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Study on Plant Hormone (Indole-3-Acetic Acid) Producing Level and Other Plant Growth Promotion Ability (PGPA) by *Asparagus racemosus* (L.) Rhizobacteria

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ABSTRACT

Plant hormones (Indole-3-Acetic Acid) regulate and influence the developmental range of cellular and physiological process of plant health. In the present study, ten PGPA bacteria were isolated from rhizospheric soil of Asparagus racemosus(L). Morphological and biochemical test showed that the isolates named KNAD1, KNAD2, KNAD6, KNAD9, KNAD10 belong to genus Bacillus. These isolates were further screened for PGPA like Phosphate Solubilization, production of ammonia and cell wall degrading enzymes. KNAD2, KNAD4, KNAD5, KNAD7, KNAD9 & KNAD10 showed high potential in Phosphate Solubilization and others PGPA. In this study,indole-3-acetic acid (IAA) production by these isolates were carried out day wises and then results showed that on Day2: KNAD6 (86.72ppm); Day3: KNAD7 (85.63ppm); Day4: KNAD2 (116.83ppm); Day5: KNAD4 (121.54ppm); Day6: KNAD1 (86.17ppm); Day7: KNAD6 (126.60ppm) of IAA (µg ml^{-1}) were produced in the presence of tryptophan (1 mg ml^{-1}) at optimum conditions of 37°C and 0.5% NaCl. Additionally, it was further observed that at 1%, 2%and 7% tryptone concentration in the nutrient broth the bacterial growth increased. These results suggest that production of IAA in presence of tryptone might be promising factor for the implementation in plant growth and development.

Keywords: *Asparagus racemosus*, IAA, PGPA, Phosphate Solubilization, rhizobacteria.

INTRODUCTION

Plant growth promoting rhizobacteria (PGPR) are very beneficial for the development of plant health and used as a Biofertilizer. Plant growth promoting activity were linked to IAA production, Phosphate solubilization, enzymes production and signal compounds by the rhizospheric bacteria of many plant [26; 18]. In our study, soil sample were collected from *Asparagus racemosus* rhizospheric zone. *Asparagus racemosus* is recommended as medicinal plant for the prevention and treatment of drug development [28]. It has an adventitious root system with tuberous roots measured about one meter in length, tapering at both ends, with roughly a hundred on each plant [25].Different plant growth promoting rhizospheric bacteria, including associative and symbiotic bacteria such as *Pseudomonas sp., Azospirillum sp., Azotobacter sp., Rhizobium sp., Klebsiella sp., Enterobacter sp., Alcaligenes sp., Arthrobacter sp., Burkholderia sp., Bacillus sp.*,and *Serratia sp.*groups have been used for their beneficial effects on plant growth [12; 7]. PGPA enhance plant health either by direct or indirect mechanisms or pathways. The direct growth promoting mechanisms involve nitrogen fixation, solubilization of minerals, production of phytohormones and the indirect approach occurs when PGPR lessen or prevent the deleterious effects of plant pathogens on plants [14]. Mainly, plant hormones regulate or influence a range of cellular and physiological process, such as cell division, cell enlargement, bud dormancy, flowering, fruit ripening, seed dormancy, seed germination and leaf abscission [14; 17]. IAA play a central role in plant health and development as a regulator of biological processes of cell division, elongation and differentiation to tropic responses, fruit development and senescence. Auxins are employed to induce

rooting, callus formation and flowering. They can also prevent abscission of leaves, flowers and fruits. The action and interaction of some growth regulators like auxins regulate most of the physiological activities and growth in plants. Naturally occurring substances with indole possessing growth promoting activity are referred to as auxins, chemically it is Indole acetic acid [23]. Indole-3-acetic acid (IAA) is one of the main physiologically active auxins. IAA is a common product of L-tryptophan metabolism by numerous microorganisms including PGPR [10; 30]. Microorganisms inhabiting rhizospheres of various plants are likely to synthesize and release auxin as secondary metabolites because of the rich victuals of substrates exuded from the roots compared with non rhizospheric soils [16; 5]. Colorimetric method is the simplest method and has long been employed for the detection of indole-3-acetic acid (IAA) produced by plants and microorganisms and this hormone production was detected in 80% of bacteria isolated from the rhizosphere [4]. Scientifically,it was also proved that rhizospheric bacterial population mainly promote plant healthby production or change the concentration of IAA [21; 13; 1]. So we have focused in effect on isolated bacteria from the rhizosphere of *Asparagus racemosus* and studied various PGPA mainly the day wise production of IAA.

