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Bioproduction of Indole Acetic Acid (IAA) by *Rhizobium* Strains Isolated from Root Nodules of Green gram, *Vigna radiata* L.

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The phytohormone auxins play a central role in plant growth and development as a regulator of numerous biological processes, from cell division, elongation and differentiation to tropic responses, fruit development and senescence. 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 nucleus possessing growth-promoting activity are referred to as auxins, chemically it is Indole acetic acid. (IAA) Not only plants but also microorganisms can synthesize auxins and cytokinins. IAA production was measured under varying tryptophan concentration, carbon sources, nitrogen sources and at different pH. Four *Rhizobium* strains were isolated from root nodules of *Vigna radiate* L collected from Narsanda farm (Dist.Kheda, Gujarat). All the four *Rhizobium* strains produced indole acetic acid (IAA), but maximum amount was produced by only two strains in yeast extract mannitol (YEM) medium supplemented with L-tryptophan. The *Rhizobium* strains M1 and M2 were found to elaborate maximum IAA when fed with 15μg /ml L-tryptophan. Cultural requirements were optimized at different pH for maximum growth and IAA production. The strains differ in their growth and production of IAA on different carbon and nitrogen sources. The significance of the study could be stated as the potential of these IAA producing isolates and optimization study for IAA production will flourish the growth and ultimately IAA production in the field and prevent environmental pollution by avoiding excessive applications of industrially produced fertilizers to cultivated fields.

Key words: IAA, *Rhizobium*, Carbon source, Nitrogen source, *Vigna radiata* L.

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. Because roots are underground rhizosphere activity has been largely overlooked. The rhizosphere is a centre of intense biological activity due to the food supply provided by the root exudates.

However, there are some microorganisms that do interact with specific plants. These interactions can be pathogenic, symbiotic, harmful, saprophytic or neutral. Interactions that are beneficial to agriculture include mycorrhizae, legume nodulation and production of antimicrobials compounds that inhibit the growth of pathogens. Rhizosphere microorganisms produce vitamins, antibiotics, plant hormones and communication molecules that all encourage plant growth. Microbial population in rhizosphere may benefit the plant in a variety of ways including increased recycling and solubilization of mineral nutrients, synthesis of vitamins, amino acids, auxins, cytokinins and gibberellins which stimulate plant growth and antagonism with potential plant pathogens through competition and development of amensal relationships based on production of antibiotics.

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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 nucleus possessing growth-promoting activity are referred to as auxins chemically it is Indole acetic acid. The ability to synthesize phytohormone is widely distributed among plant associated bacteria. Bacteria has abili80% of the bacteria isolated from plant rhizosphere are to produce IAA. According to Halda-Alija, up to 74% of rhizobacteria identified and tested to produce IAA.

The plant growth regulator indole acetic acid (IAA) has long been postulated to play a role in one or more aspects of nodule growth and development and the detection of increased levels of IAA in nodule tissue supports this hypothesis [Datta, and Basu(1988)]. According to Gosh and Basu (1998) the associative nitrogen-fixing bacteria tested produced IAA, especially with tryptophan as a precursor.

The phytohormone auxins plays a central role in plant growth and development as a regulator ofnumerous biological processes, from cell division, elongation and differentiation to tropicresponses, fruit development and senescence [Rodriguez *et al.* (1981)] .Not only plants but also microorganisms can synthesize auxins and cytokinins. The role of phytohormone biosynthesis by microorganisms is not fully elucidated. But it was indicated that there might exist a symbiotic association between plants and microorganisms. Hence the present study was undertaken to isolate organisms from nodules of leguminous plants and its rhizosphere and to study IAA production under laboratory condition.

