OPTIMIZATION AND PURIFICATION OF INDOLE ACETIC ACID (IAA) PRODUCED BY RHIZOSPHERIC PSEUDOMONAS SPP.

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ABSTRACT:

Indole-3-acetic acid (IAA, 3-IAA) is the most common, naturally occurring, plant hormone of the auxin class. Microorganism have ability to produce IAA. The present work deals with isolation, characterization, optimization and purification of Indole acetic acid (IAA) producing bacteria form rhizospheric soil sample from wheat plant. The IAA producing bacteria were isolated, screened and purified on Nutrient agar with tryptophan plates. Screening resulted in selection of bacterial isolate grow on the plates were they able to utilize tryptophan and produce the IAA. The morphological characterization showed the bacteria to be a Gram negative, motile, short rod-shaped bacterium. The biochemical tests indicated that the bacteria belonged to the *Pseudomonas Spp.* (According to Bergey's Manual of Determinative Bacteriology). The bacterial isolate was then subjected to IAA production, which was further optimized with various parameters like incubation temperature, pH, salt concentration and also with different carbon and nitrogen sources.

INTRODUCTION:

Indole acetic acid (IAA) is one of the most physiologically active auxins. IAA is a common product of L- tryptophan metabolism produced by several microorganisms including Plant Growth Promoting Rhizobacteria (PGPR). Various microorganisms present in soil capable of producing IAA include various bacterial and fungal species. The rhizospheric bacteria utilise the root exudates to produce IAA as part of their secondary metabolism. [8] IAA is produced in young leaves, stems, roots and seeds from transamination and decarboxylation reactions of tryptophan. Most of the field crops lacks in IAA due to different causes. Therefore, the IAA supplementation is a challenge for the crop production especially during the vegetative growth periods. Plant root system is a vital zone which could enhance the growth of soil microbes, the term rhizosphere first coined by Lorenz Hiltner. [2] The mechanism of plant growth promotion by the PGPR may be direct or indirect. PGPR could directly promote plant growth by producing various phytohormones or enhancing phosphate availability etc. [16]

IAA is a metabolite derived from Trp by many Trp dependant and Trp-independent pathways in plants and bacteria. [18] IAA is a heterocyclic compound containing carboxymethyl group (acetic acid) that belongs to the most studied phytohormone, and is involved in numerous mechanisms in plant physiology. They are responsible for division, extension and differentiation of plant cells and tissues. [9] IAA production is a major property of rhizosphere bacteria that stimulate and facilitate plant growth. In general, plant growth

promoting bacteria (PGPB) have been shown to improve plant health by increasing nutrient availability, providing defence against pathogens, protection from abiotic stresses, and producing phytohormones such as auxin. Therefore, our aim in this work was to focus on indole related compounds as an initial screen to identify IAA producing bacteria.

MATERIALS AND METHODS:

Isolation of IAA producing bacteria:

Soil sample were collected from the rhizosphere of wheat in the Bhorawadi, Ahmednagar, Maharashtra. The isolation of the microorganisms was done as follows. 1g of rhizosphere soil in 50 ml flask was taken and 50 ml sterile distilled water was added then 1 ml sample was serially diluted up to 10^{-10}. 0.1 ml of diluted sample was plated on sterile NA agar medium and incubated for 24hrsrs at 37^0C. Single colonies were picked up and streaked on sterile NA agar with tryptophan plates to get pure culture.

Screening of IAA production:

To determine the amounts of IAA produced by isolate, a colorimetric technique was performed with Van-Urk's (Salkowski reagent) using the Salkowski's method [7]. The isolates were grown in NB with tryptophan and incubated at 37 °C for 4 days. The broth was centrifuged after incubation. Supernatant was reserved and 1ml was mixed with 2ml of Salkowski's reagent (2% 0.5 M FeCl3 in 35% HClO4 solution) and kept in the dark. Development of pink colour shows IAA production.

Characterization and identification of isolate:

The isolate was subjected to morphological identification by Gram staining and microscopic observation. The colony characters on the NA with tryptophan agar plates were also recorded. Motility test was performed according to the standard protocol. The isolates were further identified biochemically by enzymatic profiling (catalase and oxidase test) and biochemical test using Sugar fermentation test, IMViC test, Nitrate reduction test, Sugar utilization. With the help of the test results, the isolate was identified using Bergey's Manual of Determinative Bacteriology 9th edition.

