Chapter 5

Production of indole-3-acetic-acid (IAA) by an edible white-rot fungus *Pleurotus ostreatus* using *Jatropha* seedcake as a raw material

This part has been communicated as:
5.1. Introduction

Phytohormones are the signal molecules, acting as chemical messengers that control plant growth and development. Apart from their role in plant development (Davies 2010), hormones are also the principle agents that regulate plant response to changes in environmental conditions (Morgan and Drew 2006, Tuteja 2007, Malhotra and Srivastava 2009). Nowadays their use in a variety of agronomic applications for controlled plant development has gained momentum. One of such commercially important phytohormone is indole-3-acetic acid (IAA), a principle auxin. Apart from its synthesis by plants, they are also produced by bacteria (Apine and Jadhav 2011), yeast (Xin et al 2009) and fungi (Ünyayar and Ünyayar 2000, Yürekli et al 2003, Maor et al 2004). Although the discovery of IAA in yeast extracts goes back to more than half a century, its production by filamentous fungi is relatively new. However, such reports on the production of phytohormones using fermentation are sparse and only a little data on the actual quantity of IAA synthesized by fungi is known (Yürekli et al 1999, Chung et al 2003). Researches in biotechnology have led to an increased awareness of the importance of industrial fungi for the production of plant growth hormones (Berry 1988).

Several fungi including white-rot species are widely used in biotechnological and biochemical applications such as bioremediation and delignification. However, there are limited studies on white rot fungi for production of plant growth factors. Yesilada et al (1990) and Crocoll et al (1991) reported the abscisic acid production by white rot fungi. Later on, Yurekli et al (1999) demonstrated Funalia trogii ATCC 200800 and Trametes versicolor to produce gibberellic acid, abscisic acid, indole acetic acid, and cytokinin employing olive oil mill and alcohol factory wastewaters as raw materials for fermentation. Unyayar et al (2000, 2002) and Yurekli et al (2003) reported exogenous IAA production by Phanerochaete chrysosporium strain ME446, Funalia trogii and Lentinus sajor-caju, respectively.

The physiological role of auxins in fungi is not well understood. One of the roles suggested for production of IAA by fungus is to mediate fungal-plant interaction. High concentrations of IAA can inhibit the hypersensitive response (Robinette and Matthysse 1990, Jouanneau et al 1991) and may suppress expression of plant defence genes (Yamada et al 1985, Shinshi et al 1987). However, direct evidence for the involvement of IAA in plant diseases was available only for plant pathogenic bacteria (Patten and

The aim of the present study was to determine the effect of culture conditions for production of IAA by an industrially important white rot fungus, *Pleurotus ostreatus*. Although few agricultural residues have been shown as suitable substrates for microbial IAA fermentation (Yurekli *et al* 2003, Swain and Ray 2008), there is no report on synthesis of IAA by *P. ostreatus* employing Jatropha seedcake as raw material so far to the best of our knowledge.

### 5.2. Materials and Methods

#### 5.2.1. Materials

Jatropha seedcake (JSC) was obtained from Anand Agriculture University, Anand, Gujarat, India. IAA and L-tryptophan were purchased from HiMedia, India. Malt extract was procured from SRL, India. All other solvents and chemicals used during the experiment were of analytical grade.

#### 5.2.2. Culture and inoculum

Three fungal strains, *Trametes versicolor* (TV), *Pleurotus ostreatus* (PO) and *Phanerochaete chryosporium* (PC), used in this study were a gift from Institute of Forestbotanisches, Gottingen, Germany. They were grown on 2% malt extract agar plates at 27°C, preserved at 4°C and maintained by sub-culturing once in 2 months. For inoculation, two agar blocks (1 cm × 1 cm) were removed from actively growing fungi on 2% malt extract agar plates and aseptically inoculated into the flasks containing liquid medium.

