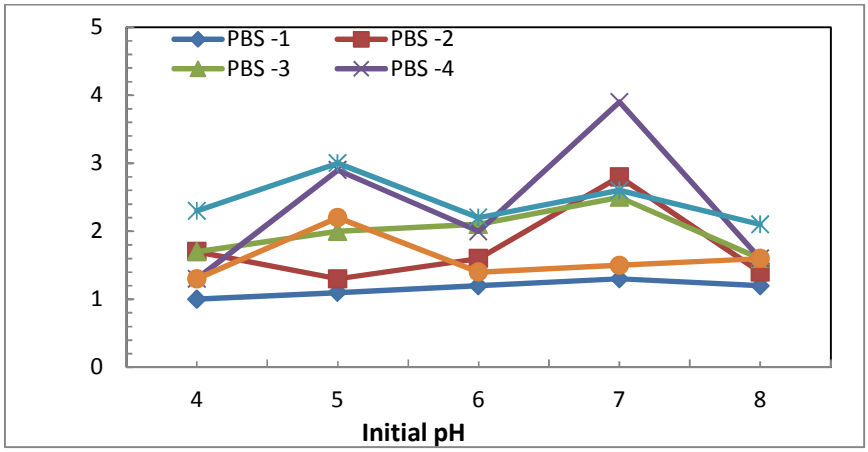


FIGURE 2. Effect of initial pH on IAA production.

Effect of Temperature

All six isolates were assessed for IAA production under varying temperature viz., 30, 35 and 45. After incubation the produced IAA was determined using Salkowski's method. The result obtained were mentioned in figure 3. The optimum temperature for PBS-2, PBS-5 and PBS-6 was 35^{0}C. For PBS-3 and PBS-4 it was 30^{0}C. PBS-1 gave maximum IAA production at 45^{0}C.

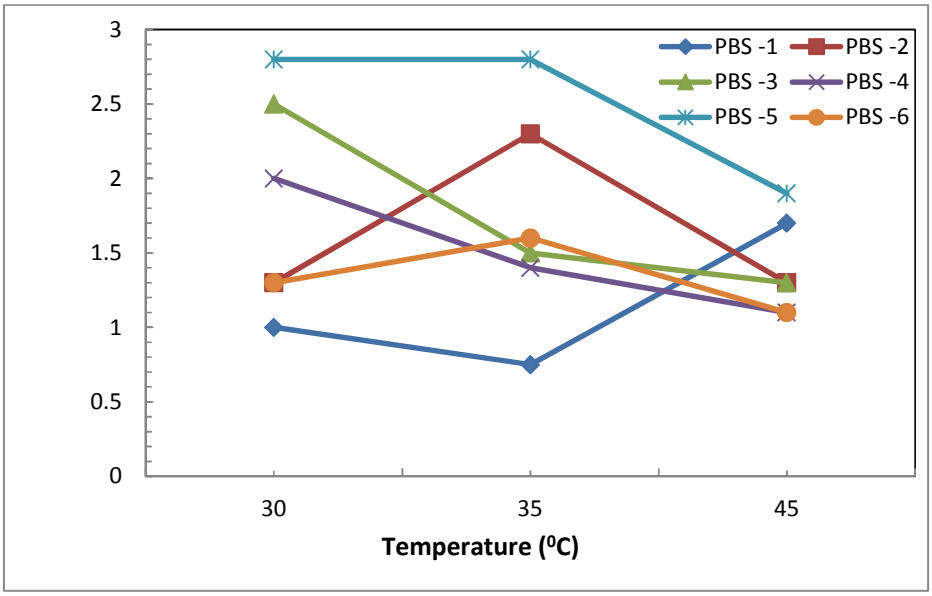


FIGURE 3. Effect of temperature concentration on IAA production

Effect of Pesticide concentration

All six isolates were assessed for IAA production under varying concentration of pesticide (Tafgor) viz., 0, 10, 50, 100, 150, 200, 250, 300, 350 and 400 µl/100ml. After incubation the produced IAA was determined using Salkowski's method. The results obtained were mentioned in Figure 4.

