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Effect of Tryptophan Concentration An Production of Indole Acetic Acid by Azatobacter Chroococum Isolated from Purple Plant Soil.

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Abstract: *Plant growth promoting rhiz bacteria [PGPR] exhibit a variety of characteristics responsible for influencing plant growth.The common traits include production of plant growth regulators [auxin, gibberellin IAA etc.].siderophores, HCN and antibiotics. Indole acetic acid [IAA] is one of the most physiologically active auxins. IAA is a common product of tryptophan metabolism by several micro organism. Rhizospheres of bacterial various plant are likely to synthesize and release auxin as secondary metabolites because of the rich supplies of substrates exuded from the roots compared with non-rhizospheric soils plant morphogenic effect may also be result ofdifferent ratio of plant bacteria. Diverse soil microorganisms including bacteria, fungi, and algae are capable of producing physiologically active quantities of auxins,which may extract pronounced effects on plant growth and establishment.*

Keywords: Biofertilizers, Plant growth promotion, Eco-friendly fertilizer, soil microorganism.

I. INTRODUCTION

Plant growth promoting rhiz bacteria [PGPR] exhibit a variety of characteristics responsible for influencing plant growth.The common traits include production of plant growth regulators [auxin, gibberellin IAA etc.].siderophores, HCN and antibiotics. Indole acetic acid [IAA] is one of the most physiologically active auxins. IAA is a common product of tryptophan metabolism by several micro organism. Rhizospheres of bacterial various plant are likely to synthesize and release auxin as secondary metabolites because of the rich supplies of substrates exuded from the roots compared with non-rhizospheric soils plant morphogenic effect may also be result ofdifferent ratio of plant bacteria. Diverse soil microorganisms including bacteria, fungi, and algae are capable of producing physiologically active quantities of auxins,which may extract pronounced effects on plant growth and establishment.

I Azotobacter

Azotobacter spp these are free living bacteria which grow well on nitrogen free medium. These bacteria utilize atmospheric nitrogen gas for their cell protein synthesis.This cell protein is then mineralized in soil after the death of Azotobacter cells there by contributing towered the nitrogen availability of the crop plants

1.2 Classification:-

Kingdom: Bacteria
Phylum : Proteobacteria.
Class : Gammaproteobacteria
Order : Pseeudomonadle
Family : Azotobacteraceae
Genus : Azotobacteracea

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1.3 Characteristics of Azotobacter:-

Azotobacter is Gram negative bacteria, polymorphic i.e. they are of different sizes and shapes.Their size ranges form 2-10x1-2.5m younger cell possess peritrichous flagella and are use also comotive organs Old population of bacteria includes encapsulated forms and have enhanced resistant to heat, desiccation and adverse condition. Theycyst germinates under favorable condition to give vegetative cells. They also produce polysaccharides. Azotobacter spp. Are sensitive to acidic Ph high salts, and temperature above 35°c There are four Important species of Azotobacter. However Azotobacter is a poor competitor for nutrients in soil. Most efficient strains of Azotobacter would need to oxidizeabout 1000 kg of organic matter for fixing 30kg of N/ha.This dose not sound realistic for our soils which have very low active carbon status .Besides, soil is inhabitated by a large variety of other microbes, all of which compete for the active carbon.

1.4 Ecology:-

Diazotrophic organisms such as Azotobacter play a vital role in every ecosystem, working to make nitrogen available to all oraganisms Azotobacter and similar bacteria turn nitrogen into ammonia through the process of nitrogen fixation, after which the ammonia is turned into proteins.

Nitrogen fixation is used in agriculture in relation to crop rotation and fertilization; soil- dewlling diazotrophs such as Azotobacter are especially useeful in gauging the health and virility of the ground.Azotobacter are found world wide, in climates ranging from extremely northern Siberia to Egypt and India

1.4.1 Azotobacter in Soil:-

In Indian soil ,the population of Azotobacter is not more than 10 thousand to 1 lakh /gm of soil. The population ofAzotobacter is mostly influenced byother micro-organisums present in soil .there are some micro-organisum which stimulate the Azotobacter population in soil there by increasing the nitrogen fixation by Azotobacter.on the other hand there are some micro- organisum which adversly affect the Azotobacter population and hence nitrogen fixation process is hampered . For example cephallosporum is most commonly found organisms in soil which restricts the growth of Azotobacter. Azotobacter also produces some substances which check the plant pathogen such as Alternaria ,Fusarium and Helminthosporium .Hence Azotobacter also act as biological control agent.

