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RESEARCH ARTICLE

ISOLATION AND IDENTIFICATION OF PHYTO HORMONE FROM WHEAT GRASS FUNGUS

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ABSTRACT:

Indole acetic acid (IAA) production is major properties of certain funguses that stimulates and facilitate plant growth. The present work deals with isolation, characterization and identification of indole acetic acid producing bacteria from the *Aspergillus* sp. Partial purification of IAA was done and purity was confirmed with Thin layer chromatography. Qualitative analysis by HPLC carried out for identification of Indole acetic acid. In conclusion, fungal can further be initiated towards purifying and standardizing for cell suspension cultures for enhanced production of secondary metabolites.

Keywords: Indole acetic acid, Isolation, *Aspergillus* sp.

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INTRODUCTION

Indole-3-acetic acid (IAA, 3-IAA) is the most common, naturally-occurring, plant hormone of the auxin class. IAA is a derivative of indole, containing a carboxymethyl substituent. It is a colorless solid that is soluble in polar organic solvents. IAA is also produced from tryptophan through indole-3-acetaldoxime in *Arabidopsis thaliana*. [1]

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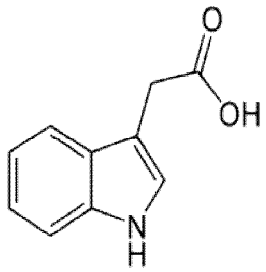


Figure: Chemical structure of indole-3-acetic acid

IAA is predominantly produced in cells of the apex (bud) and very young leaves of a plant. Plants mainly produce IAA from tryptophan throughindole-3-pyruvic acid.[2]. IAA is also produced from tryptophan through indole-3-acetaldoxime in Arabidopsis thaliana.[3]

Method and material

Sample collection:

Rotten wheat seeds were collected as sample from Crop field. The sample was collected from wheat plant which has fungal infection on upper. Sample was collected by using clean and dry sterile spatula in clean polythene (Fig.1).

Figure 1: wheat seeds

Sampling Procedure

For the purpose of sample collection, sampling kit consisting of several sterile polythene bags, a marking pen, spatula, alcohol, knives, etc. were taken. Sufficient amount of sample were collected from each site; kept in a polythene bag and tagged. At each time of collection, hands were sterilized with alcohol (70%) and then the bags were properly tied and labeled. Special care was always taken to avoid contamination as far as practicable.

Preservation of the sample

After collection, the samples were brought to the laboratory carefully and preserved in the refrigerators for immediate use; however for long- term usage, samples were stored at 4ºC.

Isolation of fungal cells for Indole acetic acid production: [4]

Media preparation & fungal culture

[A] Potato dextrose agar media: It's a common media for the isolation of Fungal cells this media allows the growth of almost all type of fungus, and it may be used to culture, subculture or storage of pure isolated Fungal colonies. The composition of media is;

Patato extract: 200gm

Dextrose : 20gm

Agar : 18.8gm

D/W : 1000ml

Potato extract: 200 gm of pealed potato are boiled in one litre of distilled water for 20-25 minutes then obtaining the drained water, adding the other component and making the final volume 1000ml.

Fungal culture

The sample was cultured in PDA plates under aseptic condition and then incubated 37^0c for 48 hours.

Sub culturing:

The separate colonies of master plates were sub cultured on separate PDA plates and again incubated for 3 days at 37^0C, these procedure was repeat three times to the get the pure cultures of the Fungus of interest (Fig. 2).

Figure 2: Inoculation for isolation of fungus

Preservation

The purified isolates were then transferred to the slants of potato dextrose agar media. The one -dram vial containing purified isolate, potato dextrose broth medium and sterilized glycerol was kept in the polyethylene bags, properly tied and preserved as stock culture.

Identification of fungus

Colony morphology and Microscopic study:

Fungal colony was observed after 48 hrs on PDA plates and further will be confirmed with lacto phenol blue staining method.

