PRODUCTION AND CHARACTERIZATION OF INDOLE ACETIC ACID (IAA) FROM *AGROBACTERIUM TUMEFACIENS.*

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Abstract

Plant hormones play an important role in controlling plant growth and development. Auxin was the first plant hormone discovered by Darwin in 1880. Auxin is produced by plants, another potential and economic important soil microorganisms like Rhizobium, Azotobactor and Agrobacterium have also been reported to synthesize auxin. Agrobacterium tumefaciens is soil born plant pathogen responsible for crown gall disease. The Agrobacterium tumefaciens produces auxin which is responsible for such type of disease. Because of which Agrobacterium spp. can be selected for the isolation and production of auxin i.e. Indole 3-acetic acid by using yeast extract broth containing different concentrations of tryptophan. Tryptophan act as a substrate for the indole acetic acid synthesis; and also the isolated species compared with pure culture of Agrobacterium tumefaciens collected from NCL (National Chemical Laboratory), Pune. The produced IAA can be obtained by centrifugation; the supernatant can be estimated by using Salkowski's reagent. The extraction of IAA can be done by diethyl ether, and IAA can be detected by TLC.

Keywords: *Agrobacterium tumefaciens, Indole acetic acid, L-tryptophan, Salkowski's Reagent, TLC.*

INTRODUCTION

Plant hormones play an important role in controlling plant growth and development. Among the phytohormones, auxin was the first plant hormone discovered by Darwin in 1880, auxin. These are the group of indole ring compounds which improve the plant growth by stimulating cell elongation, root initiation, seed

ISBN – 978-81-927211-9-2

germination and seedling growth (El Tarabily 2008). Indol-3-acetic acid (IAA) is the most important plant hormone which actively stimulates plant growth and development. Although there are certain microorganisms that produces Indole acetic acid (IAA) through their L- tryptophan metabolism. Plant rhizosphere soil is a unique biological niche with diverse micro flora such as bacteria, fungi, protozoa and algae (2010). Generally these organisms take the nourishment from plants and continue growth (Lynch 1990). Most of the organism shows pathogenic invasion to the plant. The isolation of IAA from plant tissues presents particular difficulties that automatically reduces recoveries. (Little et al, 1978). However the composition and quantity of IAA in plant tissues varies depending on the plant species and physical environmental conditions such as humidity, temperature etc (Smith 1976, Martin and Kemp 1980).There are about 80 % of rhizosphere bacteria has ability to secrete IAA, it includes Streptomyces's also(Bhavdish et al. 2003). Like other micro organisms including *Agrobacterial species* have also been reported to synthesize auxin.

Agrobacterium tumefaciens is a ubiquitous soil borne plant pathogen. *Agrobacterium tumefaciens* is the causal agent of crown gall disease (the formation of tumor's) in over 140 species of dicot. It is a rod shaped, Gram negative soil bacterium (Smith et al., 1907). It is a nitrogen fixing symbiotic and do not beneficial to the plant. The bacterium is surrounded by a small number of peritricious flagella. It contains large plasmid which carries the genes for tumor induction called as Ti (tumor inducing) plasmid. Ti plasmid also contains onco genes that determine the host range and symptoms which the infection will produce. It is responsible for Crown Gall disease, affecting on many higher plant species. The plant pathogen is problem for agriculture all over the world. To transfer the DNA of *Agrobacterium tumefaciens* to plant cell is the only known example of interkingdom DNA transfer. *Agrobacterium tumefaciens* is most well known for its ability to integrate a small part of the Ti plasmid into the host plant genome, which causes the plant cells to become cancer cells and produce specific compounds called Opines, which the bacterium utilize as a carbon source. These secreted opines also led to secretion of IAA. IAA is heterocyclic compound synthesized chemically by reaction of Indole with glycolic acid at 250^0c. Biologically IAA is synthesized from the universal precursor, amino acid L- tryptophan it is the principle component of plant root exudates and *Agrobacterium* utilize this exudates tryptophan to synthesize IAA for the plant.

Overcome this property of disease commonly the *Agrobacterium tumifaciens* is used to transfer foreign genetic material and to create genetically modified plants.

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But in current issue we use this property of *Agrobacterium* crown gall formation to produce IAA and develop general protocol at commercial level production.