It was found that the concentration of IAA decreased with increase in concentration of Tafgor initially. If the concentration is further increased the IAA production was completely inhibited. IAA production by PBS-2 and PBS-4 was inhibited at the concentration of 200 µl/100ml. IAA productions by PBS-1, PBS-3, PBS-5 and PBS-6 was inhibited at the concentration of 250 µl/100ml. It is evident from data that pesticide residue persisted in the agricultural soil may hamper the plant growth promoting ability of bacteria.

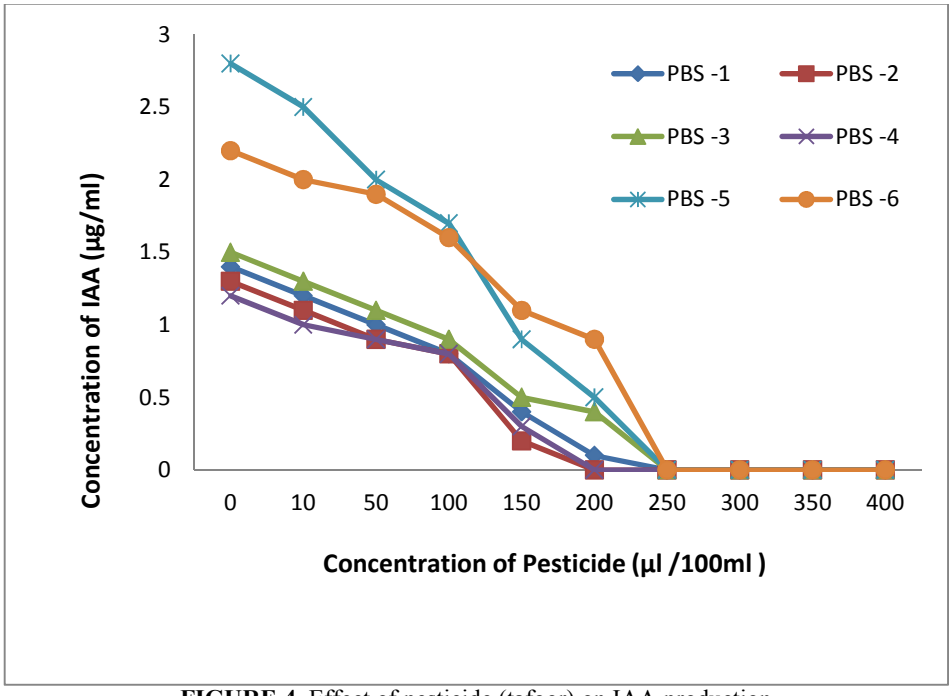


FIGURE 4. Effect of pesticide (tafgor) on IAA production

Assessment of other plant growth promoting activities of the isolates

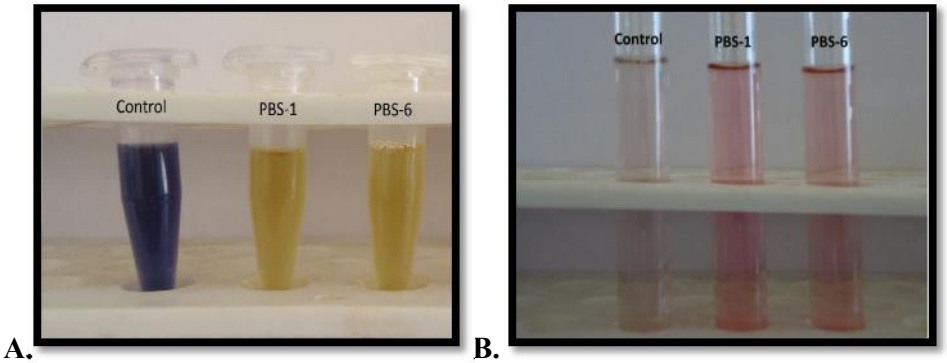
All six isolates viz., PBS-1, PBS-2, PBS-3, PBS-4, PBS-5 and PBS-6 were studied for assessing the presence of other plant growth promoting abilities such as phosphate solubilisation, nitrogen fixation, ammonia production, HCN production, siderophore production and antifungal activity against the *Alternaria alternata.* The results obtained were mentioned in table 1.