Lactophenol cotton blue staining:

1. Place a drop of 70% alcohol on a microscope slide.
2. Immerse the specimen/material in the drop of alcohol
3. Add one or at most two drop of lacto phenol cotton blue stain before the alcohol dries out.

4. Holding the cover slips between forefinger and thumb, touch one edge of the drop of stain with the coverslip edge, and lower gently, avoiding air bubbles. The preparation is now ready for microscopic examination.

Inoculation of fungal strain in Potato dextrose broth medium:

After the confirmation of isolated strain the fungal strains, the pure strain was recultured on potato dextrose agar medium for their cultivation.

Inoculation in Broth medium:

After the growth on Potato dextrose agar media plate the pure culture of fungal colony was inoculated in sterile potato dextrose medium for further study.

Desining of fermentation medium for production of Indole

For the production of IAA, a fermentation media of 100ml was prepared and inoculated with *Fungal spp.* and incubate for five days with regularly shaking for the mass cultivation of bacteria. Indole is the product or compound of tryptophan Compositions of fermentation media is as follows:

Composition:	Beef extract	3 gm
	Peptone	5gm
	Sodium chloride	5gm,
	Distilled water	1000ml.

Screening of Fungal isolates for Indole production:

To determine the production of Indole acetic acid in prepared fermentation medium, 3ml of culture were taken in a sterile culture tube and added 3-4 drop of Kovac's reagent and observe the ring formation on upper surface of culture medium.

Cell harvesting and separation of fermentum:

After the 5 days incubation of fermentation media the cells were harvested by centrifugation process. The fungal culture were taken in centrifugal tubes and centrifuge at 5000rpm for 30mins. After this extracted suspension was collected separately and apply triple filtration technique to avoid any impurity in this suspension.

Bio assay of Indole Acetic acid in Extracted suspension:

To examine the production of Indole acetic acid in extracted suspension a modified method of Loper and Scrowth (1986) was used. Procedure of this method IAA as follows:

- 2ml of supernatant was mixed with 2-3 drop of O-phosphoric acid and 4ml of Salkouski reagent solution.
- Samples incubate for 25 minute at room temperature in dark condition.
- Development of pink color was observed and Optical Density taken at 530nm with the help of spectrophotometer.

Detection of IAA by thin layer chromatography

Thin layer chromatography: T.L.C. is based on the adsorption phenomenon. In this type of chromatography mobile phase containing the dissolved solutes passes over the surface of stationary phase.

Steps involved in T.L.C.

1) Preparation of plates
2) Activation of plates
3) Preparation and saturation of chamber
4) Sample application and development
5) Detection and calculation of R_f Value TLC of extract was performed and reported.

Preparation of Plates

1. Silica gel, the most frequently used stationary phases, was employed as such for adsorption T.L.C.

2. To reduce the band broadening the stationary phase should consist of small particles of uniform size so as to provide a large surface area for interaction and a small void volume.
3. Silica Gel was mixed with water and made into slurry.
4. Coated the slurry by spreading the slurry on the plate uniformly.
5. Firstly air dried the plate for some time and then kept for activation.

Activation of Plates

1. By heating the plates in an oven at 100 to 110^{o}c for 30 minutes

2. Activation is necessary for linear movement of solutes over stationary phase.

Preparation and Saturation of Chamber

1. Prepared the solvent system.

2. Poured it into the chamber and saturated the chamber by lining the chamber with a piece of filter paper that has been wet with the mobile phase

Sample application and development

1. After plates were activated, the sample, which may be range from a few µg to mg. was applied on the plates with the help of capillary tube.

2. Plates were placed in the chamber that contains developing solvent to a depth of about 0.5 cm.

3. Plates were then removed from the chamber, the mobile phase front is marked by scratching the surface, and the solvent was evaporated in an oven.