1.4.2 Significance of Azotobacter in soil:-

Azotobacter naturally fixes atmospheric nitrogen in the rhizosphere There are different strains of Azotobactor each has varied chemical biological and other characters. However, some strains have higher nitrogen fixing ability then others. Azotobacter uses carbon for its metabolism from simple or compound substances of carbonaceous in nature. Besides carbon, Azotobacter also requires calcium for nitrogen fixation. Similarly, a medium used for growth of Azotobacter is required to have presence of organic nitrogen, micro-nutrients and saltin order to enhance the nitrogen fixing ability of Azotobacter.

II. METHODOLOGY

2.1 Enrichment of Azotobacter

Soil sample wa s collected from purple plant from vita in Sangli dist. 1 gram of soil was inoculated in sterile 100ml. nitrogen free mannitol broth.the flask was incubated at 28°c four days. After incubation pellicle smear made for grams staining for presence of gram negative bacteria.

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2.2 Isolation of Azotobacter

One loop full culture from enriched medium was streaked on Nitrogen
Free mannitol agar plate.The plates were incubated at 28°c at 4 days.Brown colonies are observed. A single colony was sub cultured on N2 free mannitol agar slant

2.3 Characterization of Azotobacterchroococcum:-

Colony characters was studied on N2 free mannitol agar Azotobacter was characterized on the basis of indole production, methyl red test, voges proskauer test, citrate utilizing test sugar fermentation test,H2S production, catalase test.

2.4 Inoculums Preparation:-

Inoculum was prepared by inoculating A.chroococcum from slant into nitrogen free mannitol medium and incubated at 28°c for2days.

2.5 Production of IAA

2% of inoculums of A.chroococcum was inoculated in N2 free Mannitol broth.The broth was kept for IAA production at 28°c for 7 days

2.6 Effect different concentration tryptophanon IAA production:-

Nitrogen free mannitol broth with different concentration of tryptophan ranging from 50mg/ml, 100mg/ml, 150mg/ml, 200mg/ml 250mg/ml were prepared and inoculated with 2% inoculums. The flasks were kept for production of IAA at 28°c for 7 days

2.7 Extraction of IAA.

From the production medium the bacterial cells were separated from the supernatant by centrifugation at 10000 rpm for 30 min and the pH of supernatant was adjusted to 2.5 with1N HCL the double amount of methyl acetate was added and evaporated at 40°C. Weight of IAA was determined.

2.8 Qualitative Detection of IAA by TLC.

IAA powder obtained was dissolved in methanol. TLC Plates where prepared silica gel G Dropmethanol solution of IAA and standard IAA on TLC plates. Solvent system was prepared as. Benzene:n-butanol :Acetic acid(70:25:5) and saturated the solvent chamber. TLC plates were kept in solvent system and run for 6 hrs. Plates were sprayed with solakwaskis reagent It was dried at 60°C for 10 min. Spot were observed by exposing to U.V.ray's.

2.9 Quantitative estimation of IAA

2ml of fermented broth + 2 drops of orthophosphoric acid +4 ml of solawaskis reagent were mixed and kept at room temp. for 15 min Development of pink color was observed. Optical density was read at 530 nm.The level of IAA produced was estimated by a solawaskis. Was added in 2ml of each concentration of IAA. It was kept at Room temperature for 15 min. The optical densitywas observed at 530 nm. Standard graph of optical density Vs concentration of IAA was plotted.

2.10 Effect of Azotobacter on seed germination:-

Azotobacter was streaked on N2 free mannitol agar and seeds were placed at equidistance on plates and were incubated at28°c for72 hrs. The roots of the germinated seeds were measured.