MATERIALS AND METHODS

Isolation of *Rhizobium* species

Mung bean (*Vigna radiata* L.) root samples were collected from different locations of Narsanda (Dist.Kheda, Gujarat). The root samples were saved in polythene bags and transferred to the laboratory. In the laboratory, the roots were washed gently with tap water to remove the soil. Then nodules were separated from the roots and placed in petri-plates. The collected nodules were surface sterilized by momentaring dipping in 95% ethanol solution followed by dipping in 0.2% $HgCl_2$ solution for 3- 5 minutes and 6-7 times washings with sterilized water[Barbara *et al* .(1989)]. The surface sterilized nodules were crushed in a minimal volume of sterilized water with the help of a sterilized glass rod to obtain a milky suspension. A loopful of the suspension was streaked out on yeast extract mannitol (YEM) agar medium [yeast, 0.5 g; mannitol, 10.0 g; K_2HPO_4, 0.5 g; $MgSO_4.7H_2O$, 0.2 g; NaCl, 0.1 g; distilled water, 1000 mL; pH 6.8] plates and incubated at 28 ± 1^0C [Normanly *et al.* (1993)]. Well isolated, colourless, single colonies, shiny in appearance, were picked and re-streaked on clean plates to obtain the pure cultures. In this way, forty four fast growing colonies of bacteria were selected, isolated and purified from the mung bean nodules. The purified rhizobial cultures were stored at 4±1^0C on slants and maintained for further experimentation.

Effect of tryptophan concentration on IAA production

The isolates were transferred on fresh media when needed. Sterilized yeast mannitol broth (YMB) was inoculated with rhizobial isolates in the presence or absence of filter (0.2 µm)- sterilized L-Trp @ 5,10,15(µg/ml) of broth in glass tubes. These tubes were incubated at 28±1^0C for 48 hours in shaking incubator at 110 rpm. After incubation the contents were filtered through Whatman filter paper No. 2 before measuring auxin production in terms of Indole-3-acetic acid (IAA) equivalents. The broth was centrifuged at 7000rpm for 10 minutes. Supernatant was collected. To 1ml supernatant, 2ml of Salkowaski reagent was added and extent of red colour i.e. IAA produced was measured spectrophotometrically at 530 nm. [Apine *et al.*(2011); Bhattacharya *et al.*(1999)]

Effect of Carbon sources on IAA production:

IAA production was studied by replacing mannitol from YEMB by glucose, sucrose, lactose, Arabinose, Xylose and mannitol 1% w/v. supplemented with 10 µg/ml of tryptophan. IAA production was studied by using Salkowaski reagent after 24, 48 and 72 hrs. Cultures selected for the study were M1, M2 and M3 i.e. *Rhizobium* isolated from Mung bean[Pedraza *et al.*(2004)]

Effect of Nitrogen sources on IAA production

IAA production was studied by changing nitrogen sources like YEMB by KNO_3, $(NH_4)_2SO_4$, $NaNO_3$, $NaNO_2$, L-asparagine, L-glycine, Casamino acid, L-glutamic acid, Cystine, Tyrosine 1% w/v.

supplemented with 10 µg/ml of tryptophan. IAA production was studied by using Salkowaski reagent after 24, 48 and 72 hrs.[Rubio *et al*. (2000)]

Effect of pH on IAA production

To study the extent of IAA produced by the different isolates at different pH, YEMB with 10µg /ml of tryptophan were adjusted to different pH as 5, 6, 7, 8 and 9. Media were inoculated with 1% inoculum of O.D.600 1.0 and incubated at 28^{0}C for 24 hrs. IAA production was studied by using Salkowaski reagent after 24hrs. [Russell *et al*. (1982).

RESULTS

Isolation and identification of *Rhizobium*

White colored, mucoid, and like a drop of water colonies observed on YEMA with congo red were the characterisiitcs of *Rhizobium* sp. The representative colony used for further biochemical chcracterization also reflected the similar biochemical characteristics to that of *Rhizobium* sp. From the results of morphological, cultural and biochemical characters and the host from which it was isolated, the isolates were identified as *Rhizobium sp.* from Mung bean. (Table 1)

A total four isolates were collected from the nodules of *Vigna radiate* L. district of Kheda of Gujarat. These isolates were designated as Rhizobia on the basis of their colony characteristics, cell morphology and cultural characteristics. Differences between isolates were verified using morphological parameters. All isolates were gram negative, rod shaped and non motile.

Temperature is one of the most important factor affecting the survival of rhizobia in soil. In the present investigation all three isolates could be able to grow at 39-40°C.