Preparation of Standard Graph of IAA: Range between standard of IAA: - 10 – 100 ug/ml

Different IAA concentrations are prepared as aqueous solution of IAA range from 10 ug/ml to 100 ug/ml. make the 2 ml of the standard working solution, and add 2-3 drop of orthophosphoric acid and 4 ml of 2% 0.5 M $FeCl_3$ in 35% perchloric acid i.e. Salkowski's reagent is added and readings are taken after 25 minutes at 530 nm by spectrophotometer.

Optimization of IAA production:

The IAA production was optimized by growing bacteria under various conditions of the culture media with various parameters such as temperature, carbon and nitrogen sources, pH and salt concentration.

a) **Effect of incubation temperature on IAA production:**
   Isolate on NB with tryptophan was incubated at different temperature; Refrigerator, Room temp, 37^0C and 60^0C. IAA production was measured at 24 hrs.
b) **Effect of carbon sources on IAA production:**
   Carbon sources, such as sucrose, dextrose, fructose and mannitol, were added to the media (NB with tryptophan) to obtain optimum IAA production. The culture was incubated at 37^0C for 24 hrs.
c) **Effect of nitrogen sources on IAA production:**
   Tryptone, beef extract, ammonium chloride and ammonium nitrate were used to substitute peptone in NB with tryptophan to investigate the effect of various Nitrogen sources on IAA production. The culture was incubated at 37 ^{0}C for 24 hrs.
d) **Effect of pH on IAA production:**
   Isolate was inoculated in NB with tryptophan at different pH; 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9 to obtain optimum pH for IAA production. The culture was incubated at 37 ^{0}C for 24 hrs.
e) **Effect of salt concentration on IAA production:**
   The effect of salt concentration on IAA production was observed on NB with tryptophan in different salt concentration of 0.25%, 0.5%, 1%, 1.25%, 1.5%. The culture was incubated at 37^0C for 24 hrs.

Extraction and purification of IAA:

Isolates were cultivated in NB with tryptophan and it was centrifuged by micro-centrifuge 10000 rpm for 10 min. The supernatant was collected and mixed with ethyl acetate (1: 2). After vigorous shaking it was allowed to stand for 10 min. IAA was extracted within

solvent layer. The ethyl acetate fraction was evaporated to dryness. The dried extract was dissolved in phosphate buffer for further use.

Thin layer chromatography:

TLC slide was prepared with silica gel G and calcium carbonate. Propanol: Water (8:2) was used as Solvent system. The extracted sample and standard IAA (10mg/100ml) were spotted on TLC plate. Chromatogram was developed with the Salkowski's reagent. (12)

Infrared spectroscopy Analysis for Confirmation of IAA Production:

In order to confirm the production of IAA by bacterial isolate based on information about its chemical bonds and molecular structure, Infrared spectroscopy analysis was carried out. Ethyl acetate extract was completely dried and mixed with spectral grade potassium bromide, and Infrared spectral analysis of IAA was recorded at the transmission mode from 400–4000 cm^{-1}.

RESULTS:

Isolation and screening of IAA producing bacteria:

After incubation at 37^0C for 24hrs the growth was observed. The screening was carried out by using Salkowski reagent. Pink colour development was first visible at the highest IAA concentration within minutes and continued to increase in intensity for a period of 30 min.

Characterization and Identification:

After incubation at 37°C for 24hrs, on Nutrient agar with tryptophan plate, the morphological and biochemical results were recorded as shown in table no. 1 and 2.

Table: 1 Colony character

Size	Shape	Colour	Margin	Elevation	Opacity	Consistency	Gram character	Motility
1mm	Short rod	Yellowish	Translucent	Convex	Opaque	Sticky	Gram negative short rod	Motile

Table: 2 Sugar utilization

Sugar	Result
Sucrose	Positive
Dextrose	Positive
Lactose	Positive
Mannitol	Positive
fructose	Positive

Table: 3 Sugar Fermentation

Sugar	Result	
	Acid	**Gas**
Dextrose	Positive	Negative
Fructose	Negative	Negative
Mannitol	Negative	Negative
Sucrose	Negative	Negative
lactose	Negative	Negative

Table: 4 Biochemical tests

Test	Result
Indole	Negative
Methyl red	Positive
Voges Proskauer	Negative
Citrate utilization	Positive
Nitrate reduction	Positive
Oxidase	Positive
Catalase	Positive

After reference from Bergey's manual of determinative bacteriology, the isolate was tentatively identified as *Pseudomonas spp.*

Preparation of Standard Graph of IAA:

Standard graph is prepared by plotting concentration of IAA in ug/ml Vs Optical Density at 530 nm. Standard graph of IAA (Fig. 2).