III. OBSERVATIONS AND RESULTS

3.1 Enrichment of Azotobacter:- Pellicle growth was observed on the surface of the N2-free mannitol broth after 4days at 28°c. The gram staining performed gram negative morphology was observed.

3.2 Isolation of Azotobacter:- Black-brown colonies were observed on N2 free mannitol agar plate It was typical for Azotobacter chroococcum. Gram negative morphology was observed after gram staining. Organism was motile. The organism identified as Azotobacter chroococcum .(observation table1,fig1&2)

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3.3 Biochemical Characterization of Azotobacter chroococcum:-Isolated Azotobacter chroococcum was tested for biochemical characters. Organism showed Catalase, Indole, Methyl red, voges Proskauer, Citrate utilization as well as Glucose, Xylose, Lactose,Mannitol,maltose Positive characters (observation table 2).

Observation Table No.1

Colony characters of well isolated colony from enriched culture on nitrogen free mannitol agar plate incubated at 28°c for 4days.

Sr.no	Size	Shape	Colour	Margi n	Elevation	Opacity	consistency	Gra m nature	Motilit y	Name
1	2m m	Circ ular	Blac k-Bro wn	Regul ar	Lowc onve x	Opaque	Mucoid	Gramn egativ e	Motile	Azotobacter Chroo cocum

Figure no 2: Gram Staning of well isolated Colony

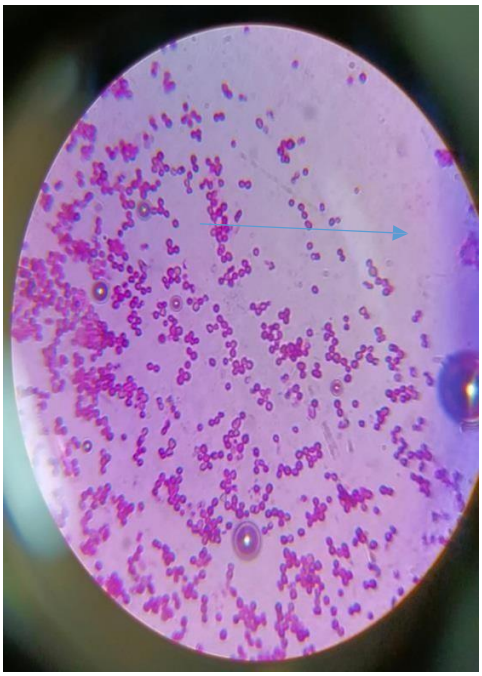

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Figure No2

Isolation of Azotobacter chroococcum on N2 free

Observation Table No 2.

Biochemical characterization of Azotobacter Chroococcum.

Sr.no	Test	Inference
1	Glucose	A
2	Xylose	A
3	Lactose	A
4	Maltose	A
5	Mannitol	A
6	Indole	+
7	Methylred	+
8	V.P	+
9	Citrate	+
10	Catalase	+

A: Acid production +:Positive -:Negative

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3.4 Effect different concentration of tryptophan on IAA production:- IAA production increased with addition of tryptophan in medium. Maximum IAA production was observed when N2-free mannitol medium supplemented with tryptophan at the concentration at 200mg/ml and incubated 28 for 7days was 5.4ug/ml (observation table 3&4, fig 2).

3.5 Qualitative detection IAA by TLC :- Blue coloured spots was observed under U.V.light by Sprayed with Solawaskis.reagent which matched the spot developed with standard IAA.

3.6 Effect of Azotobacter on seed germination:- It was observed that in the presence of Azotobacter seed germination rate was higher (90%) than without Azotobacter germination rate (40%) (observation table 6 fig no 3).