Material and Method:-

Isolation of *Agrobacterium* from soil

Soil sample was collected from the rhizoshere of the leguminous plant like ground nut and soybean from the agricultural field of Kolhapur area. *Agrobacterium* strains were isolated by plating the serially diluted soil sample on the CRYEMA medium (Manitol -1gm, K_2HPO_4- 0.05gm,$MgSO_4.7H_2O$ -0.05gm,NaCl-0.01 gm,$CaCO_3$ -0.03gm,Yeast extract -0.1gm,1:100dil. Congo red -0.2ml,Agar-2.5gm,D/W -100ml, pH -7) and incubated at 28^0c for 24hrs-48 hrs. After incubation the isolated *Agrobacterium* colonies were sub cultured on YEB medium (yeast extract 0.1gm, Beef extract 0.5gm, Sucrose 0.5gm, Peptone 0.5gm, $MgSO_4.7H_2O$ 30mg-, D/W 100ml Agar 2 gm) (NCL, Pune) until pure culture were obtained.

Morphological characteristics and biochemical analysis

The Agrobacterium isolates colonies were studied for their morphological characteristics like size, shape, appearance, gram nature, motility etc. Also biochemical tests were performed for the identification of *Agrobacterium tumifaciens* like sugar fermentation (L-arabinose, D-xylose), growth in NaCl, effect of temperature, oncogenecity to plant (Bergey's Manual of Systematic Bacteriology Second Edition- Volume two- Springer)

Screening for IAA production

The production of IAA by all *Agrobacterium spp*. Isolates was determined according to the method of , The isolated colonies was grown on YEB medium containing 2% L-tryptophan and incubated at 28 ^{0} C for 8 days with shaking at 120 rpm. Then few ml of broth was harvested and centrifuged at 8000 rpm for 20mins. 1ml supernatant was mixed with 4 ml of Salkowski's reagent (50 ml per chloric acid (35%) and 1ml of 0.5 $FeCl_3$). The appearance of pink color indicated the IAA production. Optical density (O.D.) read at 530 nm. The level of IAA produced was estimated against the IAA standard.

Extraction and purification of IAA

The isolated spp. Of Agrobacterium was cultivated in 100 ml of YEB broth containing 2 % L- tryptophan at a pH of 7. Then IAA was extracted with diethyl ether method. In this the broth was harvested and centrifuged at 8000 rpm for 20 min. Supernatant was collected and acidified with 6N HCL (2.5 to 3.5pH), the supernatant containing IAA dissolved in diethyl ethers at 4^0c for 1hr in separating funnel. Diethyl ether fractions were collected and exposed at 30-40^0c to reduce ¼ t of the original volume. Then equal volume of Sodium bicarbonate was added twice to extract more

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auxins. The bicarbonate fraction was again acidified with 6N HCl and resulting solution was treated with Diethyl ether to dissolve more IAA from the bicarbonate fractions. The diethyl ether fractions were exposed to air to get IAA in powder form.

Detection of IAA by Qualitative and Quantitative method

Qualitative method:

Extracted and purified IAA was detected by the TLC plates (Silica gel GF 254LR, SD Fine-Chem Ltd. Mumbai) and developed in xylene then IAA spots were confirmed by spraying Kovack's reagent (Isoamyl alcohol - 150ml, β –dimethyl benzaldehyde - 10gm,conc.HCl-50ml). Pink spot was observed and Rf value were calculated by the following formula

$$\text{Rf value} = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

Quantitative method:

The quantitative estimation was done by using Salkowski's reagent. In this standard IAA (500µg/ml) was prepared and taken in different concentrations as 0.2,0.4,0.6,0.8,1.0 ml and adjusted the volume to 1 ml by distilled water . In that 2 drops of orthophosphoric acid and 4ml of Salkowski's reagent was added and incubated all at room temperature for 20 minutes. O.D. was measured at 530nm. Same procedure was carried out for the unknown sample and the concentration of IAA from unknown sample was determined by using std. graph.

Result and Discussion:

The rhizosphere represents a unique biological niche that supports an abundance of diverse microorganisms because high input of organic material derived from the plants roots and root exudates. (Merck et al, 1987)

Some pathogenic bacteria invade the complete plant tissue there is invasion any other pathogen that causes the disease like Agrobacterium spp. Agrobacterium is one of the plant pathogen that cause the crown gall disease in most of the dicot plants. In this pathogenicity Agrobacteria integrates its extra chromosomal plasmid T-DNA region into the plant genome that integration is the expression of onco gene causes over secretion of auxins like IAA by utilizing the plant cell secreted tryptophan as a substrate. That secreted IAA utilized by plant cell because of extra utilization of IAA cell loss it normal cell growth pattern and show crown gall disease. According to the most of the plant pathologists because such property of the Agrobacteria is not advantages to plant but in present study on the basis of obtained results. We can prove that the Agrobacterial pathogenic property used for the commercial production of IAA from Agrobacterium spp.

