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PRODUCTION OF PLANT GROWTH HORMONES INDOLE-3-ACETIC ACID (IAA) USING BACILLUS BY BATCH FERMENTATION

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ABSTRACT
Indole acetic acid (IAA) is one of the most important physiologically active auxins. IAA is a general product of L-tryptophan metabolism produced by many microbes including Plant Growth- Promoting Rhizobacteria (PGPR). Tryptophan is an essential amino acid that can undergo oxidation by the action of the bacterial enzyme tryptophanase. Not all bacteria possess this enzyme and so this test can be used as a biochemical differentiator this tryptophanase is mainly use in production of plant growth hormones using Rhizobacteria. The most important effect of indole acitic acid is to promote development of roots and stems, through stretching of the newly formed cells in the meristem. This result depends, however, the concentration of the hormone, in several tissues the IAA control cell division. Partial purification of IAA is done and purity is confirmed with thin layer chromatography. In conclusion the study suggests the IAA producing bacteria as efficient Auxins class hormones inoculants to promote plant growth. The main goal of this study is to isolate and screen indigenous Indole acetic acid producing bacteria from different rhizospheric soil.

KEY WORDS- IAA, Bacillus, Staining, Fermentation, Downstream process.

INTRODUCTION

Indole acetic acid (IAA) is the mainly common, naturally-occurring, plant hormone of the auxin class. It is the top known of the auxins, and has been the subject of general studies by plant physiologists. [Chemically, indole acitic acid is a carboxylic acid in which the carboxyl group is attached through a methylene group to the C-3 position of an indole ring]. In appearance, IAA is a colorless solid[1]**.** The rhizosphere, representing the thin coat of soil surrounding plant roots and the soil occupied by the roots, supports big active groups of microbes[2] well-known as plant growth promoting Rhizobacteria (PGPR)[3]. Indole acetic acid (IAA) is one of the mainly physiologically active auxins. IAA is a general product of L- tryptophan metabolism produced by many microbes including Plant Growth- Promoting Rhizobacteria (PGPR)[4]. Bacteria that colonize the rhizosphere can exhibit a range of characteristics responsible for influencing plant growth. The general traits include production of plant growth regulators (like auxin, gibberellins, and ethylene), siderophores, HCN and antibiotics [5]. Bacteria synthesize auxins in order to perturb host physiological activity for their own benefits[6]. The microbes is isolated from rhizosphere zone of different crop have an ability to produce Indole acetic acid as secondary metabolites due to rich supply of substrates. IAA helps in the production of longer roots with increased quantity of root laterals and root hairs which are involved in nutrient uptake[7]. Indole acitic acid stimulates cell elongation by modifying certain conditions like, increase in osmotic contents of the cell, increase in permeability of H_2O. into cell decrease in wall pressure, an increase in cell wall synthesis and inducing specific RXA and 638 protein synthesis. It promotes embial activity, inhibit It promotes embial activity, inhibit or delay abscission of leaves, induce flowering and fruiting [8]. IAA is a metabolite derived from Trp by many Trpdependant and Trp independent pathways in bacteria and plants. More than one pathway could be present in a bacterium[9]. Physiological evidence for special Trp dependent pathways for synthesis in Azospirillum brasilense has been reported[10]. In Trp dependant pathway, tryptophan is changed to indole-3 acetamide (IAM) by tryptophan-2 monooxigenase and IAM is metabolized to IAA by IAM hydrolase[11]. Horemans and Vlassak (1985) demonstrated that a brasilense could produce IAA in the absence of tryptophan when developed aerobically showed that the main levels of auxin were produced in the presence of NH_4. It appears to be of particular significance during embryogenesis, when fine control over low levels of IAA is critical to polar development. Trp-independent pathway might supply significantly to the recently synthesized IAA; however, extensive Trp-to-IAA conversion also occurs in such preparations. Indole acetic acid (IAA) - causes stem Elongation and growth - formation of adventitious and lateral roots, inhibits leaf. The most important effect of indole acitic acid is to promote development of roots and stems, through stretching of the newly formed cells in the meristem. This result depends, however, the concentration of the hormone, in several tissues the IAA control cell division [12].