3.7 Effect of Azotbacter on root length: Average root length of seeds germinated in presence of Azotobacter was found greater than that of seeds germinated without Azotobacter. It was found hat it was 4 times greater length than without Azotobacter (observation table 6fig no3)

Observation table No : 3

Standard graph of IAA

Sr.No.	Amount of Stock Solution (ml)	Amount of methanol (ml)	Amount of 2drops of orthophosphoaric + 4ml of solawask's reagent(ml)		Optical density
1	0.2	1.8	4		0.08
2	0.4	1.6	4	Kept at	0.12
3	0.6	1.4	4	room	0.16
4	0.8	1.2	4	temp for 15min	0.25
5	1.0	1.0	4		0.31
6	1.2	0.8	4		0.32
7	1.4	0.6	4		0.34
8	1.6	0.4	4		0.35
9	1.8	0.2	4		0.41
10	2.0	0	4		0.41
11	0	2	4		…

Observation Table No 4

Effect of different concentration of tryptophan on IAA production.

Production IAA in Nitrogen free mannitol medium with different concentration of tryptophan at 28°c, pH-7 for 7 days.

Sr. no.	Tryptophan Conc (mg/ml)	O.Dat530 nm	IAA production(ug/ml)
1	0	0.02	0.4
2	50	0.06	1.1
3	100	0.06	1.1
4	150	0.15	2.8
5	200	0.29	5.4
6	250	0.15	2.8

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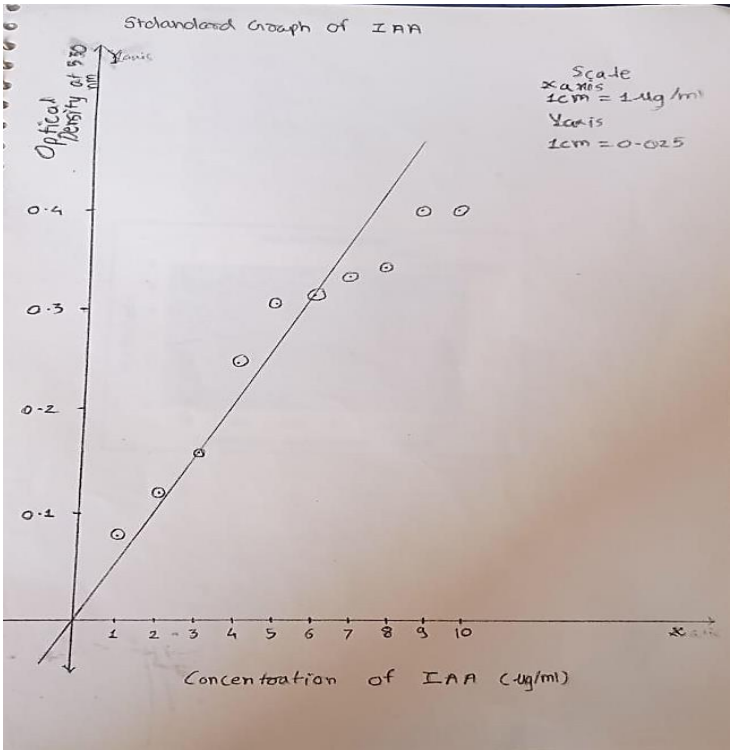

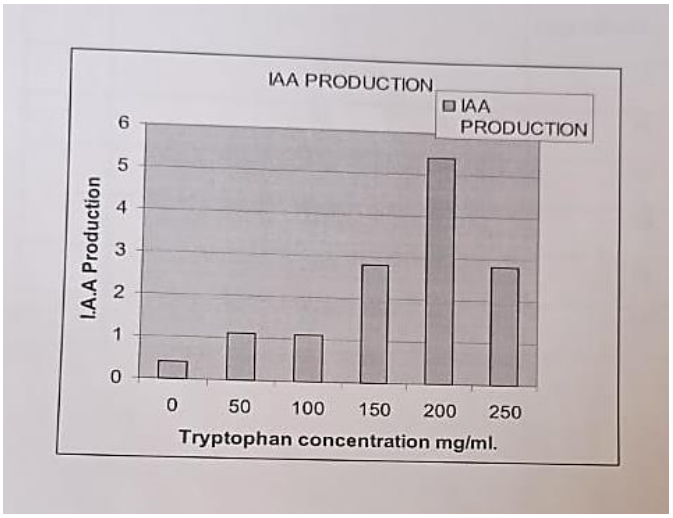

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Observation Table No 5

Sr.no.	Conc Tryptophan production medium (mg/ml)	Yield of IAA production(mg/ml)
1	0	10
2	50	10
3	100	10
4	150	10
5	200	20
6	250	10