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ISBN – 978-81-927211-9-2

Isolation of Agrobacterium spp.

The rhizosphere soil of the soybean and ground nut was taken and plated by serial dilution method on CRYEMA medium here we use directly CRYEMA medium because CRYEMA is differential medium for Agrobacterium and Rhizobium from these two rhizosphere soil samples two Agrobacterial isolates were isolated and screened for morphological identification. Morphologically Agrobacterium is a Gram negative, motile bacterium. These two isolates also there were some biochemical test was carried out for conformation of these two isolates are *Agrobacterium tumifaciens.*

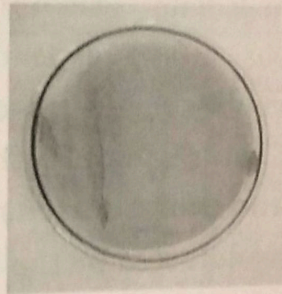
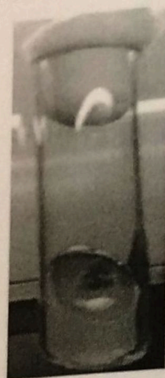
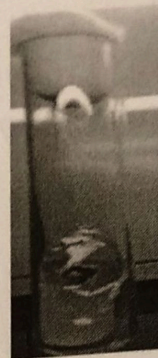


Fig :1 Isolation of *Agrobacterium tumifaciens*

One of the common confirmatory tests for *Agrobacterium tumifaciens* is the oncogensity. The isolated two Agrobacterial isolates were infected to plant leaf and cultured on the auxin free M.S. medium. After 12 days we observed the callus which is impossible without auxin. Development of callus indicates that the isolated spp. having ability to produce auxins such as IAA which confirms that the isolates are *Agrobacterium tumifaciens.*



Isolate 1



Isolate 2

Fig: 2 Oncogensity

Biochemical Tests	Isolate 1	Isolate 2
L- Arabinose	+	+
D- Xylose	+	+
1% NaCl	+	+
2% NaCl	+	+
Temp 28^{0}c	+	+
Temp 35^{0}c	+	+
Temp 40^{0}c	+	+
Oncogenecity	+	+

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Table no. 1: Biochemical confirmatory tests for *Agrobacterium tumifaciens* according to Bergey's Manual of Systematic Bacteriology Second Edition- Volume two-Springer.

Production of IAA:-

Different crop varieties or species might produce different types of root exudates, which could support the activity of microorganisms for IAA production (Frankenberger and Arshad 1995). It is possible that the high level of tryptophan will be present in root exudates of the two leguminous plants and enhance IAA biosynthesis in Agrobacterium isolated from their rhizoshere. The range of IAA production by the Agrobacterium isolates was 150- 180 µg/ mL (Table 2). The synthesis of IAA in bacteria was totally dependent on the concentration of tryptophan in the medium. If concentration of tryptophan increases their increased IAA synthesis in bacteria as that of natural mechanisms.

Detection of IAA by Qualitative and Quantitative method:-

Extracted and purified IAA was detected by the TLC plates (Silica gel GF 254LR, SD Fine-Chem Ltd. Mumbai) and developed in xylene then IAA spots were confirmed by spraying Kovack's reagent. Pink spot was observed and Rf value were calculated by comparing standard IAA spots (Table 2)

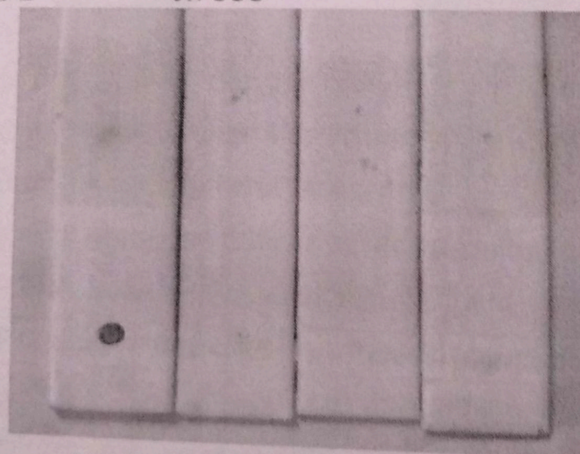
Distance travelled by solvent	Distance travelled by solute		
	Std	Isolate 1	Isolate 2
10.5cm	8cm	7.9cm	7.7cm

Rf values of

Std. IAA = 0.7619

Isolate 1 = 0.7523

Isolate 2 = 0.7333



Std. IAA Isolate 1 Isolate 2

Table- 2:

Quantitative method:

The quantitative estimation was done by using Salkowski's reagent by taking standard IAA at 500µg/ml.

