Bulletin of Environment, Pharmacology and Life Sciences
Bull. Env. Pharmacol. Life Sci., Spl Issue [1] January 2023: 403-408.

Online ISSN 2277-1808
Journal's URL:http://www.bepls.com
CODEN: BEPLAD

ORIGINAL ARTICLE **OPEN ACCESS**

Isolation and Characterization of Indole Acetic Acid (IAA) Producing *Rhizobium* Spp. from Leguminous Plants in Presence of Pure Tryptophan and Other Precursors.

Pooja S. Patil
Department of Microbiology, Smt. Kusumtai Rajarambapu Kanya Mahavidyalaya, Islampur, Sangali, Maharashtra, India.
Author email: poojapatil9414@gmail.com.

ABSTRACT:
The Phytohormone auxins plays a central role in plant growth and development as a regulator of numerous biological processes like cell division, cell enlargement, root formation, bud inhibition and abscission of leaves and fruits. Not only plants but also microorganisms can synthesize auxins. Indole acetic acid (IAA) production is a major property of rhizosphere bacteria that stimulate and facilitate plant growth. Among that Rhizobium spp. is used for the production of IAA in larger amount which can be further used as efficient bio-fertiliser. Hence the present paper discuss isolation, characterisation and identification of IAA producing Rhizobia from the root nodules of leguminous plants as well as shake flask studies on production of IAA. Four different samples were collected from the four different plant root nodules. Out of them, isolate GM is high yielding isolate produces 350µg/ml of IAA which is further used for colorimetric assay in absence and presence of pure tryptophan and other precursors like soya flour. Purification of IAA was confirmed with thin layer chromatography. Chromatogram of culture showed a pink spot of purified IAA at the Rf value (0.74).
KEYWORD: *Indole acetic acid, Rhizobia, Tryptophan, Soya flour.*
Received 12.11.2022 Revised 23.11.2022 Accepted 20.12.2022

INTRODUCTION

Indole acetic acid (IAA) is the most abundant natural auxin which is essential for growth and development of plants. . Frist Went was the first who used the term 'Auxin'. Auxin is a generic term representing a class of compounds which are characterised by their capacity to induce elongation in shoot cells in the sub apical region. Indole Acetic Acid is a frequent byproduct of the metabolism of L-tryptophan by a variety of bacteria, notably PGPR[6]. Auxins, a phytohormone, are essential for plant growth and development because they control a wide range of biological processes, including cell division, elongation, differentiation, tropic responses, fruit formation, and senescence [8]. Additionally, they can stop fruits, flowers, and foliage from falling off [8]. IAA is the most common product of Tryptophan metabolism. Plant cell synthesizes IAA from Tryptophan as well as microorganisms in rhizosphere also possess the ability of production of IAA using L-tryptophan.The microorganisms isolated from rhizosphereregion have capacity to produce indole acetic acid as secondary metabolite.Among that *Rhizobium* spp. is the most known species of group of bacteria that acts as the symbiotic fixers of Nitrogen and produces IAA by using Tryptophan is the main precursor. IAA is metabolite derived from tryptophan by Trp- dependent and Trp- independent pathways in plants and bacteria. There are two major pathways in which tryptophan may convert to IAA .The conversion of tryptophan to indole-3-pyruvic acid by using the enzyme tryptophan transaminase. It is then decarboxylated by indole pyruvate decarboxylase to indole-3-acetaldehyde, which is then subsequently converted to IAA via indole acetaldehyde dehydrogenase. The second pathway involves the decarboxylation of tryptophan to Tryptamine by Tryptophan decarboxylase which is then converted to Indole-3-acetaldehyde by amine oxidase and finally to IAA by indole-3-acetaldehyde dehydrogenase [9]. Instead of using pure tryptophan as precursor for IAA production several other crude and cheap sources of Tryptophan can be added in media. One of the sources is soya flour which is rich source of tryptophan.

The first objective of this present study was to isolate and screen the Indole acetic acid producing Rhizobia from root nodules of leguminous plants and second was production of IAA with pure Tryptophan and other precursors like soya flour and Tryptone.

MATERIAL AND METHODS

2.1) Isolation & Identification of *Rhizobium* species.