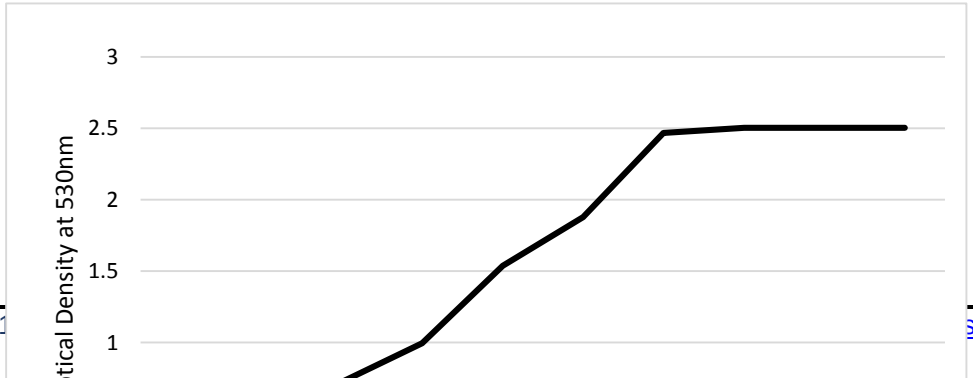


Fig. 2 Standard graph of IAA

Optimization of IAA production:

a) Effect of temperature on IAA Production:

The effect of temperature was studied in the range Refrigerator, Room temp,37^{0}C and 60^{0}C. where by maximum yield (100ug/ml) was observed at 37^{0}C by isolate (Fig. 3). Where 37^{0}C was the best temperature for IAA production for *Pseudomonas sp*. This indicates that the optimum temperature for IAA production by isolate was 37^{0}C.

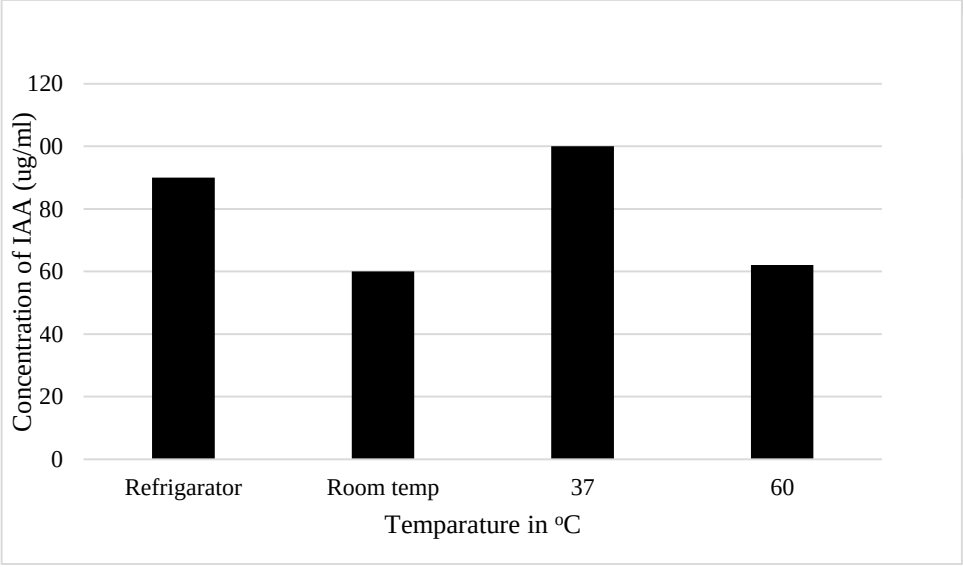


Fig. 3 Effect of temperature on IAA production

b) Effect of Carbon Source on IAA Production:

The carbon sources that were used in the biosynthesis of plant hormone during their growth in liquid culture media contribute to the overall efficiency of biosynthesis. On IAA production, different carbon sources were studied. Sucrose gave maximum production as compared to other carbon sources (Fig. 4).