EXPERIMENTAL SECTION

Rhizospheric soil sample of *Asparagus racemosus*(L.) was collected from Dehradun, Uttarakhand, 248002 India (Latitude: 30°-30°32', Longitude: 77°43'-78°24', Altitude-2,200 ft in the Doon Valley and Soil type: alluvial soil) tested for various PGPA and IAA production. The test soil sample was added in 5mL miliQ water and was centrifuged at 250rpm. The fresh upper part water sample was serially diluted (10^{-6}) and dilution sample (100µl/1plate) was plated onto HiVeg Nutrient agar medium (HiMedia, India) (Incubation temperature: 30±0.2°C for 48hrs.) [15].

Morphological and biochemical test was carried out. For the acid-alkaline and gas-sulfur reaction, TSI (Triple Sugar Iron) medium (HiMedia, India) was prepared and all isolate were streaked onto TSI tubes. Test tubes are incubated at 28^0C for 24 hours [6; 8].

Hydrogen sulfide (H_2S) production by the isolates were detected using Kligler's agar (HiMedia, India) by the formation black precipitate at the stabbing side. It can be examined by using the pH indicator phenol red in the medium [15].The citrate utilization test were performed using Simmons Citrate Agar (HiMedia, India) [15; 27]. Starch Hydrolysis test were performed by Starch Agar (Bacterial Peptone: 1.00gm, NaCl: 5.00gm, Starch: 5.00gm, Beef extract: 3.00gm, agar: 15.00gm, Distilled water: 1lit.) [27]. All plates are incubated at 28^0C for 24 hours. Hydrolyzing ability was determined by the development of the clear zone around bacterial colony using iodine solution (1.8% iodine and 3.0% KI).

Casein degradation was determined from clear zone around the colony in Casein hydrolysis agar (Casein: 100.00gm, peptone: 5.00gm, beef: 3.00gm, NaCl: 5.00gm, agar: 15.00gm, pH: 7.2) [21]. Esculine hydrolysis test were performed using bile esculine hydrolysis agar (HiMedia, India) [29].An inoculum from a pure culture was transferred aseptically to a sterile tube of phenylalanine agar to streak the slant. After 5 days of incubation, five drops of 10% ferric chloride ($FeCl_3$) and five drops of 0.1N HCl were added. All tubes were then gently shaken. A positive result was indicated if a green color develops within 7 minutes (11; 27).The purpose was to see if the microbes has catalase, with protective, chemical hydrogen peroxide (H_2O_2). Firstly, 100µl of 24hours bacterial culture was added on a microscopic slide and 50µl hydrogen peroxide (3%) was also added (27).NO^{3-} reduction test was carried out by nitrate broth. Bacterial culture was inoculated and incubated at 28^0C for 48 hours [11].

Test for Plant growth promoting ability (PGPA) were carried out. Phosphate solubilization (PS) ability of the isolates were evaluated in PKV medium (HiMedia, India) incorporated with tri-calcium phosphate (TCP) [$Ca_3(PO_4)_5$] as insoluble phosphate. PKV media plate was prepared and point inoculation was transferred in each plate at 28^0C for 48 hours. After incubation, positive phosphate solubilizing bacteria which give clear zone around the colony were measured [22; 3]. Cellulase enzyme production was determined from clear zone test in CMC (Carboxyl methyl cellulose) agar (CMC: 100.00gm, peptone: 5.0, agar: 15.00gm, pH: 7.2) and Czapek-mineral salt medium ($NaNO_3$-2.2g, K_2HPO_4-1.0g, $MgSO_4.7H_2O$-0.7g, KCl- 0.5g, CMC-5.0g, peptone-2.5g, agar-18.0g, distilled water-1000ml, pH-6.0) [21]. Two days after incubation, 2ml of Congo red solution (0.1% congo red in 1M NaCl) was added in each plates. All the plates were then gently shaken. A positive result was indicated when a clear zone & light develops[31].Protease activity (casein degradation) was determined from clear zone in skim milk agar (skim milk powder-100.0g, peptone- 5.0g, agar-15.0g, and pH-7.2). After the preparation of media, one a single colony was transferred in the plates and was incubated at 28^0C for 48 hours [20]. Detection of ammonia production was done by adding 1.5ml Nessler's reagent (Mercuric chloride: 10.0gm, Potassium iodide: 7.0gm, Sodium hydroxide: 16.0gm, Water (ammonia free): 100.0 ml, pH:13.2±0.05) to a 24 hours culture in 4% peptone broth (5ml) and recording the presence of the yellowish brown colour precipitation [24].