#### 5.2.3. Screening of growth medium and fungal strain for IAA production

Three different liquid media viz., (i) 2% Malt extract broth (pH 5.5); (ii) 2% (w/v) JSC in basal salt medium (BSM) (5 g/L of glucose, 1 g/L of KH₂PO₄, 0.5 g/L of MgSO₄, 0.5 g/L of KCl; pH 5.5) and (iii) 2% (w/v) JSC in distilled water (pH 5.5), all supplemented with 0.1% (w/v) L-tryptophan, were used for IAA production in submerged condition. 100 mL sterilized media in 250 mL Erlenmeyer flasks were
inoculated with two blocks (1 cm × 1 cm) of TV, PO or PC mycelial growth cut from malt extract agar plates. The flasks were incubated on a rotary shaker at 30°C and 150 rpm. The uninoculated media served as abiotic controls. The 2 mL aliquots were withdrawn at regular interval during incubation and monitored for IAA production.

5.2.4. **Profile of IAA production by P. ostreatus**

Two blocks of uniform growth *P. ostreatus* was inoculated in 500 mL Erlenmeyer flasks containing 250 mL of 2% (w/v) JSC in distilled water amended with 0.1% (w/v) L-tryptophan and incubated on rotary shaker at 30°C, 150 rpm for 22 d. Uninoculated media served as a control. The 2 mL aliquots were withdrawn regularly after every 2 days during the course of fermentation and analyzed for IAA content.

5.2.5. **Effect of physico-chemical parameters on IAA production by P. ostreatus**

To study the effects of various physico-chemical parameters for maximum IAA production by *P. ostreatus*, 250 mL Erlenmeyer flasks with 100 mL media were inoculated with 2 blocks (1 cm × 1 cm) of uniform growth of fungus. Uninoculated media served as controls. The production media with varying JSC (0.5, 1, 2, 3, 4, 5 and 6%; w/v) and L-tryptophan concentrations of (0.05, 0.1, 0.5 and 1%; w/v) were used to study their effect on IAA production by *P. ostreatus*.

The IAA production by *P. ostreatus* over a pH range of 4.0 to 9.0 (adjusted using appropriate buffers) was studied. The initial pH of medium was adjusted using different buffers: Sodium acetate buffer (100 mM) for pH in range of 4.0 to 5.0, sodium phosphate buffer (100 mM) for pH in range of 6.0 to 7.0, Tris-Cl buffer (100 mM) for pH 8.0 and glycine-NaOH (100 mM) for pH 9.0. The optimum production temperature was determined by monitoring IAA production by *P. ostreatus* at 20, 25, 30, 37, 42 and 50°C. Unless otherwise mentioned, experiments were carried out by incubating the inoculated media and uninoculated controls on rotary shaker at 30°C and 150 rpm for 18 d. Upon incubation, the fermentation broth was centrifuged at 6,797 ×g for 15 min and supernatant was assayed for IAA. All experiments were done in triplicates.

5.2.6. **Extraction of IAA from fermentation broth**

The culture supernatant containing IAA obtained from a 18 d fermented broth of *P. ostreatus* was acidified to pH 2.0 using (1N) HCl and extracted twice with double
volumes of ethyl acetate each time. The ethyl acetate fractions were pooled and evaporated dry in a rotary evaporator at 40°C. The extract was dissolved in minimum amount of methanol and stored at -20°C till further use.

5.2.7. Analysis of IAA by High Performance Thin Layer Chromatography (HPTLC)

The concentrated fungal IAA (1 µL) was applied to TLC (Silica gel G f254, thickness 0.25 mm, Merck, Germany), using LINOMAT (CAMAG, Germany) and developed with n-butanol:ammonia:water (10:1:10 v/v/v, upper phase). The developed TLC plate was then sprayed with Salkowaski reagent in order to visualize IAA band. The authentic IAA standard (1 µL) was also simultaneously run on TLC in order to identify the IAA band in sample on the basis of Rf value.