High salt concentrations may have a detrimental effect on soil microbial populations. The root nodule-colonizing bacteria (*Rhizobium*) are more salt tolerant than their legume hosts, they show marked variation in salt tolerance. The results of the present studies revealed that salt concentration in the growth medium had significant effect on the growth of *Rhizobium*. M1 and M3 strains grew lightly at 2 % but M2 could not able to grow at this concentration. In the present study, M1 and M3 isolates were able to grow on 2 % NaCl and growth rate was increased from I week to II week whereas in III week, growth was decreased for both isolates. All the four isolates were not produced H_2S and could not able to hydrolyse casein. All three isolates utilized all carbohydrates as a sole source of carbon. Positive results were obtained from the starch hydrolysis assay as well as alkaline phosphatise test. On subjecting inoculated plates to iodine test, clear zones around the colonies were seen and the colonies turned yellow in appearance, whereas blue color appears on no growth areas. It indicates the isolates have the potential to hydrolyze starch present occur in the medium.

Table 1. Results of Biochemicals

Chracteristics	M1	M2	M3
Root nodules produced	+	+	+
Fast growth on YEMA	+	+	+
Growth at 39-40^{0}C	+	+	+
Growth in presence of 2% NaCl	+	-	+
H_2S production	-	-	-
Growth at pH			
3.5	-	-	-
4.0	-	-	-
4.5	-	-	-
5.0	+	+	-
8.0	+	+	+
9.0	+	+	+
9.5	-	-	+
Alkaline phosphate activity	+	+	+
Starch hydrolysis	+	+	+
Casein hydrolysis	-	-	-
Utilization of sugars			
Glucose	+	+	+
Sucrose	+	+	+
Lactose	+	+	+
Maltose	+	+	+
Mannitol	+	+	+
Xylose	+	+	+
Fructose	+	+	+
Rhmnose	+	+	+
Arabinose	+	+	+
Raffinose	+	+	+
Dulcitol	-	+	-
Antibiotic sensitivity			
Penicillin	+	+	+
Streptomycin	+	+	+
Tetracycline	+	+	+

M1, M2 and M3 : *Rhizobium* strains isolated from Mung bean

The present observation, four isolates were able to grow well in the presence of glucose, sucrose, lactose, maltose, mannitol, xylose and fructose In the present investigation, all four strains showed good growth on carbohydrates. From the present study it is evident that all the strains effectively utilized a wide range of carbohydrates, it is one of the important criteria to be considered as plant growth promoting bacteria.

Table 2. Effect of L- Tryptophan concentration on the growth of *Rhizobium* strains

	IAA concentration (µg/ml)		
	3DAI	5 DAI	7 DAI
5µg			
M1	50.6	71.0	60.2
M2	25.4	49.0	39.4
M3	17.5	39.0	29.7
10 µg			
M1	37.9	80.3	71.2
M2	33.2	68.0	52.4
M3	23.6	52.0	39.3
15µg			
M1	51.3	103.5	96.4
M2	49.6	98.5	77.8
M3	27.4	75.9	63.6

Table 3. Growth of *Rhizobium* strains in absence of L-Tryptophan

	IAA concentration (withouttryptophan) (µg/ml)		
	3DAI	5 DAI	7 DAI
M1	20.3	35.9	26.7
M2	12.3	24.9	22.6
M3	8.7	22.5	18.5

Table 4. Effect of nitrogen sources on growth and IAA production

Nitrogen Sources	IAA concentration (µg/ml)		
	3DAI	5 DAI	7 DAI
Control	2.06	2.59	2.74
KNO_3	149.2	125.8	108.5
$(NH_4)_2SO_4$	27.8	32.4	40.5
$NaNO_3$	80.6	84.3	46.3
$NaNO_2$	69.7	54.6	59.7
L-asparagine	8.9	9.4	4.6
L-glycine	10.1	12.7	11.5
Casamino acid	21.7	23.9	36.4
L-glutamic acid	20.6	25.2	21.8
Cystine	15.9	17.6	14.7
Tyrosine	6.4	7.8	13.6

Soil acidity is a significant problem facing agricultural production in many areas of the world and limits legume productivity. pH is an important parameters for the growth of the *Rhizobium*. Slight variations in pH of medium might have enormous effects on the growth of organism. *Rhizobium* has been reported to grow the best at neutral pH i.e., 7. In the present observation, pH 7 recorded maximum absorbance for the broth experiment except *Rhizobium* strain M1. *Rhizobium strains* M1 and M2 were showed good growth between pH 5 and 9.5, but less growth was observed at pH 3, 4 and 4.5. These observations are in line with the reports of In the present study, all rhizobial isolates growth rate was increased at all the pH level on II and III week of growth. Rhizobial strains M1 and M2 was tolerant to pH 5 and 9. Rhizobia with a higher tolerance to acidity and alkalinity will be of great impacts in acidic or alkaline soil conditions in the field.

