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Research Article

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Production of Indole Acetic Acid by a Wood Degrading Fungus *Phanerochaete chrysosporium*

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Abstract

Auxin is a plant hormone, which mainly includes indole acetic acid (IAA). The study was designed to evaluate and compare different techniques used to check the purity of IAA produced by *Phanerochaete chrysosporium*. Colorimetric method was used for the qualitative and quantitative determination of crude IAA. Partial purification of IAA was carried out by silica gel column chromatography. UV-Visible spectrophotometric analysis revealed the high similarity of the purified compound with standard IAA. Besides having a comparable UV-Visible spectrum, high performance liquid chromatography (HPLC) analysis showed two peaks, which also confirmed the presence of IAA. Thus the article describes efficient production of IAA by *Phanerochaete chrysosporium* and explores laboratory level experiment design to produce such bioactive compound with different methods to check its purity, which is simple, accurate and rapid to be employed for screening of new compounds.

Keywords

Indole-3-aceticacid, Auxin, *Phanerochaete chrysosporium*, HPLC, UV-vis spectrum

Introduction

Phytohormones are the signal molecules which act as chemical messengers to facilitate plant growth and development. Use of these hormones in agronomic applications has gained importance recently. One of such commercially significant phytohormone is indole-3-acetic acid (IAA). Indole-3-acetic acid is a monocarboxylic acid and one of the methyl hydrogens has been replaced by a 1H-indol-3-yl group in acetic acid having molecular formula $C_{10}H_9NO_2$ corresponds to a molecular weight of 175.187 g mol^{-1} (Figure 1).

Researches in biotechnology have explored the use of white rot fungi including *Phanerochaete chrysosporium* for the production of plant growth hormones [1]. These fungi can easily be grown on agricultural lignocellulosic waste like wheat or rice straw. Beside lignin, these agro residues contain water soluble fractions (soluble sugars, some pectin component, some soluble proteins and organic acids) and complex polysaccharides (hemicellulose and cellulose) which may be utilized by fungus as energy source for its growth and produce IAA. Due to their selective degradation of lignocellulosic biomass white rot species are widely used in biotechnological and biochemical applications such as bioremediation and delignification [2]. Extracellular auxin production by lignin degrading white rot fungi, *Phanerochaete chrysosporium* has been reported earlier [3].

Although there are numerous sensitive biological assays for auxins, most

are not specific for IAA, are time-consuming and have poor precision. Reliable and sensitive quantification methods for IAA have been developed over the last decade; however little amount of information is available on the levels of IAA produced by white rot fungi [4-6]. Prior to the development of chromatographic methods many color reagents were available for detection of IAA like Salkowski reagent (strong mineral acid plus a mineral oxidant), which has found extensive use in bacteriology [7]. This reagent yields a pink color with IAA while the intensity of the color diminishes in proportion to the IAA oxidized. The decrease in color intensity may be entirely due to the decrease in IAA concentration, or dependent upon the formation of peroxides during the oxidation of IAA by peroxidase [8]. Although the Salkowski assay may continue to be valuable, particularly on chromatograms but its application in solution is limited.

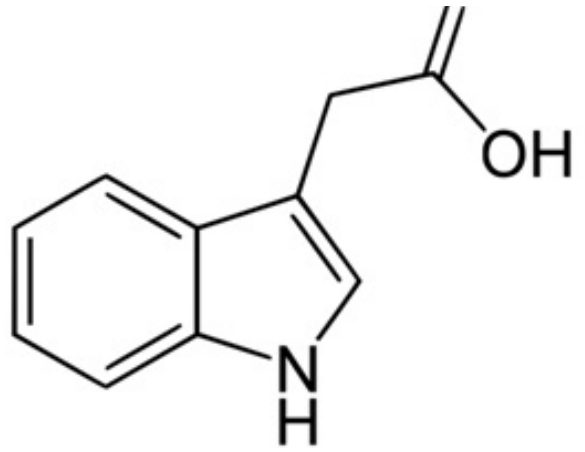

Figure 1: Chemical structure of indole-3-acetic acid.

The UV-Visible spectrophotometric determination is one of the most widely used methods for quantification of IAA produced due to its simplicity, low cost of implementation and wide availability in laboratories for quality control [9]. On the other hand, the HPLC analysis is an analytical procedure more sensitive and selective in the area of natural products to quantify isolated substances [10]. Moreover, multi-component formulations are difficult to separate via UV as they might have similar lambda max values but HPLC will clearly separate them via retention time.

Hence, in the present study, *Phanerochaete chrysosporium* was used to produce IAA using wheat straw under submerged conditions. The product was isolated and purified using column chromatography and further confirmed by UV/Vis spectrophotometric and HPLC analysis.