For IAA production, 48 hours bacterial isolate(tryptone containing broth) was taken, 1 ml of Kovac's reagent was added and after 5 minutes result was appeared with pink bands [19].

Standard graph of IAA was prepared as described by Gordon & Weber (1951). Different IAA concentrations were prepared as aqueous solution of IAA ranging from 5-150 µg/ml (ppm). To each 1 ml of the standard, 2ml of 2% 0.5 M $FeCl_3$ in 35% perchloric acid i.e. Salkowaski reagent was added and readings were taken after 30 minutes at 535 nm using UV-Visible spectrophotometer(Thermo Scientific™ GENESYS 10S UV). Standard graph wasfinally prepared by plotting concentration of IAA in µg/ml Vs Optical Density at 535 nm.

Production of IAA was quantified using the Quantitative analysis method [9] on different day. The assay absorbance data were taken after Day2, Day3, Day 4, Day5, Day6 and Day7. Bacterial cultures were grown for 48 hrs in tryptone containing HiVeg nutrient broth. Fully grown cultures were centrifuged (Eppendorf, 5810R)at 4000 rpm for 20minutes at 4^0C. The supernatant (1 ml) was mixed with 4 ml of Salkowaski reagent (50 ml, 35% of perchloric acid, 1 ml 0.5 M $FeCl_3$ solution). Samples were left at 37^0C for 30 minutes. Absorbance was taken at 535 nm with a spectrophotometer.

Bacterial growth were monitored using different concentration of tryptone (0.1%, 0.5%, 1%, 2%, 5%, 7% & 10%) in nutrient broth and absorbance was taken at 660nm with a UV/visible spectrophotometer (Thermo Scientific™ GENESYS 10S UV).

RESULTS AND DISCUSSION

The soil samples are collected from rhizospheric zone of *Asparagus racemosus* L. Then, these samples were plated on Nutrient agar medium (Bacterial Peptone: 5.00gm, Beef extract: 3.00gm, NaCl: 5.00gm, Agar: 15.0gm, pH: 6.8). After 48hours on 30±0.2°C, in this culture plate's 175-190 bacterial colony appeared on 10^{-6} fold serial dilution. Ten bacteria isolates were screened and re-plated in culture media for morphological and biochemical characterizations. [Table 1 & Table 2].

Table. 1. Morphology characterization of different bacterial isolates

MORPHOLOGICAL TEST				
Isolates Name	**Gram Staining**	**Shape**	**Margin**	**Elevation**
KNAD1	+	Bacilli	Undulate	Flat
KNAD2	+	Bacilli	Filamentous	Flat
KNAD3	-	Sarcinae	Entire	Flat
KNAD4	-	Coccobacilli	Undulate	Raised
KNAD5	-	Coccobacilli	Erose	Flat
KNAD6	+	Streptobacilli	Entire	Flat
KNAD7	-	Bacilli	Entire	Flat
KNAD8	-	Diplococcic	Undulate	Flat
KNAD9	+	Bacilli	Entire	Flat
KNAD10	+	Staphylococci	Entire	Convex

IsolatesName	**Form**	**Motility**	**Pigment**	**Colony Colour**
KNAD1	Irregular	+	-	Light greenish
KNAD2	Irregular	-	-	Creamy
KNAD3	Circular	-	-	Creamy
KNAD4	Irregular	+	-	White
KNAD5	Circular	-	-	Creamy
KNAD6	Circular	+	-	Light yellowish
KNAD7	Irregular	+	-	Light white
KNAD8	Irregular	-	-	Creamy
KNAD9	Circular	+	-	Light greenish
KNAD10	Punctiform	+	Yellow	Yellow

Legend: [+]: Positive; [-]: Negative

Morphological characterization of different rhizobacteria, out of the 10 isolates, 5 belongs to the genus bacilli, 2 belongs to the genus coccobacilli, 2 cocci and 1 sarcinae with KNAD10 having a yellow colour pigment.