Examination

The extracted methanol fraction of crude compounds was performed using pre-coated silica gel TLC plates of grade F274 (E Merck, Germany) to detect IAA compounds produced by *Aspergillus sp.* The crude extract was spotted with capillary tube and solvent front was allowed to run for approximately 80% of the plate. The crude was eluted with butanone-ethyl, acetate-ethanol-water (3:5:1:1) solvent system. Spots with Rf values coincide with that of authentic IAA Was identified under UV light (254 nm).

Detection and Calculation of R_f Value

1. Once the chromatogram was developed the R_f Value of the spot was calculated using the formula and results was depicted in Table.

$$R_f = \frac{\text{Distance traveled by solute}}{\text{Distance traveled by solvent}}$$

Determination of IAA BY Spectroscopy

Indole-3-acetic acid extraction and determination Indole-3-acetic acid production was determined. Culture media were filtered, and then samples were acidified to pH 3.0 with 1 N HCl and extracted using liquid-liquid (diethyleter/ NaHCO3) extraction. The samples were concentrated and dissolved in methanol. Indole-3-acetic acid in methanol was determined with Salper reagent (1 mL of 0.5 M FeCl3 in 50 mL of 35% perchloric acid). Absorbance was read at 535 nm using a Jenway 6105 UV/VIS spectrophotometer. The amount of indole-3-acetic acid was calculated from the standard curve (Fig. 4).

Detection of IAA by High performance Liquid Chromatography (HPLC)

Selection of stationary phase -

The column used in this method C_{18} grace. The configuration of the column is 4.6 x 250 mm, particle size 5 μm.

Selection analytical wavelength -

The sensitivity of HPLC method depends upon proper selection of detection wavelength. An ideal wavelength is one that gives good response for the drug that is to be detected. Appropriate dilution of each stock solution with mobile phase, various concentrations of IAA was prepared separately. Each solution was scanned in the spectrum mode between the range 200 to 400 nm were overlaid. The wavelength selected for the analysis was 220 nm at which both drugs showed significant absorbance.

Selection of mobile phase -

Based on sample solubility, stability and suitability various mobile phases and compositions were tried to get good resolution and sharp peak. The standard solution containing IAA as was run in different mobile phases. Acetonitrile and water in the ratio 40:70 % v/v, selected as mobile phase. Mobile phase was filtered through 0.45μm membrane filter and degassed by sonication for 20 minutes. Acetonitrile -water system containing 0.1% trifluoroacetic acid was selected, since it gave sharp completely resolved peaks with symmetry within limits and significant retention times for all the drugs.

Degassing the mobile phase-

The mobile phase prepared was degassed by ultrasonication for about 20 min so as to avoid the disturbances caused by dissolved gases.

Filtration of mobile phase

The mobile phase prepared was filtered through 0.45µg membrane nylon filter to remove the smaller particles that may be present in the mobile phase and which may caused by dissolved gases.

Preparation of standard stock solutions

10mg of IAA was weighed accurately and transferred to separate 10ml volumetric flask, dissolved in sufficient quantity of methanol and diluted to 10ml with the same solvent to give a stock solution of 1000ppm.

Loading of mobile phase

Filtered & degassed mobile phase was loaded in the reservoir. Priming was done for each freshly prepared mobile phase.

Baseline stabilization

The detector was tuned for an hour before the actual run in order to obtain the stable UV light. The mobile phase run was started at desired flow rate & the run was continued until the stable baseline was obtained.

Loading of samples

A well prepared & filtered sample of IAA was loaded into Rhenodyne injector port using a syringe & the sample was then injected.

Washing the column

Once the analysis of samples was finished, the column was first washed by flushing with the mobile phase for half an hour, afterwards with double distilled water & methanol 1:1 proportion for another one hour.

Selection & Optimization of HPLC method

After the selection of suitable mobile phase, it was then optimized for its reproducibility, sensitivity & accuracy. The optimized parameters for selected method are as below.

Table 1 - Optimized parameters for HPLC method.