Materials and Methods

Substrate and organism

Wheat straw (WS) collected locally was ground (particle size 2 mm ± 0.5). *Phanerochaete chrysosporium* (BKM-F-1767) was received from the Center for Forest Mycology Research, USDA Forest Products Laboratory, Madison, Wisconsin. YPD [2% (w/v) peptone, 1% (w/v) yeast extract and 2% (w/v) dextrose] was used to grow the pure cultures of *P. chrysosporium.*

Screening of IAA production by *P. chrysosporium*

Flasks containing 1 g wheat straw and 50 ml of 0.5% (w/v) malt extract were sterilized and amended with 0.05% (w/v) tryptophan. *P. chrysosporium* (2 mycelial discs of 4 days old culture) was inoculated aseptically and incubated upto 10 days at 30 °C along with uninoculated control flask. Two ml aliquot was aseptically taken out from the flask at one day interval and centrifuged at 8000 rpm for 10 min. The supernatant was used for IAA estimation.

Analytical methods

Estimation of IAA

IAA produced was estimated according to the method described earlier [11]. Briefly, 1 ml of the supernatant was mixed with 1 ml of Salkowski's reagent and the OD was recorded at 530 nm after 30 min of incubation. Uninoculated sample flask was used as control. The standard IAA was used to prepare a standard curve for quantitative comparison.

Purification of IAA

Partial purification of IAA from crude extract was carried out by silica gel column (22 × 5 cm) using the methanol:water (9:1) as a mobile phase. The flow rate was kept at 1 ml/min and the fractions (2 ml) were collected up to 50 fractions. Each fraction was tested for the presence of IAA using Salkowski's reagent.

UV-Visible spectrum

The fraction (collected during column chromatography) showed maximum presence of IAA was used for spectrum analysis. The spectrum was run from 190 nm to 600 nm using appropriately diluted sample and compared with the standard IAA (0.01 mg/ml).

Confirmation of IAA produced by HPLC analysis

After partial purification, the positive fractions were pooled together and evaporated to dry in a rotary evaporator at 60 °C then solubilized in 2 ml of methanol. Presence of IAA produced was further confirmed by HPLC due to its higher sensitivity and accuracy using C18 column (5 µm; 25 x 0.46 cm) with elution performed at the ratio 9:1 of methanol and water, containing 0.5% acetic acid with a flow rate of 0.5 ml/min and the detection was monitored at 220 nm at 40 °C.

Statistical analyses

All the experiments were performed in triplicate and repeated. The values were represented as mean with standard deviation.

Result and Discussions

IAA production by *P. chrysosporium*

Auxin (IAA) is known as plant growth regulator which has hormonal functions. It is mainly produced by plants, but they are also produced by fungi as primary or secondary

metabolites [12]. In sterile conditions many of fungi can produce auxin mainly through indole-3-pyruvate acid and tryptamine pathway, most species uses tryptophan to produce IAA [13]. IAA production using a variety of fungal isolates like *Trichoderma harzianum* (68 µg/ml), *Penicillium citrinum* (52 µg/ml) and *Aspergillus niger* (85 µg/ml) has been reported earlier [14]. However, *P. chrysosporium* produced a significant amount of IAA (34.4 µg/ml) in medium supplemented with 0.5% (w/v) malt extract and 0.05% (w/v) tryptophan on 7th day of incubation (Figure 2). *Phlebia* species and *P. chrysosporium* were reported to produce similar amount of IAA (31-20 µg/ml) in complex yeast extract glucose broth [3]. Previous studies indicated that increased level of IAA inhibits the growth of fungi [15, 16]. Gradual decrease in IAA produced can be seen after reaching threshold (7th day), this decrease in level of IAA might be due to aging and death of the fungal biomass.

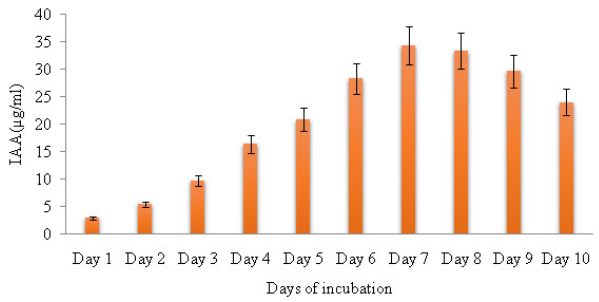

Figure 2: Production of IAA (µg/ml) by *P. chrysosporium*.

Purification of IAA

To obtain utmost extraction of IAA and minimize its destruction caused due to chemical reactions with impurities or oxidation [17], partial purification of IAA from crude extract was carried out by silica gel column chromatography. Fraction number 8 to 12 showed intense pink color after treatment with Salkowski's reagent, which confirmed the presence of IAA. These fractions were pooled together and used for further analyses.

UV-Visible spectrum

Plant hormones are difficult to analyze because they occur in very low amounts and are very rich in interfering substances, especially secondary metabolites. UV- visible spectrometry provides a quantitative assay and some assurance as to sample purity and identity. The λ_{max} of purified IAA was determined and compared with the λ_{max} of standard IAA. The wavelength (nm) at which the O.D. was maximum, that nm is considered as λ_{max} of the sample standard under study. Detection of IAA by UV- visible spectrometry had been studied earlier with comparable peaks [18]. The lambda max (λ_{max}) of purified IAA (100 times diluted fraction) that is 219 nm and 280 nm was same as that of standard IAA which confirmed the production of IAA by *P. chrysosporium* (Figure 3). However, the absorption maxima at 280 nm in purified sample may depict the presence of unutilized tryptophan or formation of tryptophan conjugate with indole-3-acetic acid (IAA-Trp) as high molecular weight conjugates with peptides and proteins also has been reported earlier [19, 20].