Table. 2. Biochemical characterization of different isolates

BIOCHEMICAL TEST						
Isolates Name	**TSI**	**H_2S Production**	**Citrate Utilization**	**Ph-ala Deaminase**	**Starch Hydrolysis**	**Peroxidase Test**
KNAD1	K/K	+++	-	-	-	-
KNAD2	K/A	-	-	-	+	-
KNAD3	K/K,G	-	-	-	-	+
KNAD4	K/A	-	-	+	-	+++
KNAD5	A/A,G	-	-	-	+++	+
KNAD6	K/K	++	+++	+	-	++
KNAD7	K/A	-	-	-	-	++
KNAD8	A/A	-	-	+	-	+
KNAD9	K/A,G	+	++	-	+	-
KNAD10	K/A,G	-	+	-	-	-

IsolatesName	**Esculine Hydrolysis**	**Nitrate Reduction**	**Casein Hydrolysis**	**EMB**	**MacConkey's agar**
KNAD1	-	+	-	-	-
KNAD2	-	+	+	+	+
KNAD3	+	-	-	+	++
KNAD4	+++	+	-	+	+
KNAD5	++	+	+	±	+++
KNAD6	++	+	+	-	+
KNAD7	+++	+	-	+	+
KNAD8	-	-	-	+	++
KNAD9	++	-	+	+	++
KNAD10	-	-	-	+	++

Legend: [+++]: High; [++]: Medium; [+]: Low, [-]: Negative; [A]: Acid; [K]: Alkaline; [G]: Gas production. Ph-ala: Phenylalanine

Biochemical characterization of different isolates, out of the 10 bacterial culture, 3 are positive for H_2S production, citrate utilization while 3 are positive for phe-ala deaminase and starch hydrolysis. 6 out of 10 isolates showing peroxidase test positive. Six isolates shows positive result for esculine hydrolysis and 8 isolates shows nitrate reduction positive.

Plant growth promoting activity are known to produce antibiotics, antifungal metabolites, enzymes, phenolics, signal compounds and other determinants of defense in response to pathogen attack [26]. In this study, selected bacteria are tested for Phosphate solubilization, Cellulose enzyme, protease enzyme, ammonia production. Results are shown in figure. 1& Table. 3.

Table. 3. Plant Growth Promotion Ability (PGPA) Test

Plant Growth Promotion Ability					
Isolates Name	**Phosphate Solubilization**		**Cellulose Ability**	**Protease Ability**	**Ammonia Production**
	Ability	**Zone Diameter (mm)**			
KNAD1	-	-	-	++	-
KNAD2	+	10mm	+	+	++
KNAD3	-	-	-	+	+
KNAD4	+	12.5mm	+	-	+
KNAD5	+	11mm	+++	++	++
KNAD6	-	-	+++	-	+
KNAD7	+	13mm	+	+	+++
KNAD8	-	-	-	+	+
KNAD9	+	26mm	-	-	++
KNAD10	+	13mm	-	-	-

Legend: [+++]: High; [++]: Medium; [+]: Low, [-]: Negative

Indole acetic acid (IAA) production is a vital property of rhizobacteria that stimulate and facilitate plant growth and development. The present work deals with isolation, characterization and identification of indole acetic acid producing bacteria from the rhizospheric soil of *Asparagus racemosus* [2]. At first, selected ten bacteria were screening for IAA production with adding of Kovac's reagent (Figure. 6).

Standard graph of IAA was prepared as described by Gordon & Weber (1951). Standard graph is prepared by plotting concentration of IAA in µg ml^{-1} Vs Optical Density at 535 nm. Straight-line graph indicates direct proportional concentrations of IAA and indole production. R^2 value of the graph was found to be 0.9327 that showed the validity of the graph. Rf values of the standard IAA production and IAA produced by isolates showed same value (Figure. 2).