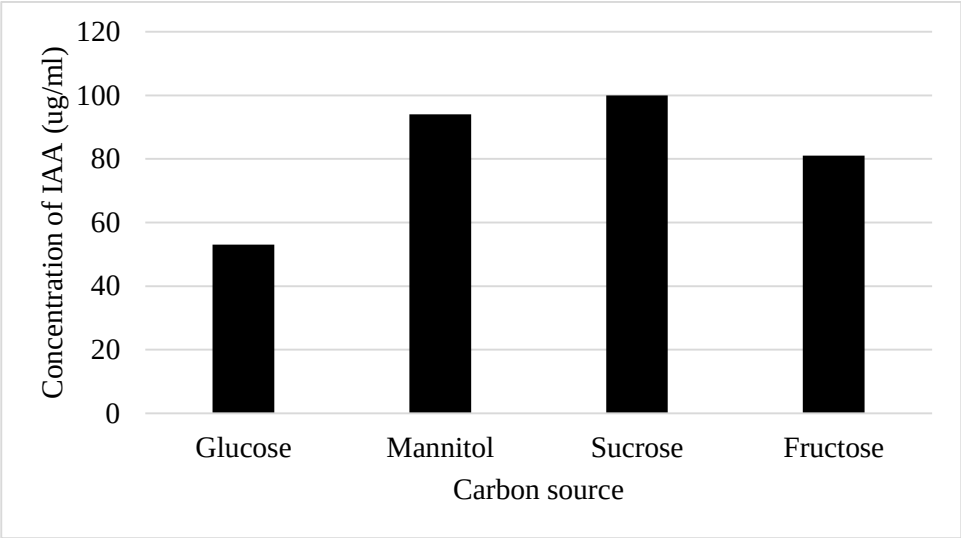
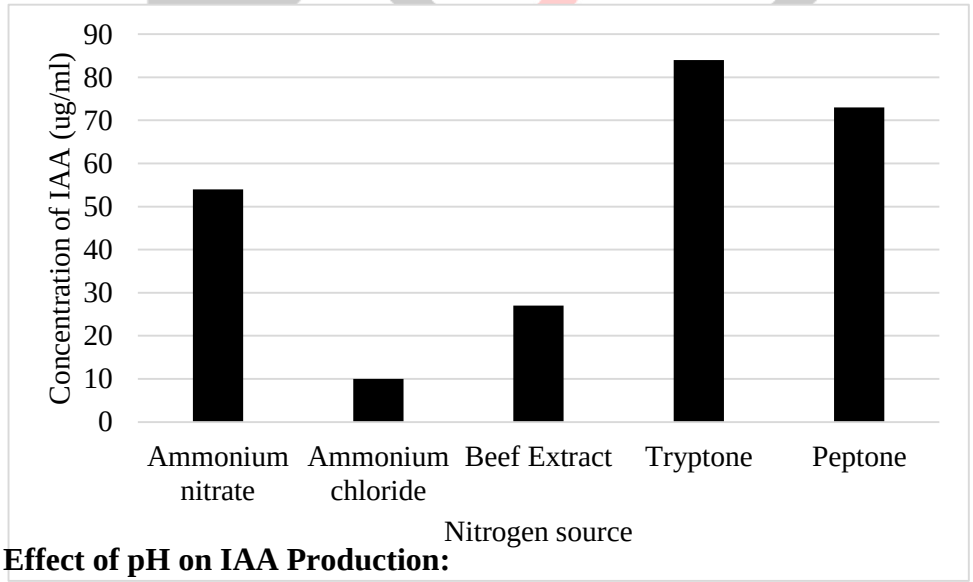


Fig. 4 Effect of Carbon source on IAA production

c) Effect of Nitrogen Source on IAA Production:

Impact of nitrogen sources on IAA production were studied by addition of various nitrogenous compounds to the tryptophan supplemented medium. Among all the nitrogen sources used, tryptone was found to be the best nitrogen source for IAA production (Fig. 5). The nitrogen source of the production medium affects IAA production.



d) Effect of pH on IAA Production:

Fig. 5 Effect of Nitrogen source on IAA production

One of the most important parameters for the growth of IAA producing organisms and their metabolic activity is the pH of the IAA production media. In our investigation, maximum IAA production was observed at pH 8 (Fig. 6). Where 8 was the best pH for IAA production for *Pseudomonas sp*.