Antibiotic resistance in *Rhizobium* strains was tested against different antibiotics. M1, M2 and M3 rhizobial isolates were resistance to penicillin, streptomycin and tetracycline In general, data obtained from this study clearly show that certain concentration of antibiotics used in the medium (YEMA) was suitable for differentiation between members of the rhizobial groups. Depends on the differences between strains of rhizobia towards the different antibiotics, this technology could be successfully, employed for the field of ecological studies of rhizobia.

Effect of tryptophan concentration

Most of the organisms produce IAA in presence of tryptophan. In present study it was observed that as the concentration of tryptophan in the medium increases, the amount of IAA produced increased. All three *Rhizobium* strains M1, M2 and M3 was found to be the efficient producer of IAA. Using 15 µg/ml concentration of tryptophan in the medium, IAA production within 24 hrs was found to be 103.5 µg/ml, 98.5µg/ml and 75.9µg/ml respectively M1, M2, and M3 (Table.2). IAA production was found to be decreased in 5 DAI. This may be due to its degradation by the

isolate. *Rhizobium* strain M1 isolate showed maximum production at 103.5 µg/ml of tryptophan concentration after which amount of IAA produced decreased. The same organism showed maximum IAA production at all concentrations. *Rhizobium* strain M2 isolates gave graded response to tryptophan concentration.

All organisms showed little amount of IAA production in absence of tryptophan, which the requirement of tryptophan as a precursor for the synthesis of IAA (Table3). Various researchers reported variable IAA production ability of bacteria produced 20-90.8mg/L of IAA where as *Azotobacter chrococcum, Azotobacter vinelandii, Pseudomonas aeruginosa, Pseudomonas putida, Serratia* sp. and *Klebsiella pneumoniae* strains

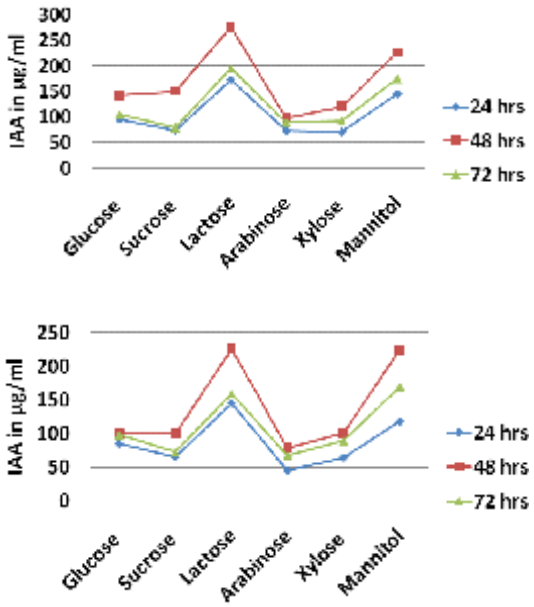

Fig 1(A-B). Effect of carbon sources on IAA production

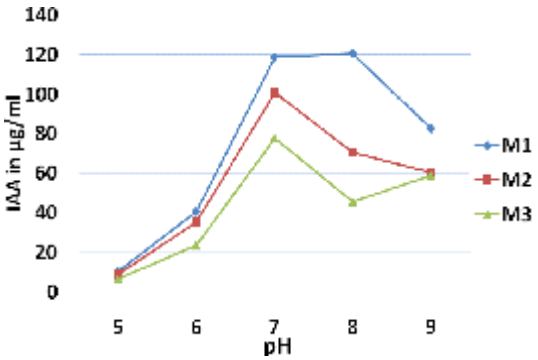

Fig. 2. Effect of pH on IAA production

shown to produce IAA from 3.5 mg/ml to 32.2mg/ml. *Azospirillum brasilense* produced 26.1µg/ml of IAA[Rubio *et al*.(2000)]. The bacteria produced a high amout(107 µg/ml) of IAA in culture from tryptophan supplemented yeast extract mannitol medium.[Russell *et al*. (1982)] Selected cultures showed much higher IAA production than reported.