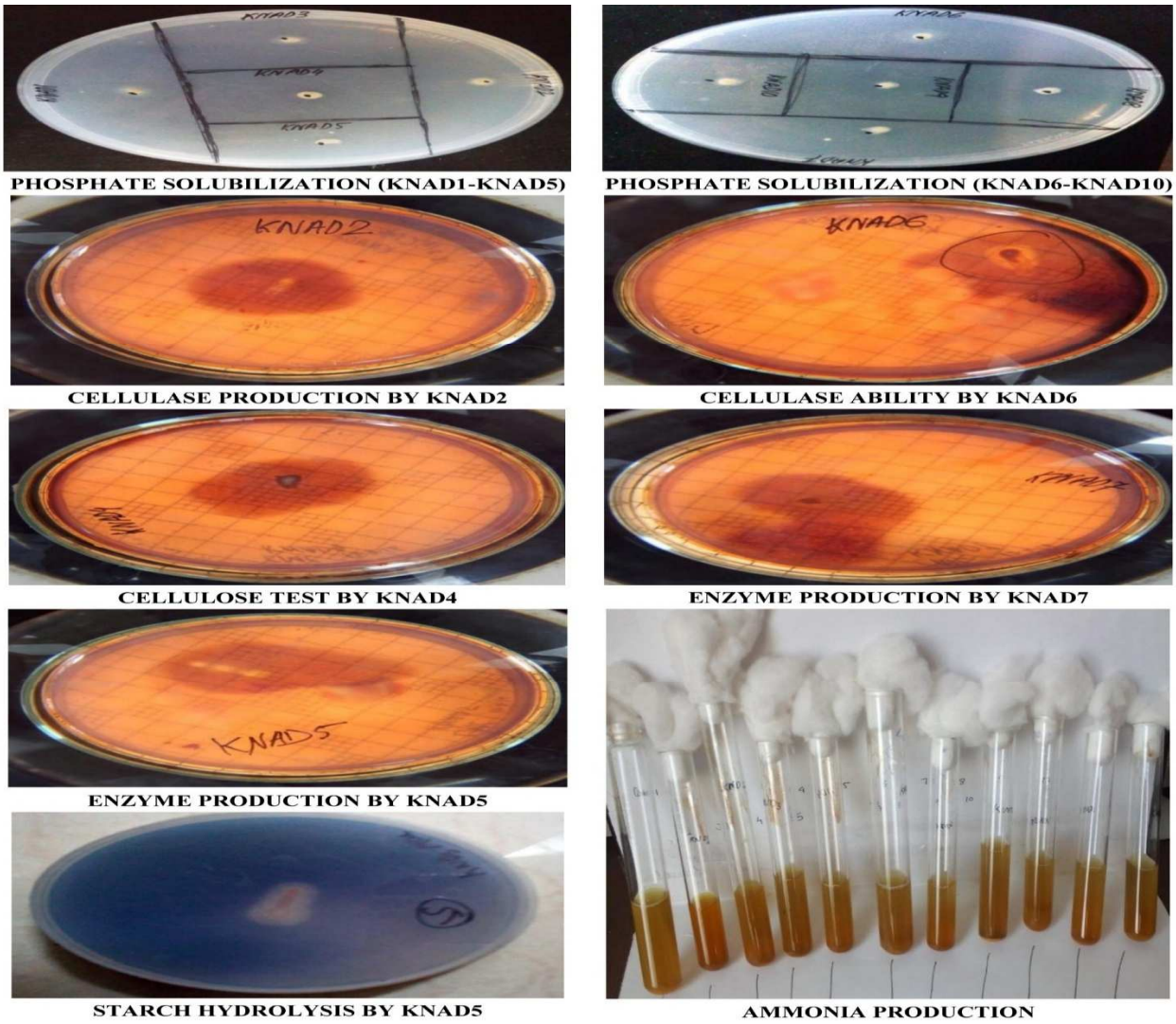


Figure. 1. Plant Growth Promoting ability (PGPA) test

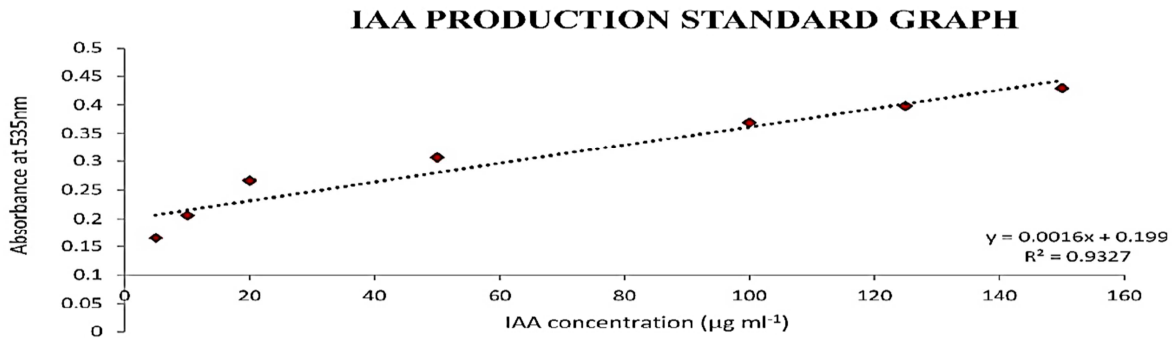


Figure. 2. Indole-3-Acetic Acid Production standard graph

Quantitative assay of day wise (Day2, Day3, Day 4, Day5, Day6 and Day7) IAA production was done. Isolated bacterial cultures were grown for 48 hrs in tryptone containing HiVeg nutrient broth (Figure. 4) and centrifuged at 4000 rpm for 20minutes at 4^0C. 1ml supernatant was mixed with 4 ml of Salkowaski reagent and kept at 37^0C for 30 minutes. Development of pink color indicates production of IAA. Optical density was taken at 535 nm with a spectrophotometer. Concentration of IAA produced by bacterial cultures was measured with the help of standard graph of IAA. In this study, concentration