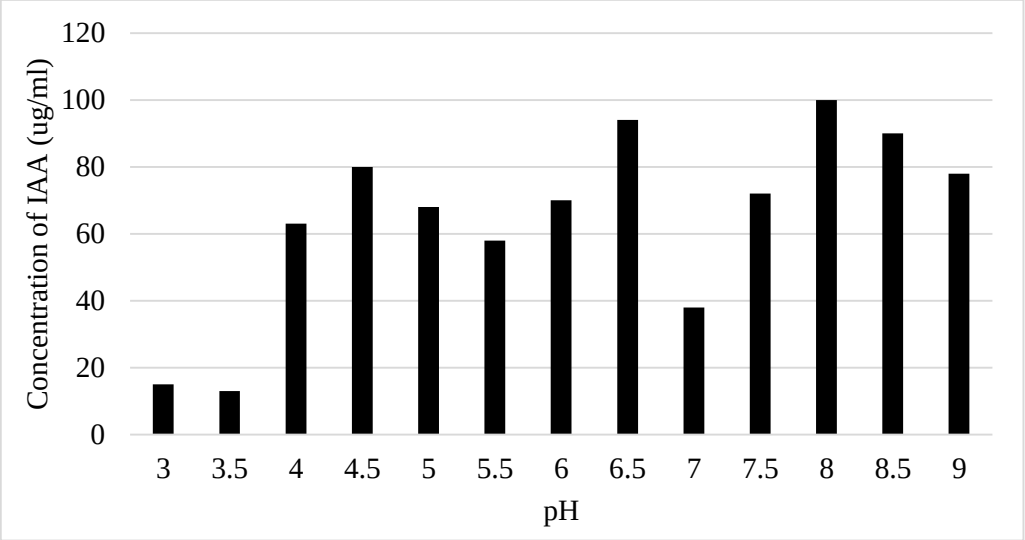
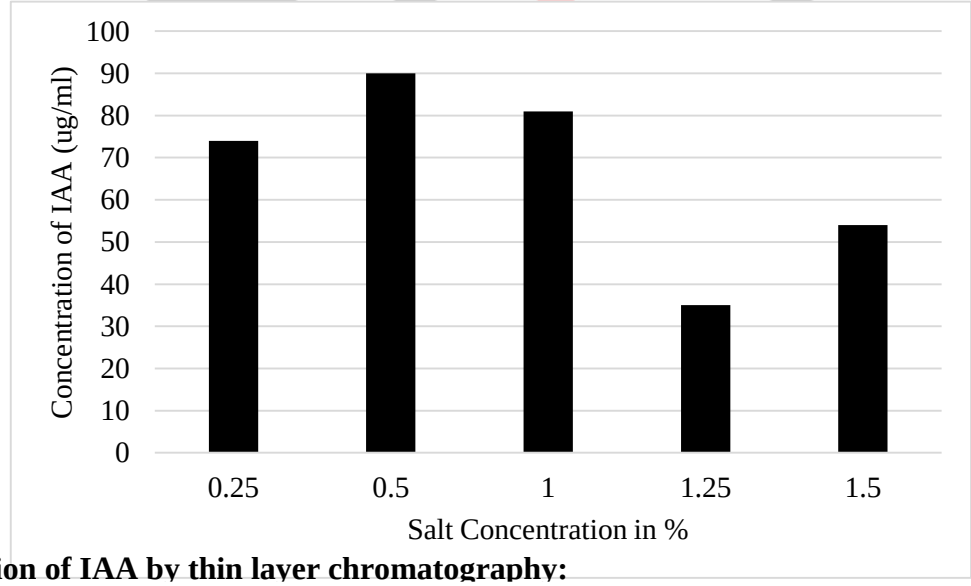


Fig. 6 Effect of pH on IAA production

e) Effect of Salt Concentration on IAA Production:

Organic salt in the media was helps to retain the osmotic balance and help in regulating membrane potential by providing sodium. Different concentrations of Salt were studied to see the effect on IAA production. The spectrophotometric analysis showed gradual in the IAA production with in salt concentration. 0.5% of salt concentration in the medium showed maximum IAA production (Fig. 7).