Sr. No.	Parameter	Description
1	Stationary Phase	C_{18} column with 250 mm x 4.6 mm i.d and 5 µm particle size
2	Mobile Phase	Acetonitrile: water system containing 0.1% trifluoroacetic acid
3	Flow Rate	1.0 ml/min
4	Detection wavelength	220 nm
5	Detector	UV detector
6	Injector	Rheodyne Injection
7	Injection volume	20µl
8	Column Temperature	Ambient
9	Run Time	30 min

Checking the resolution:

The column was saturated with the mobile phase. Standard solution of Indole acetic acid was injected to get the chromatogram. The retention times for the two drugs were found to be: IAA: 8.312 min.

RESULT AND DISCUSSION

Sample collection

A rotten wheat seed was successfully collected as sample from Crop field. The sample was collected from wheat plant which diseased by fungal infection on upper body. The sample was collected by using clean and dry sterile spatula in clean polythene.

Screening of fungal cells on PDA media

Primary screening was carried out on potato dextrose agar media plates. On the basis of morphological expression isolates shows ***Aspergillus*** species which are ***Aspergillus niger***

and ***Aspergillus flaves*** exhibited higher growth of so these strains was selected for further study.

Colony Morphology and microscopic analysis

On the basis of culture characteristics ,colony morphology and lactophenol cotton blue staining the isolated fungus is recognised as ***Aspergillus niger*** and ***Aspergillus flaves.***

Effect of Temperature, pH and supplementation with carbon source

Temperature and pH are the most important factors, which markedly influence the growth of micro organism. Maximum indole activity is noted at 30^0C. Further increase in temperature, resulted the decrease in the indole activity. The effect of pH on the indole production activity indicates that the indole is more active at the pH 6.5-7.4 this suggested that the enzyme would be useful in process that requires slight acidic to slight basic range.

Screening of Indole activity in fermentation medium

To determine the production of Indole acetic acid in prepared fermentation medium, 3ml of culture were taken in a sterile culture tube and added 3-4 drop of Kovac's reagent. The cherry red color ring formation is appearing in the tubes that indicate the presence of indole in medium.

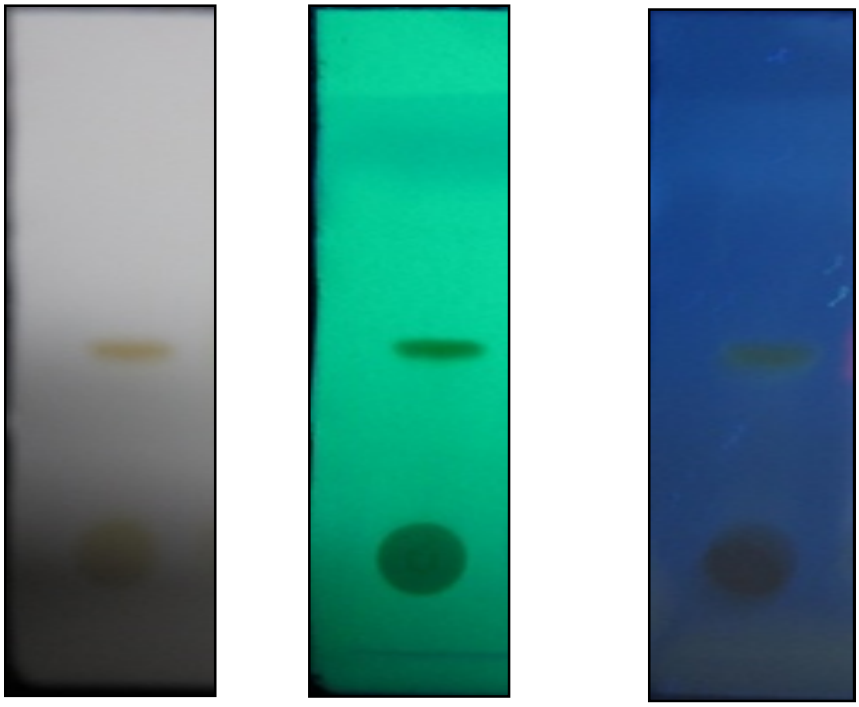
Bio assay of Indole Acetic acid in Extracted suspension:

Results of Thin Layer Chromatography

From the Rf value it was confirmed the presence of indole acetic acid in extracted Indole.