of IAA produced maximum day2: KNAD6, KNAD7, KNAD8; day3: KNAD7, KNAD6, KNAD2; day4: KNAD2, KNAD7, KNAD8; day5: KNAD3, KNAD4, KNAD2 day6: KNAD1,

KNAD3, KNAD4; day7: KNAD2, KNAD6, KNAD4 in the presence of tryptophan (1 mg ml^{-1}). Quantitative estimation all results are show in Table. 4 &figure. 3.

Table. 4. Highest IAA producer isolates

DAY2		DAY3		DAY4	
Isolates Name	*IAA (ppm)*	Isolates Name	*IAA(ppm)*	Isolates Name	*IAA(ppm)*
KNAD6	*86.72*	KNAD2	*47.38*	KNAD2	*116.83*
KNAD7	*46.89*	KNAD6	*48.30*	KNAD7	*99.18*
KNAD8	*44.11*	KNAD7	*85.63*	KNAD8	*96.47*

	DAY5	DAY6		DAY7	
Isolates Name	*IAA(ppm)*	Isolates Name	*IAA(ppm)*	Isolates Name	*IAA(ppm)*
KNAD2	*45.09*	KNAD1	*86.17*	KNAD2	*122.17*
KNAD3	*120.60*	KNAD3	*48.36*	KNAD4	*120.09*
KNAD4	*121.54*	KNAD4	*45.75*	KNAD6	*126.60*