Detection of IAA by thin layer chromatography:

Fig. 7 Effect of Salt Concentration on IAA production

Purified IAA sample was compared with standard IAA on TLC chromatograms. TLC of ethyl acetate extract showed pink colour spot at the Rf corresponding to the authentic IAA (0.8) as shown in Fig.1. It confirmed IAA producing potential of rhizospheric isolate.

Fig. 6 Thin layer chromatogram of bacterially sized IAA detected by Salkowski's reagent compared with standard

Infrared spectroscopy Analysis for Confirmation of IAA Production:

Figure shows the infrared spectra of Extracted IAA from isolate. IR absorption at 3406.29 cm^{-1} indicates presence of (N-H) bond, absorption at 1666.50 cm^{-1} indicates presence of (=O) bond and absorption at 1801.51 cm^{-1}, 1845.88 cm^{-1}, 1884.45 cm^{-1} and 1919.17 cm^{-1} respectively for indicates presence of aromatic ring. Infrared spectra confirm the presence of IAA.

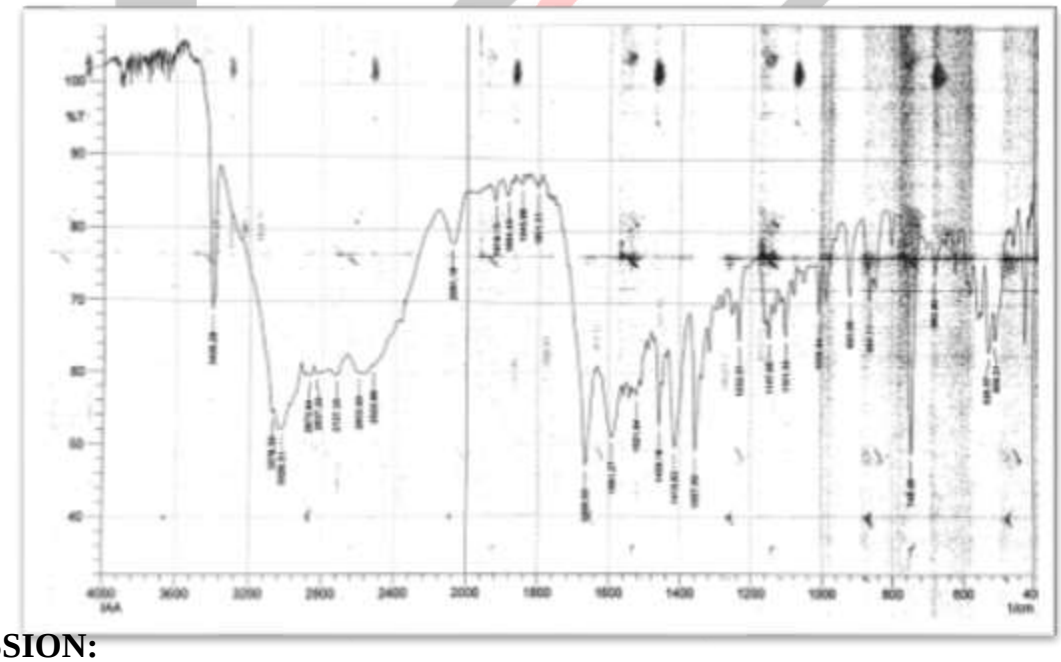


Fig No.7 Graph of infrared spectroscopy

DISCUSSION:

The present reports on IAA producing microorganisms indicate that similar media (Nutrient agar with tryptophan medium) was used for the isolation [B. Mohite. 2013]. By Salkowaski's Method was determined based on development of pink colour [J. Agr. Sci. Tech. 2016]. Optimum production of IAA from *Pseudomonas spp.* was achieved at incubation temperature 37˚C, pH 8, salt concentration 0.5%, sucrose as carbon source, tryptone as nitrogen source [Hariharan, H., Vellasamy, S. and Natesan, B., 2014].

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