Four different samples of root nodules of leguminous plants selected for the study are Groundnut i.e. *Arachis hypogea*, Chick pea i.e. *Cicer arietinum*, Soybean i.e. *Glycine max*, Fenugreek i.e. *Trigonellafoenum graecum.*Healthy root nodules were detached from the root & further isolation of root modulating *Rhizobia* was carried out. Washed with tap water &surface sterilized by 0.1% $HgCl_2$ (mercuric chloride) solution for 30 sec. and washed with sterile distilled water several times. These nodules were crushed in few drops of sterile water, to obtain suspension of bacteriods and inoculated on sterile yeast extract mannitol (YEMA) agar with Congo red and Incubated at Room temp for 48 hrs[1].*Rhizobium* colonies were white, opaque, mucoid and convex. Colony characters of well isolated colony were studied. Gram staining and motility was carried out. All isolates of *Rhizobium* spp. biochemically characterized for amylase and gelatinase, catalase like enzyme production, H_2S production, citrate utilization, Indole test, sugar fermentation etc[10].

2.2) Characterization of IAA Production.

To determine the amount of IAA produced by isolate, colorimetric technique was performed.

Rhizobium culture was inoculated into nutrient broth with 5mg/ml tryptophan and incubates at room temp for 3 days on shaker. The 1.5ml broth was centrifuged at 12,000 rpm for 5 minutes. 1ml of the supernatant was added to 4ml. of Salkowaski's reagent and 2 drops of o-phosphoric acid. Incubated at 30 min[2] observe the intensity of colour and optical density (O.D.) was recorded at 530nm.Development of pink color indicated IAA production. The amount of IAA produced per milliliter culture was estimated using standard curve.

2.3) Production of IAA using Pure Tryptophan & other precursors (By using shake flask method).

High yielding isolate was used for further production studies. Production of IAA was done using shake flask method.Test bacterial culture was inoculated in nutrients broth without Pure tryptophan and with Pure tryptophan, soya flour and tryptone. Incubated at R.T. Readings were taken after 2^{nd}, 4^{th}, 6^{th} days of incubation. Then broth was centrifuged at 12,000 rpm. For 5min. then 4ml. Salkowaski reagent & 2 drops of o phosphoric acid was added in supernatant. Incubated at 30 min. optical density was recorded at 530 nm.

2.4) Preparation of standard graph of IAA.

Different IAA concentrations are prepared as IAA aqueous solution ranging from10 µg/ml to100 µg/ml and 50 µg/ml to1000 µg/ml.To each 1ml of standard, 2 ml of salkowaski reagent added readings are taken after 30 min. at 530nm by UV- visible spectrophotometer. Standard graph is prepared by plotting conc. of IAA in micrograms / ml VS optical Density at 530 nm.

2.5) Confirmation of IAA using TLC

Supernatant was collected after centrifugation and mixed with ethyl acetate (1:2) after shaking it was allowed to stand for 10 min. IAA was extracted within solvent layer. Procedure was repeated 3 to 4 times.TLC slide was prepared with silica gel G and calcium carbonate (thickness 0.25mm.)

Benzene: n-butanol: acetic acid (70:25:50) IAA (10mg/100ml) were spotted on TLC plate. Spots were detected by spraying the plates using salkowaski reagent. Rf value of standard & IAA produced by the isolate was calculated.

RESULT AND DISCUSSION

Isolation and Identification of *Rhizobium* species.

Four different bacterial isolates were obtained using Yeast extract Mannitol agar (YEMA) with Congo red. The isolates were coded as AH, CA, GM, TF from *Arachis hypogea*, *Cicer arietinum*, *Glycine max* and *Trigonell*a *foenumgraecum* respectively. White, entire, convex, mucoid colonies were observed. The isolates were identified based on biochemical characterization (Table1).They show Gram negative, motile characteristics. Bergey's manual of determination of bacteriology was used as reference to identify the isolates.From the results, isolates were identified as *Rhizobium leguminosarum* from Groundnut, *Rhizobium loti* from chick pea, *Rhizobium leguminosarum* from Soybean, *Rhizobium meliloti* from Fenugreek[10].

Similarly, Sahasrabudhe Madhuri, 2011 isolated *Rhizobia* from the different leguminous plants[8]

Table 1: Biochemical characterization of Isolates.

Characteristics	Isolates from Leguminous plants			
	AH	CA	GM	TF
Catalase	+	+	+	+
Oxidase	-	-	-	-
Urease	-	+	+	-
Amylase	+	-	+	+
Caseinase	-	-	+	-
H2S Production	-	-	-	-
Indole production	-	-	-	-
Citrate utilization	+	+	+	+
Gelation liquification	-	-	-	-
Utilization of Sugar				
Glucose	+	+	+	+
Sucrose	+	+	+	+
Mannitol	+	+	+	+
Lactose	-	-	-	-
Xylose	+	+	+	+
Rhamnose	+	+	+	+
Arabinose	+	+	+	+
Growth at 2% NaCl	-	-	-	+

3.2)Characterization of IAA production.