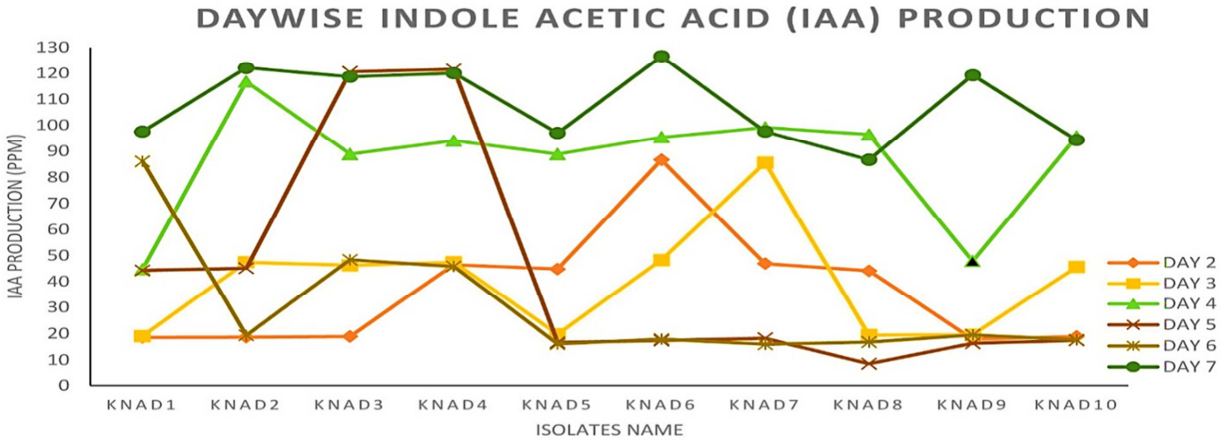


Figure. 3. Day wise Indole-3-Acetic Acid Production at 535nm

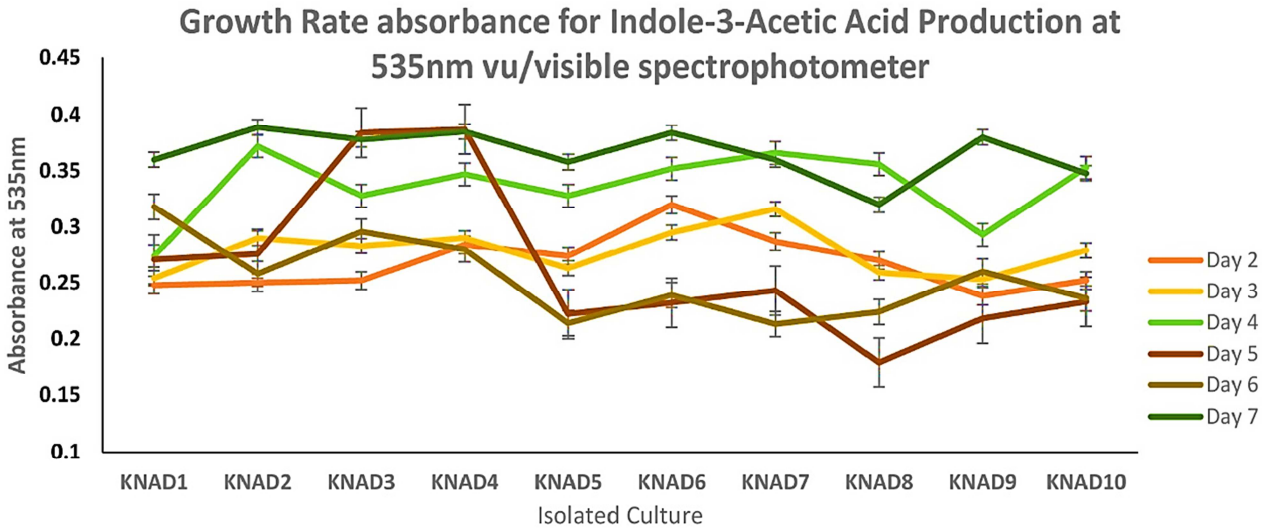


Figure. 4. Growth rate for IAA production in tryptone broth

When bacterial growth was measured as against the 0.1%, 0.5%, 1%, 2%, 5%, 7% & 10% tryptone concentration, result was found that in 0.1% (KNAD1, KNAD9, KNAD10), 0.5% (KNAD9, KNAD6, KNAD10), 1% (KNAD7, KNAD9, KNAD3), 2% (KNAD6, KNAD9, KNAD10), 5% (KNAD2, KNAD9, KNAD7) and 7% (KNAD9, KNAD7, KNAD5) isolated growth rate was high (Figure. 5). So, KNAD7 was considered to the best producer of IAA.