MATERIALS & METHODS

Sample collection

Soil samples were collected from Rhizospheres of different Field of different locations of nearby regions of Bhopal (M.P.) India. At each sites, 2 samples were taken randomly. The samples were then transported in sealed

aluminum foil to the laboratory, where stones in the samples were removed and the soils were homogenized through a 2 mm sieve. The samples were store in the dark bottles till further analysis.

Isolation of microbes (Bacillus)

Firstly prepare of bacillus selective media and then after sterilize media and glasswares. pH should be 7.2 to 7.4. For isolation of Bacillus strains, a serial dilution technique was followed and a 10^3 dilution of each sample was prepared, after serial dilutions were prepared and a volume of 200 µl was spread on bacillus selective medium for the isolation of IAA Producing microbes. The inoculated Petri dishes were incubated 48 hours on 37.4^{o}C. The isolates were maintained on L.B. medium for further analysis.

Identification & characterization of microbes

Morphological Characterization

Staining

The difference in staining responses to the gram stain can be related to chemical and physical difference in their cell walls. The gram negative bacterial cell wall is thin, complex, multilayered structure and contains relatively a high lipid contents, in addition to protein and mucopeptides. The higher amount of lipid is readily dissolved by alcohol, resulting in the formation of large pores in the cell wall which do not close appreciably on dehydration of cell wall proteins. Thus facilitating the leakage of crystal violet-iodine (cv-1) complex and resulting in the decolonization of the bacterium which later takes the counter stain and appears red. In contrast the gram-positive cell wall are thick and chemically simple, composed mainly of protein and cross-linked mucopeptides, when treated with alcohol, it causes dehydration and closure of cell wall pores, thereby not allowing the loss of (cv-1) complex and cells remain purple [13].

Method

First Make thin microbial smears on glass slide. Then Let the smear air dry and Heat fixes the smears. Hold the smears using slide rack or cloths pin. Cover each smear with crystal violet for 30 seconds. Then after Wash each slide with distilled water for a few seconds, using wash bottle and Cover each smear with iodine solution for 30 seconds. Wash off the iodine solution with 95% ethyl alcohol; add ethyl alcohol drop by drop, until no more color flow from the smear and Apply safranin to smear for 30 seconds. Last treated Wash with distilled water and blot dry with absorbent paper. Let the stained slides air dry and observe to microscope.

Indole production test

Tryptophan is an essential amino acid that can undergo oxidation by the action of the bacterial enzyme tryptophanase. Not all bacteria possess this enzyme and so this test can be used as a biochemical differentiator. 1% tryptone broth was prepared. The medium was sterilized by autoclaving at 121^{o}C for 15 to 20 min. When the temperature of the broth medium comes to room temperature poured the broth in deep tubes. Each tube was labeled with the name of the organisms to be tested. The tubes were then inoculated with the organism to be tested and tubes were then incubated at 37.4^{o}C for 24 to 48 hrs. After 48 hrs of incubation 200µl of Kovac‘s reagent is added. Shake the tubes gently after intervals for 5-10 min. the tubes were allowed to stand to permit the reagent to come up to the top. The formation of a cherry red reagent ring is a positive result and absence of cherry red colour as indole negetive

When cultured on peptone H_2O, a liquid medium containing tryptophan, certain bacteria will produce indole. The occurrence of this indole is readily revealed through addition of Kovak’s solution, producing a pink color. This reagent contains the organic solvent amyl alcohol that extracts the collared (pink) substance [13].

Production of IAA by fermentation

Prepare of IAA fermentation media (Triptone Yeast extract broth media) whose constituents are as - Triptone 5 gram, Yeast extract 2.5 gram, Dextrose 1gram, Distilled water 1000 ml. pH should be 7.3 before sterilization. Sterilize of medium then after some time 20 -20 ml pour media each gem bottle and inoculated to purify sample in each gem bottle. Inoculated bottle were incubated on Shaker fermenter at normal room temperature for 7 to 10 days.