The results of IAA production by all the four isolates namely AH, CA, GM, TF are presented in table no.2 It can be seen from the table that all the four isolates produced IAA. Isolate GM produced maximum IAA compared to remaining three isolates.

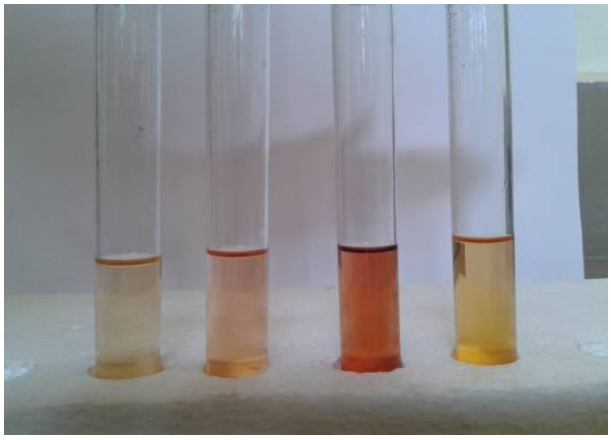


Figure 1: Detection of IAA (Sr. no 1,2,3,4)(from Left to Right).

Table 2: Detection of IAA production by four isolates.

Sr.No.	Isolate	Intensity of pink colour	OD at 530 nm	Conc. Of IAA µg/ml
1	AH	+	0.14	120
2	CA	+++	0.15	140
3	GM	++++	0.41	350
4	TF	+	0.13	110

3.3)Production of IAA using pure tryptophan and other precursors:

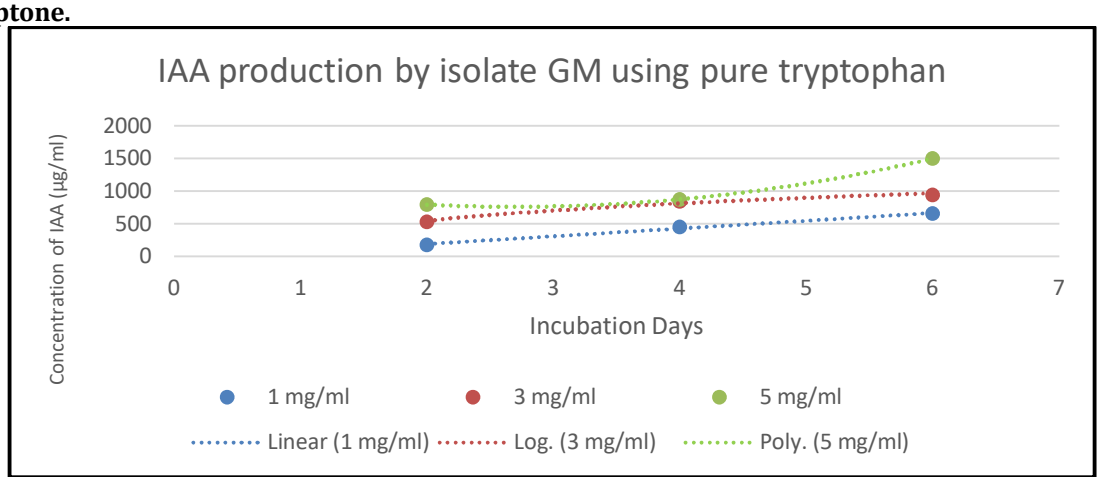
Varying levels of IAA production were recorded with different concentrations of tryptophan and other precursors like soya flour& tryptone i.e. 0, 1, 3, 5 mg per ml (Table 3). The results of IAA production using isolate GM are presented in table no 3. From the table seen that, using medium without tryptophan a yield of IAA obtained was 34 and 44 µg/ml. after 4th day & 6th day of incubation and no yield of IAA were obtained. After 2 days of incubation yield were obtained.After 2,4, 6 days of fermentation with all