Observation Table No.6

Effect of Azotobacter on seed germination and root length Duration 3 days

See d no	Root length of seed germinated with Azotobacter (cm)	Avg. root length with Azotobacter (cm)	Seed no	Root length of seed germinated without Azotobacter (cm)	Avg. root length with Azotobacter (cm)
1	8cm	3.8 cm	1	-	1.3Cm
2	2.5		2	-	
3	4		3	-	
4	4		4	2	
5	7		5	3	
6	3		6	-	
7	-		7	5	
8	2		8	3	
9	6		9	-	
10	4		10	-	

Percentage Germination = No of seeds germinate d x 100
Total no of seeds
Percentage germination
with Azotobactor = 9/10x100 = 90%
Percentage germination
without Azotobacter = 4/10x100 = 40%

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Figure No 5

Seed germination on N2 Free mannitol agar plate And effect on root length

IV. DISCUSSION

The Azotobacter chroococeum was isolated from wheat field and identified on the basis of morphological charactersas described in Bergeys manual of determinative bacteriology. The same organism was used for IAA production

IAA was produced in nitrogen free mannitol containing tryptophan at 28°C for 7 days.

The chromato grams of sample and standard IAA,sprayed with Solawaskis reagents howed almost same Rf value

The IAA production was measured for varying concentrations of tryptophan. Maximum production of IAA was observed for 200 mg/ml beyond this any increase in tryptophan concentration did not gave increase inproduction.The maximum production with tryptophan concentration at 200mg/ml was 5.4pg/ml.

The % germination of seeds was determined with Azotobacter chroococcum and was found to be 90% which was 50% more than germination in absence of Azotobacter chroococcum. 30% average root length increase was obtained with Azotobacter

The extracted IAA was maximum for 200mg/ml i.e 20mg/100ml.

Farah Ahmad and et.al reported the IAA production by A chroococcum in presence of tryptophan. IAA production by A chroococcum in range 2.72-30.37ug/ml.in presence of tryptophan

A chroococcum produces favourable amount of IAA From this investigation we say that A.chroococcum can be used for IAA production.

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Appendix A

Media And Reagent

1) Media for enrichment and isolation : Nitrogen free mannitol medium

Composition

Mannitol	-	10gm
$MgSO_4$	-	0.2gm
$MnSO_4$	-	0.02gm
K_2HPO_4	-	0.5gm
NaCl	-	0.2gm
$FeCl_3$	-	0.005gm
$CaCO_3$	-	10gm
D/W	-	1000ml.
PH	–	7

Prepration : The broth except CaCo3 was sterilized at121^0c at 15min lbs for 15 min.CaCo was sterilized Separately and then added to broth aseptically

Regent of Solution:-

1. INHCL Solution
2. Methanol.
3. Ethyl Acetate
4. Solvent Syste :[Benzeme:Nbutanol:Aceticacid
5. Silica gel-G9

Salowask's Reagent:

Prepared fresh before use by mixing of 1ml of 0.5M FeCl3 in 50ml 35%HCLO,[Perchloric acid] 7.40% KOH and 5%aNapthol.

8.Kosser's Citrate Broth. 2%tryptone Water.

Appendix-B

Protocols of Standard Procedures Protocols-

Qualitative detection of IAA by TLC

↓

Prepare TLC plate using silica gel-G

↓

Spot drop of extract and standard IAA on TLC plate

↓

Prepare solvent system benzene in butano lacetic acid (70:25:5) and saturate the solvent chamber.

Keep the plates in solvent chamberb and run for 6hrs

↓

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↓

Spray plates with solawaskis reagent dry it at 60^0C for 10 min

↓

Observe under U.V light

Protocol- 2
Estimation of IAA

Take 2ml of Supernatant

↓

Add 2drops of Orthophosphoricacid+ 4miofsolawask's reagent

↓

Kept at room temp for 15min

↓

Pink color develop in tube

↓

Measure optical density at 530nm

↓

Prepared standard IAA solution and prepared standard graph

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