Extraction of IAA from fermented media by downstream process

The procedure for separation of IAA from fermented medium is given below,

The fermented medium is centrifused at 5000 rpm for about 15 to 20 min. during the centrifugation, the mycelial fragments and spores settle down at the bottom of the centrifuge tube. The supernatant solution is seprated from the tube and treated with 1 N HCL to reduce the pH to 3 and prepare 10 ml (5%) sodium carbonate solution with distilled water. The sodium carbonate solution added 5 – 5 ml in each supernatant solution. This solution treated with equal volume of di ethyl eather. This acidified solution is transferred a separating funnel and shaken well at normal room temperature for about 30 to 45 min. the IAA gets dissolved in ether, After settle down of Di ethyl eather seprated IAA in petri plates. It is concentrated by exposing it to air (for 24 hours) to gate some crystal of IAA. It has mixture of different types of Auxins. They are seprated by chromatography.

Quantitative analysis of IAA

A sample of isolated IAA is treated with salver reagent (0.5M Fecl3 in 5 ml D\w, 35% Perchloric acid with D\w) Added of 200 µl Fecl3 in 10 ml Perchloric acid. It is a salver reagent. Sample / standard were used with different concentration with distilled water and salver reagent, whose constituents are as (Table 1).’’ Quantitative analysis was carried out by using spectrophotometer at 535 nm in this observed the good quantity of produced IAA after downstream processing.

TABLE 1- Different concentration of Sample / standard with distilled water and salver reagent.

S.No.	Sample\standard	D\W	Salver reagent
1	20 µl	980 µl	1ml
2	40 µl	960 µl	1ml
3	60 µl	940 µl	1ml
4	80 µl	920 µl	1ml
5	100 µl	900 µl	1ml

Qualitative analysis of IAA

Thin layer chromatography (TLC) slide was prepared with silica gel G. for dry of slide on heating mantle at 100^{o}C for 30 minute. Propanol and distilled Water (8:2) was used as Solvent system. The produce sample and standard indole acitic acid (10mg/100ml) were spotted on TLC plate [14].

RESULTS & DISCUSSION

Microbial Analysis

Five bacteria isolates were successfully isolated as IAA producer from rhizosphere soil among which two were selected based on IAA formation ability. The isolates were identified based on morphological study and grams staining and biochemical characterization [15].

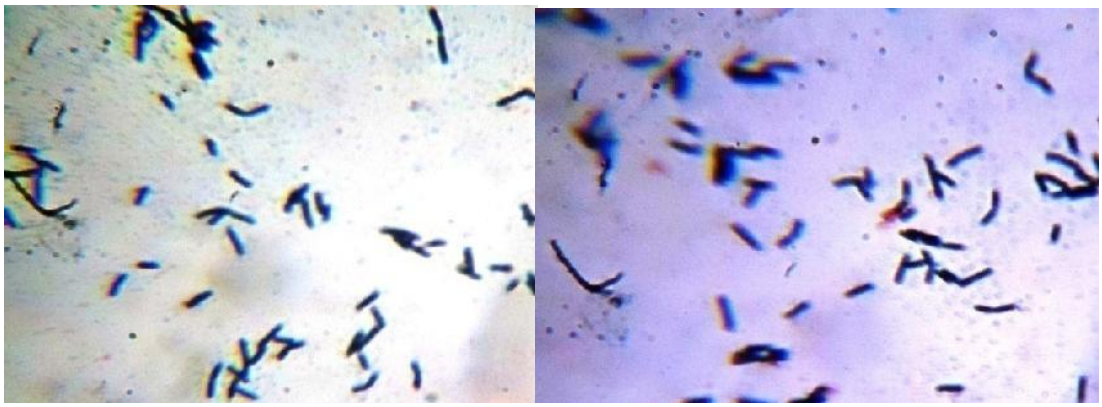
Staining

Gram's staining of all the isolated bacteria from samples of soil isolates has been done. Thus, the results of gram's staining of the pure culture isolated from sample of soil bacteriais listed below in the (Table 2).'' [13].