Effect of Carbon sources on IAA production

For finding out the most favourable carbon source giving maximum IAA production mannitol from YEMB is replaced by different sugars. Rhizobial isolates responded in varied manner to different carbon sources. *Rhizobium* strains M1 and M2 showed maximum IAA production in presence of lactose, mannitol in 48 hrs. In 48 hrs M1 isolate gave maximum production of IAA in presence of glucose. For the study of effect of carbon source on IAA production M1 and M2 were selected as these organisms are found to be the efficient producers. Isolate M1 showed maximum IAA production in presence of lactose, mannitol, glucose followed by sucrose, arabinose, xylose and in descending order. Ghosh and Basu reported 1% glucose as a preferred carbon source for IAA production. *Rhizobium* strain M2 used lactose as the best carbon source as it gives maximum IAA production in presence of Lactose as compared to all carbon sources (Fig 1 A & B). The majority of rhizosphere bacteria produces growth promoting substances like IAA, gibberillins and cytokinins. Rhizobia are known to produce IAA in the culture supernatant and the carbon sources present in the medium influence its production (Gosh and Basu, 2002).

Effect of Nitrogen sources on IAA production

Effect of different nitrogen sources (0.1%) was studied by replacing yeast extract in the original YEM medium supplemented with L-tryptophan. It revealed that the M1 utilized inorganic nitrogen sources like KNO_3, $NaNO_2$ and $NaNO_3$ and produced maximum amount of IAA, while amino acid glycine as additional nitrogen source reduced growth and IAA production. Some amino acids were shown earlier to inhibit IAA production by *Rhizobium meliloti* (Garcia-Rodriguez *et al*. 1981) due to inhibition of conversion of tryptophan to IAA. The *Rhizobium* strain M1 showed maximum growth and IAA production on organic nitrogen sources (casamino

acid, cystine, and tyrosine), while the *Rhizobium* strain M2 utilized and produced IAA on both organic and inorganic nitrogen sources (Table 4). Among the five Rhizobium strains, the maximum growth and IAA production were observed in Rhizobium strain M1 in the medium amended with L-glutamic acid as nitrogen source. When L-glutamic acid used as nitrogen source, a *Rhizobium* sp. from root nodules of Cajanus cajan was also reported to produce maximum IAA.[Bhattacharya *et al*. (1999)]

Effect of pH on IAA production

To decide the optimum pH for IAA production, the isolates are inoculated in YEMB amended with 10 µg/ml of tryptophan having different pH such as 5, 6, 7, 8, 9. (Fig 2) All rhizobia showed no or little amount of IAA production at pH 5 whereas maximum production found at pH 7. IAA production decreased at pH 9. *Pantoea agglomerans* produced maximum IAA production at pH 7. [Russell *et al*. (1982)] . *Rhizobium* strains M1 and M2 showed little amount of IAA production at pH 5 and 6 respectively. All isolates showed maximum IAA production at pH 7 expect M1, which showed peak at pH 8. Hence optimum pH for IAA production was found to be 7.

DISCUSSION

Rhizobial strains were isolated from the nodules of mung bean. These strains were screened for their auxins biosynthesis in the presence and absence of L-tryptophan. The selected strains (efficient auxins producers) were further evaluated for their ability to utilize different carbon and nitrogen sources.

Vincent (1970) reported that the *Rhizobium* grown on YEMA medium and produced small to medium sized colonies, usually smaller than 2mm. This result was confirmed by using Bergey's Manual of determinative bacteriology (Holt *et al*. 1994). Similar findings were made by Baoling *et al*. (2007).