It was observed that, PBS-6 bears all the plant growth promoting abilities except Nitrogen fixation. This isolate was not identified. PBS-5 showed three plant growth promoting abilities in addition to IAA production. It can fix nitrogen, produce ammonia and HCN. PBS-3 and PBS-4 have similar properties. Both of them can solubilise phosphate and can produce ammonia and HCN. PBS-2 is a phosphate solubilizer and can produce ammonia in addition to IAA production. PBS-1 has antifungal activity and can produce siderophore. For siderophore producing ability of the isolates cell free sample from each tube was used for CAS assay. Development of yellow colour indicates presence of siderophores (Plate 1A). Out of six only two isolates viz., PBS-1 and PBS-6 have shown siderophore producing ability. As only two isolates, PBS-1 and PBS-6 have shown siderophore production ability, only these two were used for determination of type of siderophore. Both PBS-1 and PBS-6 doesn't show development of yellow colour in Arnow's assay, it confirms the absence of cathecol group of siderophore in both of them. Both PBS-1 and PBS-6 were assessed by Gibson and Magrath assay and it was observed that both PBS-1 and PBS-6 shows development of faint pink colour (Plate 1 B). This confirms the presence of Hydroxamate group of siderophore in them. So, the type of siderophore produced by both isolates PBS-1 and PBS-6 was of Hydroxamate type. PBS-6 was found to be better plant growth promoting bacteria among these isolates. It has total 6 different plant growth promoting activities.

TABLE 1. Other plant growth promoting abilities of the isolates

Isolate	Phosphate solubilization	Nitrogen Fixation	Siderophore production	HCN production	Ammonia production	Antifungal activity
PBS-1	-	-	+	-	-	+
PBS -2	+	-	-	-	+	-
PBS -3	+	-	-	+	+	-
PBS -4	+	-	-	+	+	-
PBS -5	-	+	-	+	+	-
PBS -6	+	-	+	+	+	+

Legend: (-): Negative test (+): Positive test



A. B.

PLATE 1. A. Detection of siderophore by CAS assay, **B.** Detection of Hydroxamate group in siderophore

Identification and characterization of the isolates

The isolates were identified by morphological and Biochemical tests using the Bergey's Mannual of Determinative Bacteriology (9th edition). After incubation the cultural and morphological characteristics of individual isolates were noted. All six isolates were tested for the presence of catalase, oxidase and gelatinase activity. They were also tested for the presence of sugar (sucrose, lactose, mannitol and dextrose) fermentation ability. It was observed that none of the isolate shows gelatine liquefaction. Casein hydrolysis was shown only by PBS -1. All six isolates were showing catalse activity while only PBS- 1 was showing oxidase activity. The results of biochemical characterization and sugar fermentation are mentioned in table 2.

TABLE 2. Biochemical characterization of isolates

Isolates	Catalase	Oxidase	Gelatinase	Casein hydrolysis	Sugar							
					Sucrose		Lactose		Mannitol		Dextrose	
					Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas
PBS -1	+	+	-	+	-	-	-	-	-	-	+	-
PBS -2	+	-	-	-	+	+	+	-	+	+	+	+
PBS -3	+	-	-	-	+	+	-	-	+	-	+	+
PBS -4	+	-	-	-	+	+	+	-	+	+	+	+
PBS -5	+	-	-	-	+	+	-	-	+	+	+	+
PBS -6	+	-	-	-	-	-	-	-	+	+	-	-

Legend: (-): Negative test (+): Positive test

On the basis of cultural, morphological and biochemical characteristics according to *Bergey's Manual of Determinative Bacteriology* (9th edition), PBS-1 and PBS-5 were identified as *Pseudomonas* species and *Azotobacter chrococcum* respectively. PBS-2, PBS-3, PBS-4 and PBS-6 were not identified.

CONCLUSION

All the isolates viz., PBS-1, PBS-2, PBS-3, PBS-4, PBS-5 and PBS-6 were found to be IAA producer. The type of siderophore produced by both isolates PBS-1 and PBS-6 was found to be of Hydroxamate type. PBS-6 is the found to be better plant growth promoting bacteria among these isolates. It was found that the concentration of IAA decreased with increase in concentration of tafgor. PBS-1 and PBS-5 were identified as *Pseudomonas* species and *Azotobacter chrococcum* respectively.

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