Gram staining showed that the bacteria are Gram Positive bacteria because the cells are Purple in color under the compound microscope after staining. It has a rod shape with irregular clusters arrangement of colonies. Based on this result, the possible unknown has been narrowed down into 2 from the possible species given (Fig. 1).''

TABLE 2 - Result of Gram's staining of Bacterial culture isolated from soil samples

S.No.	Bacterial ID	Status
01.	S-1	+ve
02.	S-2	+ve
03	S-3	-ve
04	S-4	-ve
05	S-5	-ve



(A) (B)

FIGURE 1 - Gram staining test for identification of microorganism biochemical analysis.

Indole production test

The tubes were then inoculated with the organism to be tested and tubes were then incubated at 37.4^{o}C for 24 to 48 hrs. After 48 hrs of incubation 200µl of Kovac's reagent is added. Shake the tubes gently after intervals for 5-10 min. the tubes were allowed to stand to permit the reagent to come up to the top. The formation of a cherry red reagent ring is a positive result and absence of cherry red colour as indole negative**.** When cultured on peptone water, a liquid medium containing tryptophan, certain bacteria will produce indole. The presence of this indole is readily revealed through addition of Kovak's reagent, producing a pink color. This reagent contains the organic solvent amyl alcohol that extracts the collared (pink) substance (Fig. 2).'' (13).

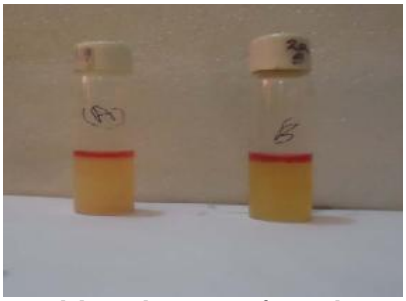


FIGURE 2: Indole production test for production of IAA.

Production of IAA by fermentation

Fermentation was done by IAA fermentation media (Triptone, Yeast extract, broth media) inoculated bottle

were incubated on Shaker fermenter at normal room temperature for 7 to 10 days. And Complete of Fermentation further analysis.

Extraction of IAA from fermented media by downstream process

Fermented solution is transferred to a separating funnel and treated with Di ethyl ether this solution is shaken well for about 30 to 45 min. The IAA gets dissolved in the ether. The fraction of ether is separated from the separating funnel and its volume is reduced to ¼ of the original of ether (Fig. 3).'' after extraction of IAA by downstream Process. In di ethyl ether solution and 20 ml Fermented media. And centrifuge media was 10 ml each solution. And mix equal volume of Di ethyl ether. After extraction produce IAA were 6 mg and 48 mg.

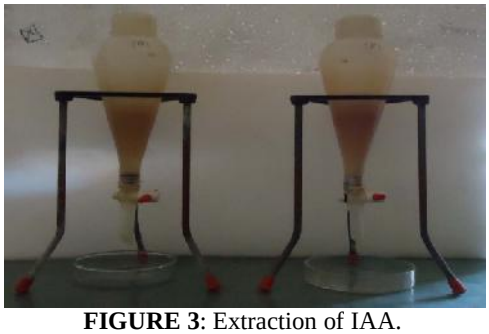
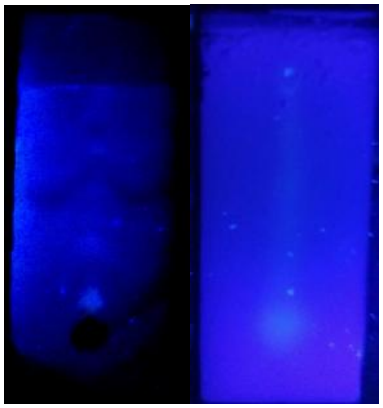


FIGURE 3: Extraction of IAA.

Qualitative analysis by TLC

The manufacture of IAA was definite by TLC. Chromatogram of culture extract mark and standard IAA when observed in UV light showed the same Rf value 0.928. The TLC findings are in agreement with other reports [16].the extracted methylated compound was further confirmed as IAA. The production of auxins also depends upon the type of microorganisms and strains and on their age. The maximum IAA was observed in the stationary phase of PSM and other species of Pseudomonas species and Bacillus species. The auxins type substances were detected by means of paper chromatography methods.