Graham (1992) reported that, for most rhizobia the optimum temperature range for growth in culture is 35 to 40°C. Kucuk *et al*. (2006) reported that the among the 30 isolates, 18 were able to grow at 37 and 40°C, whereas 5 isolates showed only minimal growth at 42 and 45°C. Embalomatis *et al*. (1994) reported that the growth of a number of rhizobia was inhibited by 100 mM NaCl, while some rhizobia, e.g., *Rhizobium meliloti* were tolerant to 300-700 mM NaCl. Previous studies reported by Talibart *et al*. (1994) have shown that the changes in osmotic potential exerted by salt concentration alter the structure of lipolysaccharides of bacteria in response to salt stress and that rhizobia accumulate several solutes to overcome the osmotic stress induced by salt when growing in association with the host plant. Lloret *et al*. (1995) also reported that the *Rhizobium* strains capable of growing at NaCl concentrations of upto 0.5 M have been isolated from melilotus plants. This similar findings were recorded by Mensah *et al*. (2006).

De Oliveira *et al* (2010) also observed that the *Rhizobium* strains utilize the starch obtained from the different sources. Similar observations were also reported in *Rhizobium* isolates from root nodules of *Sesbania sesban* (Helmish *et al*. 1993). Mensah *et al*. (2006) recorded that the optimum pH of the growth of *Rhizobium* is 7. Similar findings were made by Singh *et al*. (2008) reported that *Rhizobium* were able to grow at pH 7 and kept at 29.4°C but no growth was observed in medium with pH 3 to 4.5. Aurag and Sasson (1992) indicated that strains of *Rhizobium leguminosarum* bv. *phaseoli* grew in liquid media of pH 5. Issa and Wood (1995) reported that the use of relatively low (or) moderate concentration of the antibiotics give a more reliable information about the IAR and how far is the range of resisted antibiotics a *Rhizobium* strain can tolerate and this could be very helpful on the ecological and diversity studies of rhizobia. . This similar observations were reported by Milcic *et al*. (2006).

Effect of tryptophan concentration

In this study, all the rhizobial isolates produced auxins (expressed as IAA equivalents) in the presence and absence of L-tryptophan but with variable degrees of efficacy. However, the auxin production by all rhizobial isolates was more in the presence of L-tryptophan (an auxin precursor). The *Rhizobium* strains are known to prefer L-tryptophan for IAA production. In this study, all the rhizobial isolates produced auxins (expressed as IAA equivalents) in the presence and absence of L-tryptophan but with variable degrees of efficacy. However, the auxin production by all rhizobial isolates was more in the presence

of L-tryptophan (an auxin precursor). Auxin biosynthesis by *Rhizobium* sp. has also been reported by Bhattacharyya and Pati (2000). They reported that *Rhizobium* sp. isolated from the root nodules of *Alysicarpus vaginalis* produced higher amounts (107µg mL^{-1}) of auxin in the presence of L-TRP. Similarly, higher amount of auxins production by different bacterial strains in the presence of L-TRP has been reported by other researchers (Datta and Basu, 1998; Ghosh and Basu, 2002; De and Basu, 2007). Many earlier researchers have reported similar results in agreement to the present study who have demonstrated that rhizobia are capable of producing auxin and auxin biosynthesis increased many folds in Tryptophan supplemented medium (Sridevi and Mallaiah ,2008; Etesami *et al*., 2009).

It has been reported by Ghosh and Basu (1999) that mature stem nodules of *Aeschynomene aspera* L. contained high amount (2.54 µg g^{-1} fresh weight) of IAA which may be due to the production of higher quantities of auxin in L-TRP supplemented medium by the microsymbiont, *Azorhizobium caulinodans*. Similar results have also been reported by other scientists (Arshad and Frankenberger, 1992; Zahir *et al*., 2000; Asghar *et al*., 2002; Zahir *et al*., 2005; Ghosh and Basu, 2006).

The isolated organisms were identified as *Rhizobium* sp. It could be concluded that the IAA produced by the organisms could be used as sprays for plant growth promotion. Out of the selected Rhizobia, M1 *was* found to be the best to produce IAA. Co- inoculation of rhizobia with other plant growth promoting bacteria can be done for growth promotion. All the isolates produce only IAA in the culture medium and no other interfering substance. *Rhizobium* strain M1 showed maximum IAA production at all concentrations of Tryptophan. All organisms produced little amount of IAA in absence of Tryptophan. Optimum pH for IAA production was found to be 7.

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