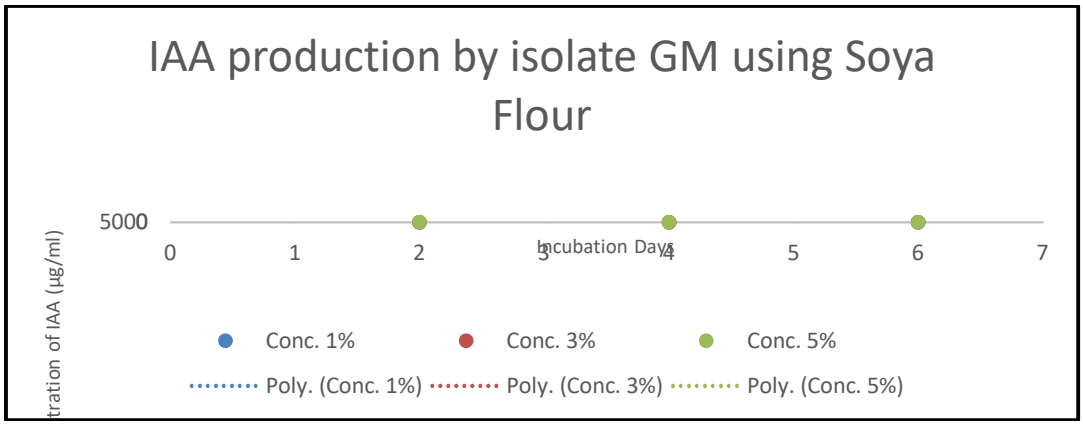
concentration of tryptophan, Soya flour and Tryptone results recorded in table 3. As per table, graph were recorded[Figure2].

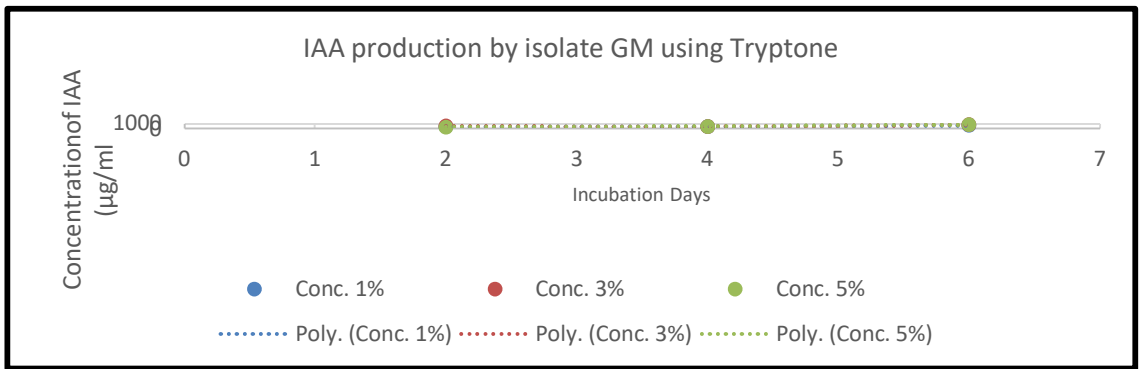
Table 3: IAA production by using different precursors with 1,3,5 mg/ml for 2,4,6 days of incubation.

S. no.	Precursors for IAA production	Amount of precursor in medium	O.D. at 540 nm after incubation time in days [for 2 ml broth]			concentration of IAA after incubation time in days (µg/ml)		
			2 days	4 days	6 days	2 days	4 days	6 days
1	Without tryptophan	-	0.00	0.04	0.05	0	34	44
2	Pure tryptophan	1 mg/ml	0.21	0.53	0.76	180	45	655
		3 mg/ml	0.63	0.99	1.10	535	850	945
		5 mg/ml	0.93	1.02	1.24	795	875	1500
3	Soybean flour (gm %)	1%	0.8	0.74	1.07	690	635	920
		3%	0.36	0.80	1.3	305	685	2010
		5%	0.36	0.86	2.01	305	740	2040
4	Tryptone (gm %)	1%	0.5	0.43	0.82	425	365	700
		3%	0.6	0.49	0.95	515	420	815
		5%	0.4	0.56	0.98	345	480	840

Figure 2: Graphs representing IAA production by isolate GM using Pure tryptophan, Soya flour, Tryptone.