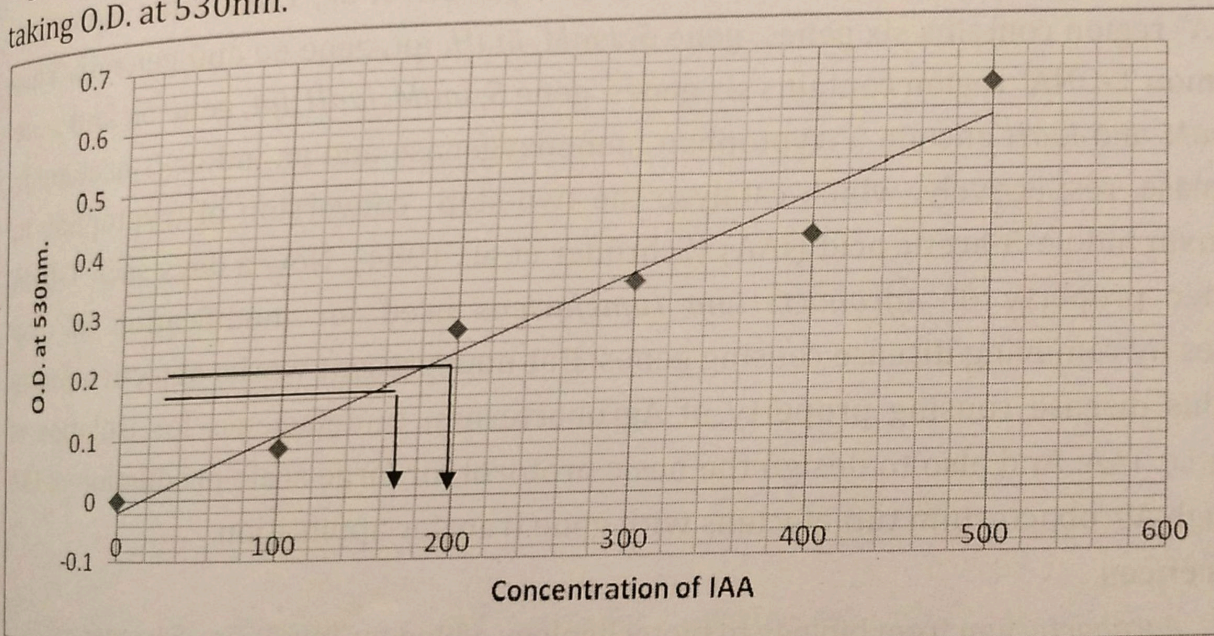
Estimation of IAA by Salkowski's reagent:

Std. IAA=500μg/ml

Sr. No.	Std. IAA.	D/W	Orthophosphoric acid	Salkowski's reagent		O.D.at 530nm
1)	0.0	1.0	2drops	4ml	Incubate	0.0
2)	0.2	0.8	2drops	4ml	At R.T	0.08
3)	0.4	0.6	2drops	4ml	For	0.23
4)	0.6	0.8	2drops	4ml	20	0.33
5)	0.8	0.2	2drops	4ml	Min.	0.40
6)	1.0	0.0	2drops	4ml		0.55

Sample no.	Quantity	D/W	Orthophosphoric acid	Salkowski's Reagent		O.D.at 530 nm
Isolate 1	1.0	0.0	2drops	4ml	Incubate at R.T. For 20 Min	0.20
Isolate 2	1.0	0.0	2drops	4ml		0.16