Table 2: Calculation of R_f Value

S. No.	Compound	Extract	Rf Value
1.	Indole acetic acid	Butanone-ethyl, acetate-ethanol-water (3:5:1:1)	0.68



Normal Light **Short U.V** **Long U.V**

Figure 3: Photograph of T.L.C.

Results of spectrophotometric estimation of IAA

Indole-3-acetic acid extraction and determination Indole-3-acetic acid production was determined. Culture media were filtered, and then samples were acidified to pH 3.0 with 1 N HCl and extracted using liquid-liquid (diethyleter/ NaHCO3) extraction. The samples was concentrated and dissolved in methanol. Indole-3-acetic acid in methanol was determined with Salper reagent (1 mL of 0.5 M FeCl3 in 50 mL of 35% perchloric acid). Absorbance was read at 535 nm using a Jenway 6105 UV/VIS spectrophotometer. The amount of indole-3-acetic acid was calculated from the standard curve.

$Y=0.011X+0.011$ with $r^2 = 0.997$

Table 3: Standard Curve of IAA

S. No.	Conc.	Absorbance
1	0	0
2	10	0.125
3	20	0.236
4	30	0.345
5	40	0.458
6	50	0.545

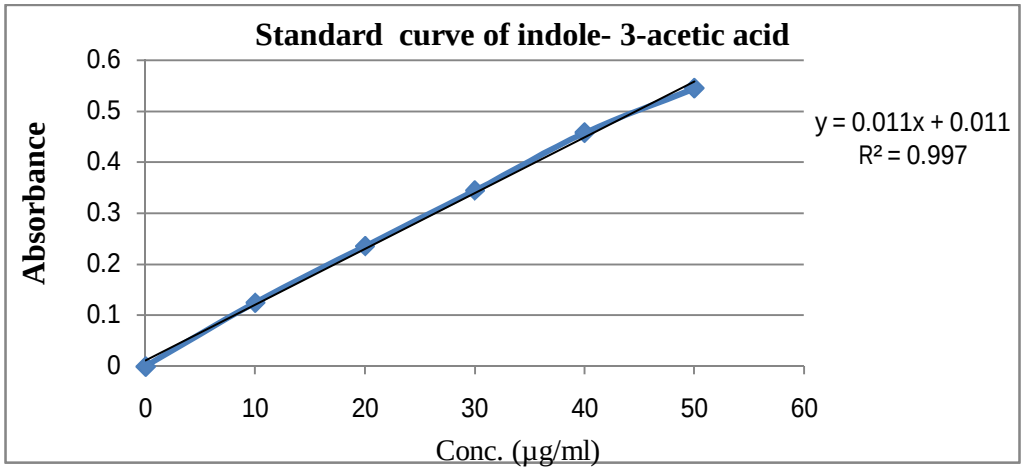


Figure 4: Standard curve of indole- 3-acetic acid

Table 4: Estimation of IAA using spectrophotometry

S. NO.	% Estimation
1.	0.356

Results of High Performance Liquid Chromatography

Identification of Marker Compound (Indole acetic acid) by HPLC

A reverse phase C-18 column equilibrated with mobile phase acetonitrile- water system containing 0.1% trifluoroacetic acid was used. Mobile phase was filtered through Whatmann filter paper and degassed. Mobile phase flow rate was maintained at 1 ml/min and effluents were monitored at 220 nm. The sample was injected using a 20 µl fixed loop, and the total

run time was 30 min. The sample solution was chromatographed and compared with standard (Fig. 5 & 6).