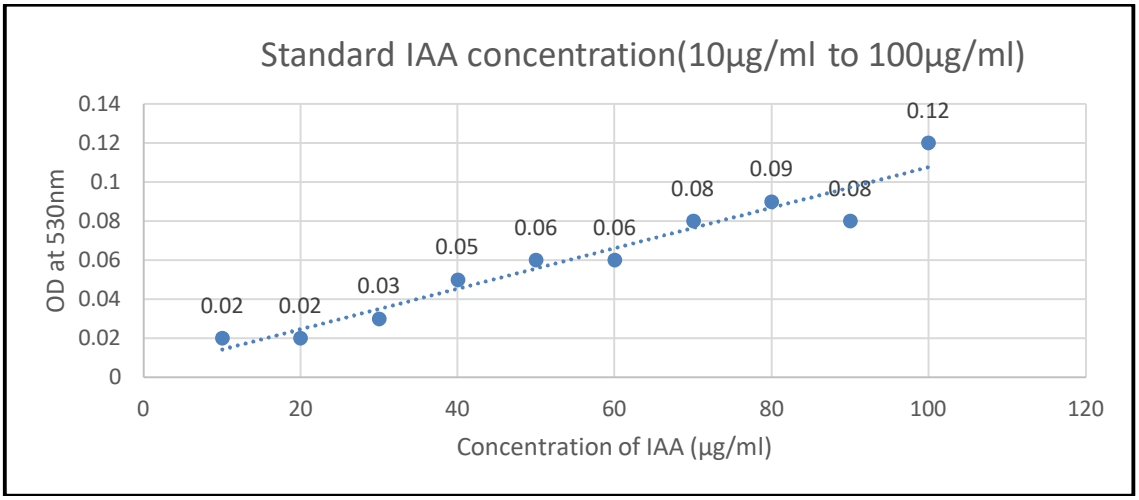
These results also indicate increase in concentration of soybean flour & tryptone from 1 to 5% increase in IAA production. Hence, optimum fermentation time is 6 days. It can be seen from table no- 3 that, isolate GM was superior not only pure tryptophan but also with Soya four and Tryptone.

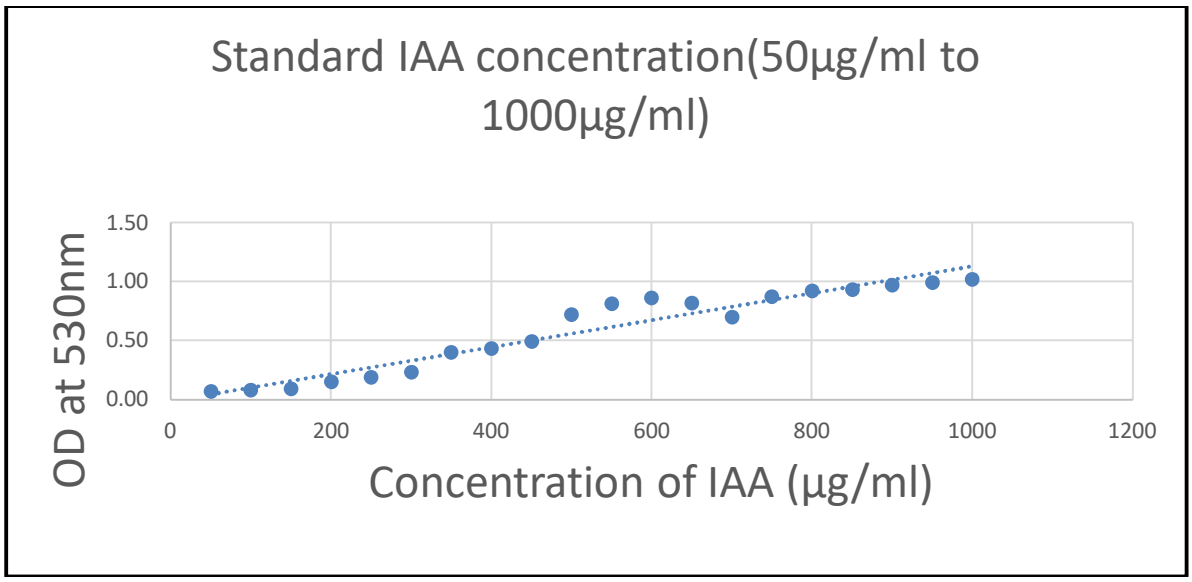
3.4) Preparation of Standard Graph:

Standard graph is prepared by plotting concentration of IAA in microgram/ml Vs option density at 530nm.Straight Line graph obtained. It indicates concentration of IAA is directly proportional to the extent of red colour developed.

Figure 3: Graph representing standard IAA concentration.

1. **10µg/ml to 100µg/ml**
2. **50µg/ml to 1000µg/ml**





3.5) Confirmation of IAA using TLC:
Thin layer chromatography was significant for the purification of Indole acetic acid produced by *Rhizobium* Spp. Chromatogram cultures shows different Rf values.Standard IAA showed Rf value of 0.88and isolate GM showed Rf value of 0.74 which was near to the standard.