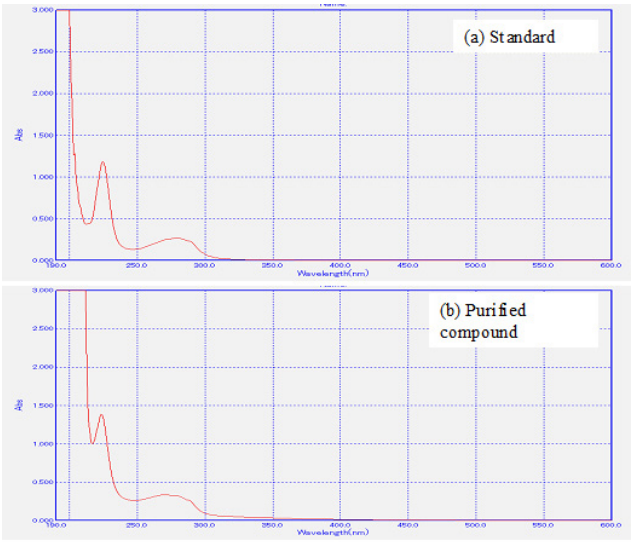

Figure 3: UV-Visible spectrum of IAA: (a) standard IAA (0.01 mg/ml); (b) purified compound from *P. chrysosporium* (100 times diluted fraction).

Confirmation of IAA produced by HPLC analysis

In order to get more precise and accurate results conformation of IAA produced was done by HPLC analysis and the detection was monitored at 220 nm as described earlier [21]. HPLC detected the peak at 6.737 min when standard IAA was run (0.1 mg/ml). A peak comparable to standard IAA confirmed the presence of IAA in the methanolic extract (Figure 4). However, an adjacent peak was also observed, which might belong to some related indole compound (indole-3-carboxylic acid) or amino acid conjugate (IAA-Ala) as described earlier during the HPLC determination of IAA [22, 23]. Similarly, HPLC analysis of IAA produced by fungus *Fusarium delphinoides* showed the comparable peaks to present study [24].

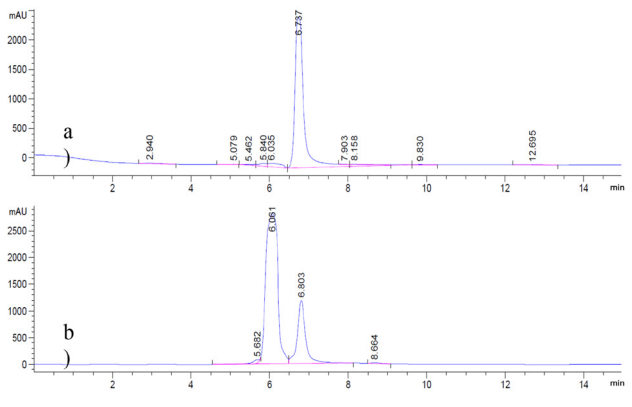

Figure 4: HPLC profile of (a) standard IAA, (b) purified IAA.

IAA comprises only up to 25% of the total amount of IAA in its free form. Various mechanisms such as transport, degradation, biosynthesis and conjugate formation regulate auxin homeostasis which later can be hydrolyzed to the active auxin. The most important forms of IAA conjugate are low molecular weight ester (with sugar moieties) or amide (with

amino acids) forms, but there is increasing evidence of the occurrence of high molecular weight conjugates with peptides and proteins (via amide bond) [25]. IAA conjugates with amino acids has been reported earlier in *Arabidopsis* [26] and *Helleborus niger* [27]. However, individual amide conjugates i.e. IAA–Glu and IAA–Asp in cucumber [28], IAA–Asp in Douglas fir [29] and IAA–Ala in spruce [30] have also been reported.

A biosynthetic pathway for the production of indole-3-acetic acid by an endophytic fungus, *Colletotrichum fructicola* also indicated that IAA production may follow indole 3-acetamide (IAM) routed for IAA biosynthesis [31]. IAM peak was also detected in the HPLC and the similar peak could also be seen the present study, so the possibility for the *P. chrysosporium* to follow the same path cannot be overlooked.

Conclusion

Phanerochaete chrysosporium produced a significant amount of IAA using wheat straw as substrate under submerged conditions. Also, IAA produced by wood degrading *P. chrysosporium* can be efficiently purified by silica gel column chromatography. UV-Visible spectrum showed a comparable spectrum of the purified compound and standard IAA. However, HPLC results revealed the presence of another related compound, which is either a related indole compound or IAA conjugated with amino acid. Thus, confirmation using HPLC analysis may provide a better insight about the purity of product. The present findings also point towards an efficient production of plant hormone by fungal cultures, which may lead to develop a cost effective production of such metabolites and their further use in agriculture field to reduce the negative impact of chemical fertilizers.

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