Standard and test chromatogram of Indole acetic acid showed same R_t value which confirm the presence of IAA.

Chromatogram of standard

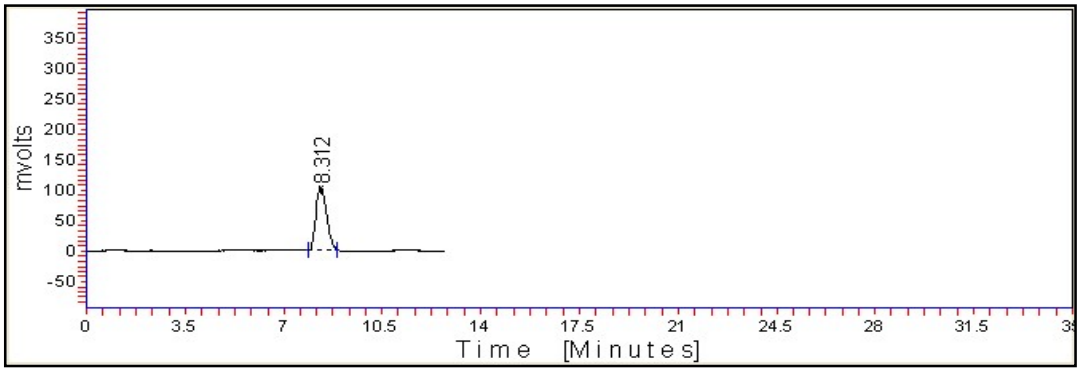


Figure 5: Chromatogram of standard IAA

Chromatogram of extracted Indole

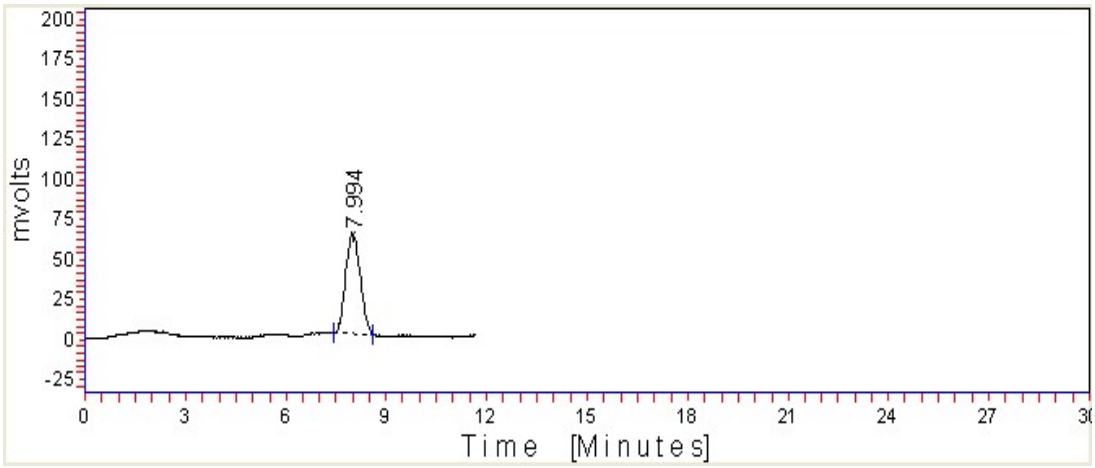


Figure 6: Chromatogram of extracted IAA

Table 5: Estimation of IAA using HPLC

S. No.	Sample	% estimation
1.	Extractred IAA	0.389

CONCLUSION

Thus indole-3-acetic acid from the cultures of *Aspergillus* sp. can be utilized for plant growth regulation. Fungal IAA could be used for further cell culture studies in order to enhance of plant secondary metabolites. If this fungal IAA further could also be a very efficient elicitor for plant secondary metabolite production, this method can also be an economical way of producing biotic elicitors. In conclusion, fungal can further be initiated towards purifying and standardizing for cell suspension cultures for enhanced production of secondary metabolites.

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