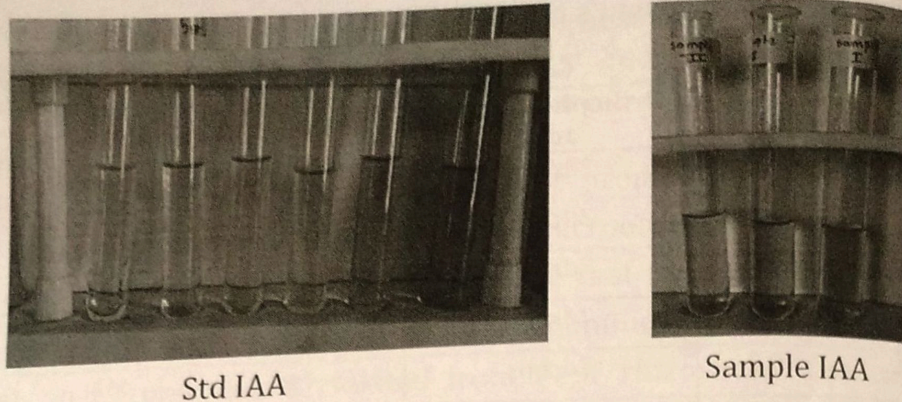
Fig. 3 Graph show the determination of concentration of IAA by colorimetric analysis taking O.D. at 530nm.



Comparison of IAA Production

	Isolate 1	Isolate 2
Produced IAA	180μg/ml	150μg/ ml

Table shows concentration of IAA from isolates 1and 2 with reference to standard concentration of IAA.



Std IAA   Sample IAA

Fig. 4 Quantitative determination of IAA by Salkowski's estimation method.

Discussion:-

Several early studies of crown gall disease suggested that *A. tumefaciens*-mediated tumor formation was initiated and maintained by alterations in plant hormone metabolism (reviewed by Binns and Costantino,1998). In 1977 Chilton et al. demonstrated that *A. tumefaciens* T-DNA is horizontally transferred to plants and integrates into the plant cell genome (Chilton et al., 1977), and later studies demonstrated that a single fragment of the T-DNA is conserved between octopine- and nopaline-type strains of *A. tumefaciens* (Willmitzer et al., 1983) This "common T-DNA" region contains six genes: gene *5*, *iaaM*, *iaaH*, *ipt*, gene *6a* and gene *6b*. This "common T-DNA" region contains six genes: gene *5*, *iaaM*, *iaaH*, *ipt*, gene *6a* and gene *6b*. *iaaM* and *iaaH* encode a tryptophan monooxygenase and an indole-3-acetamide hydrolase, respectively, which catalyze the two-step conversion of tryptophan to the auxin indole-3-acetic acid (IAA) (Schröder et al., 1984). Now a days such T-DNA transfer property of *Agrobacterium tumefaciens* used for modification of crop species by removing disease causing genes. But now present works objective was to use this disease causing property of Agrobacterium tumefaciencs for isolation of auxin i.e. IAA. And also to design the basic protocol for large scale production of IAA through Agrobacterium tumefaciens with gene transfer application.

References

- *Agrobacterium* from biology to biotechnology edited by Tzvi Tzfira, Springer.
- Bergey's Manual of Systematic Bacteriology Second Edition- Volume two- Springer.
- Bhavdish N, Johri A, Sharma J, Virdi S, (2003) Rhizobactrial diversity in India and its influence on soiland plant health advances in Biochemical Engineering and Biotechnology 84, 49-89.
- Brown ME (1972) Plant growth substances produced by microorganisms of soil and rhizosphere. Journal of applied Bacteriology 35,443-451.
- Department of Plant Sciences, University of California, One Shields Ave, Davis, CA 95616 USA; 2Department of Biological Sciences, California State University San Marcos, 333 S. Twin Oaks Valley Road,San Marcos, CA 92096 USA

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- El- Terabily KA (2008) promotion of tomato (*Lycopersicon esculentum* Mill.) plant growth by rhizosphere copetent 1- aminocyclopropane-1-carboxylic acid deaminase-producing streptomycete actinomycetes plant and soil 308,161-174.
- Lynch JM (1990) introduction: some consequences of microbial rhizosphere competence for plant and soil. In Lynch JM (ed.), the Rhizosphere, Wiley and Sons.
- P. M. Klapwijk, P. J. J. Hooykaas, H. C. M. Kester, R. A. Schilperoortand A. Rorsch *Department of Biochemistry, The University, Leiden, The Netherlands* Isolation and Characterization of *Agrobacterium tumefaciens* Mutants Affected in the Utilization of Octopine, Octopinic Acid and Lycopene.
- Smith WH (1976) character and significance of forest tree root exudates. Ecology 57, 324-331.