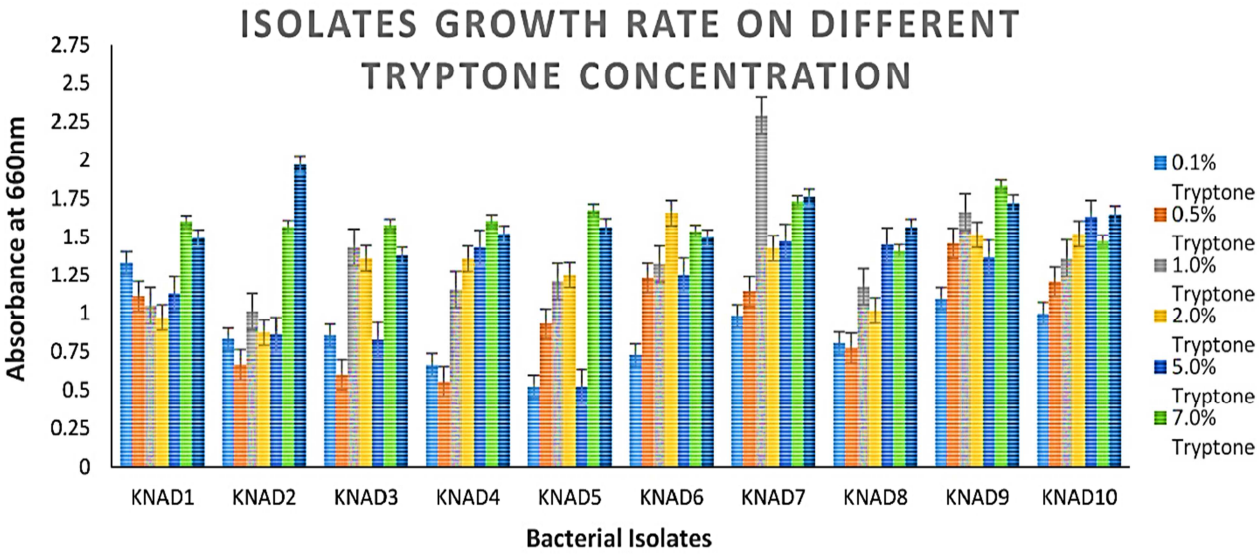
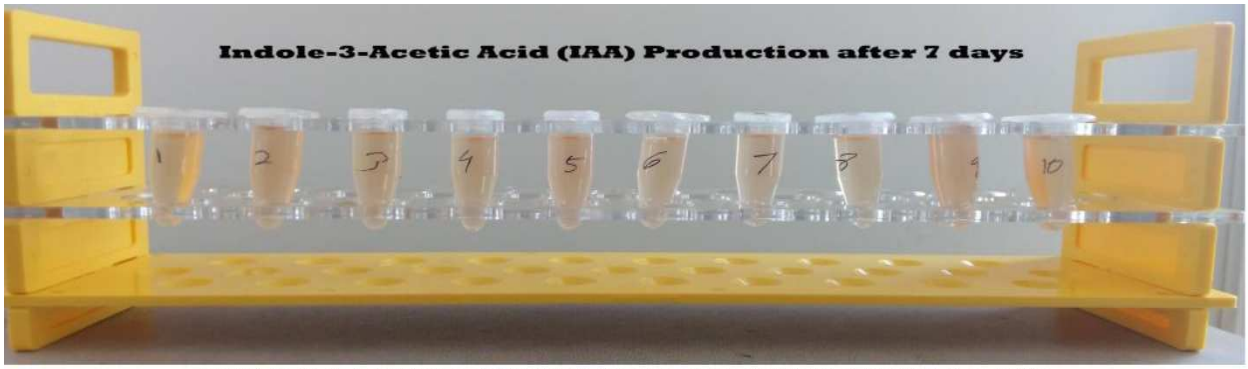


Figure. 5. Growth rate on different tryptone concentration



INDOLE-3-ACETIC ACID (IAA) PRODUCTION AFTER 7 DAYS

TSI (TRIPLE SUGAR IRON) TEST

ESCULINE HYDROLYSIS BY KNAD1-KNAD10

Figure. 6. IAA production (Assay), TSI test, Esculine hydrolysis

CONCLUSION

All the strain isolated produced IAA and out of all the isolated ten strain, KNAD2, KNAD4, KNAD6 given highest IAA production. Optimization of indole acetic acid production level and isolates growth rate in tryptone was carried out at different cultural condition. So, these isolates can be used as efficient biofertilizer inoculants for the study of plant microbes interaction.

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