CONCLUSION
From this study, it is clear that soya flour also provide rich source of tryptophan precursor for the growth of IAA producing*Rhizobium* spp. It has capability to produce Indole acetic acid, which is important for the plant growth. All the four isolates were primarily screened for their IAA production ability using 5mg/ml. Pure tryptophan as a precursor. Optimum fermentation time using pure tryptophan, soya flour and tryptone is 6 days.Yield of IAA using isolate GM was superior with pure tryptophan as well as soya flour. Use of soya flour reduces the economy in production of IAA and soya flour is the best replacement to pure tryptophan and will improve the fermentation economics drastically.

ACKNOWLEDGMENT
The author is thankful to Dr. A. K. Salunkhe, Head of Department of Microbiology,Smt. Kusumtai Rajarambapu Kanya Mahavidyalaya, Islampur, Sangali, Maharashtra, Indiafor providing the necessary facilities for this study.

REFERENCES

1. Deshmukh, A. M. Handbook of Media Stains and Reagents in Microbiology, Published by Oxford Book Company, ISBN 10: 8189473182ISBN 13: 9788189473181
2. Rahman, A., Sitepu, I. R., Tang, S. Y., &Hashidoko, Y. (2010). Salkowski's reagent test as a primary screening index for functionalities of rhizobacteria isolated from wild dipterocarp saplings growing naturally on medium-strongly acidic tropical peat soil. *Bioscience, Biotechnology, and Biochemistry*, *74*(11), 2202–2208. doi:10.1271/bbb.100360
3. Atlas, R. M., & Atlas, R. M. (2004). *Handbook of microbiological media* (3rd ed). Boca Raton, FL: CRC Press. doi:10.1201/9781420039726
4. Mohite, B. Isolation and characterization of indole acetic acid (IAA) producing bacteria from rhizospheric soil and its effect on plant growth. (2013). *Journal of Plant Nutritione*Pub, *13*(3) Temuco set, 27-Ago. doi:10.4067/S0718-95162013005000051, *versión On-line* ISSN 0718-9516
5. Ehmann, A. K. (1977). The van urk-Salkowski reagent—A sensitive and specific chromogenic reagent for silica gel thin-layer chromatographic detection and identification of indole derivatives. *Journal of Chromatography*, *132*(2), 267–276. doi:10.1016/s0021-9673(00)89300-0
6. Ahmad, F., Ahmad, I., & Khan, M. S. (2005). Indole acetic acid production by the indigenous isolates of Azotobacter and fluorescent Pseudomonas in the presence and absence of tryptophan. *Turkish Journal of Biology*, *29*(1), article 5. Retrieved from https://journals.tubitak.gov.tr/biology/vol29/iss1/5
7. Malcolm B. Wilkins, Physiology of Plant Growth and Development – 1 December 1969, McGraw-Hill Inc.,US (1 December 1969), ISBN-10 : 0070940886, ISBN-13 : 978-0070940888
8. Sahasrabudhe, M. (2011). Screening of rhizobia for indole acetic acid production. *Ann. Biol. Res.*, *2*.
9. Pandey, S. N., & Sinha, B. K. (January 1, 2005), Publisher : S Chand. *Plant Physiology* (4th ed), ISBN-13 p. 978-8125918790, ISBN-10: 9788125918790.
10. Bergey's Manual of Systematic Bacteriology (volume 2, Parts A–C, 2nd Edition). (published : April 01, 2006). *Copyright* G. George (Ed.), 2001–2005© Bergey's Manual Trust.
11. Singh, R., & Prasad, M. P. (2014). Isolation and screening of Rice rhizosphere Soil microorganisms for the production of IAA. *International Journal of Current Microbiology and Applied Sciences*, *3*(9)993-998, 993Original, ISSN: 2319-7706.
12. Gordon, S. A., & Weber, R. P. (1951). Colorimetric estimation of indoleacetic acid. *Plant Physiology*, *26*(1), 192–195. doi:10.1104/pp.26.1.192

CITATION OF THIS ARTICLE
P. S. Patil: Isolation and Characterization of Indole Acetic Acid (IAA) Producing *Rhizobium* Spp. from Leguminous Plants in Presence of Pure Tryptophan and Other Precursors. Bull. Env. Pharmacol. Life Sci., Spl Issue [1]: 2023:403-408.