Qualitative analysis had done by TLC (thin layer chromatography) using Propanol and Distilled water (8:2) solvent system (Fig. 4).'' Rf value of produced content 2.78 and 3.15 of produced IAA by selected bacterial isolates (Table 3).''



(A) (B)

FIGURE 4 - TLC of IAA.

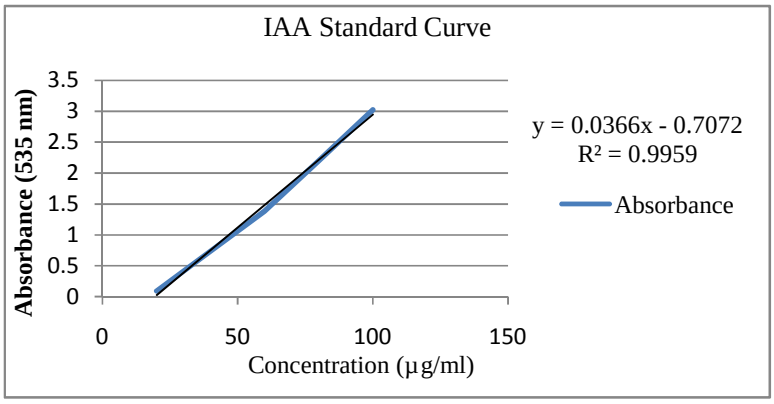
TABLE 3: Rf value of produce IAA.

S.No.	Sample	Rf Value
1	S-1	2.78
2	S-2	3.15

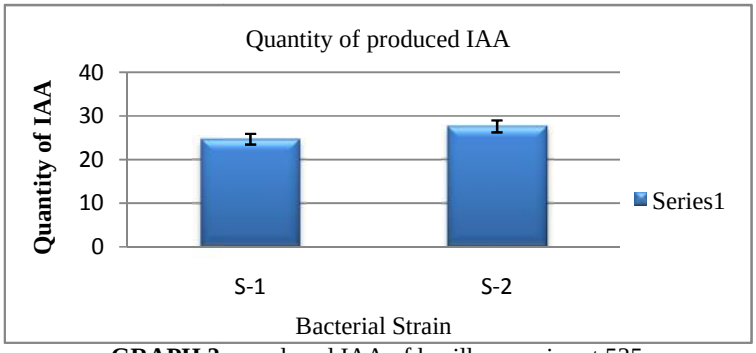
Quantitative analysis of IAA

Quantitative analysis was carried out by using spectrophotometer at 535 nm in this observed the good quantity of produced IAA after downstream processing. This analysis had done by standard IAA with different concentration of standard IAA (Table 4).''

A sample of isolated IAA is treated with salver reagent. It gives characteristic coloration, which indicates the presence of IAA in the solution. The help of standard graph (Graph. 1) and sample graph (Graph. 2) were simply quantified of produce IAA after downstream process. And produce IAA Were 24.68 µg/ml and 27.62 µg/ml (Table 5).''



GRAPH 1: Standard curve of IAA



GRAPH 2 - produced IAA of bacillus species at 535 nm

TABLE 4: Standard IAA

Concentration	Absorbance
20 µg/ml	0.093
40 µg/ml	0.741
60 µg/ml	1.38
80 µg/ml	2.197
100 µg/ml	3.021

TABLE 5: Produce IAA

Bacterial Strain	Quantity of IAA (µg/ml)
S-1	24.68
S-2	27.62

CONCLUSION

The first goal of this study was to isolate and screen indigenous Indole acetic acid producing bacteria from different rhizospheric soil. The next was to purify the IAA and screen their abilities of plant growth promoting rhizobacteria attributes. IAA is several special effects as every auxins do, such as inducing cell division and cell elongation with every subsequent product for plant growth and development. I found the good quantity and quality of Indole acitic acid. Uses of IAA different but results are very good because IAA is auxins class plant growth hormones. This Research can help to easily produce of IAA in few conditions, because IAA is secondary metabolite of microbes.

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