5.2.8. GC-MS analysis of IAA produced by P. ostreatus

The 10 µL aliquot of concentrated ethyl acetate extracted fraction of IAA was analyzed by GC-MS (Pekin Elmer Autosystem XL Gas Chromatograph-Turbomass Mass Spectrometer) equipped with 30 m T5-MS capillary column (film thickness of 0.25 µm). Helium served as carrier gas at a flow rate of 1 mL/min and column temperature ranging from 70 to 250°C. A temperature program of 70-250°C at the rate of 10°C/min was started upon injection. The temperature of injector and detector was 250°C. The electron impact mass spectrum was recorded at an ionizing voltage of 70 eV with a source temperature of 250°C.

5.2.9. Etiolated wheat coleoptiles bioassay for P. ostreatus indoleacetic acid

Wheat seedlings (Triticum aestivum L.) were grown in dark for 4 d on moist sand at 25 ± 1°C, and then harvested. The apical 2 mm was cut and discarded. The next 4 mm sections were used for the bioassay. These sections were floated on distilled water to remove the endogenous IAA. Five such sections were then placed into test tubes containing phosphate-citrate buffer (1.794 g/L K2HPO4 + 1.019 g/L citric acid monohydrate; pH 5.0) supplemented with 2% (w/v) sucrose, in three sets: (1) Control (without IAA), (2) serial dilutions (5, 2, 1, 10⁻¹, 10⁻², 10⁻³ and 10⁻⁴ µg/mL) of extracted fungal IAA, and (3) serial dilutions (5, 2, 1, 10⁻¹, 10⁻², 10⁻³ and 10⁻⁴ µg/mL) of synthetic IAA standard. The assay tubes were incubated for 20 h at 25°C in dark with a roller tube apparatus (10 rpm) to avoid curling of coleoptiles (Nitsch and Nitsch 1956). At
termination of the bioassay, coleoptile sections were measured using micrometry, and the length of each coleoptile was recorded. All assays were done in triplicates.

5.2.10. Analytical methods

**Indole acetic acid assay**

The production of IAA was determined as described by Gordon and Weber (1951). The fermented broth was centrifuged at 12,310 ×g for 15 min to separate mycelial growth. One mL aliquot of appropriately diluted culture supernatant was mixed with 0.2 mL of o-phosphoric acid and 2 mL of Salkowski reagent (50 mL of 35% of HClO₄ and 1 mL of 0.5 M FeCl₃ solution). This assay system was allowed to stand in dark at room temperature for 20 min. The development of pink colour indicated IAA production and was quantified by reading absorbance at 535 nm using Systronics Spectrophotometer 106, Ahmedabad, India.

**Laccase assay**

Laccase activity was determined by monitoring oxidation of o-dianisidine as described by Palmieri *et al* (1993). All enzyme reactions were started by adding 200 µL of culture filtrate, and the enzyme activities were calculated from the linear phases of the reaction. One unit of enzyme activity was defined as the amount of the enzyme required to oxidize 1 µmol of o-dianisidine/mL.min under specified assay conditions.

5.3. Results and Discussion

5.3.1. Screening for growth medium and fungal strain for IAA production

The JSC contains approximately 49% carbohydrate, 22% of protein and 14% of fiber (Rakshit *et al* 2008). This composition is suitable to support good microbial growth. The use of JSC as feedstock for fermentation, thus appears logical and worth exploring. In the present study, we investigated the suitability of different media composition for IAA production under submerged fermentation by three white rot fungi, viz., TV, PO and PC.

All the three white rot cultures were able to produce IAA in 0.1% L-tryptophan supplemented test media (Fig. 5.1a). All the three fungal cultures produced maximum IAA after 15 d of incubation but the extent of IAA produced varied depending on the fungal culture and production medium. In 2% (w/v) malt extract medium PO produced maximum IAA (563 µg/mL) by 20 d of incubation whereas PC produced a maximum of
(840.46 µg/mL) IAA in JSC+BSM by 15 d of incubation. In JSC+D/W, PO and TV produced almost equal amount of IAA upon 15 d of fermentation.

Figure 5.1 (a) IAA production by three different white rot fungi on three different media and (b) Detection of fungal IAA in ethyl acetate extract of culture supernatant by silica gel TLC (In Fig. 5.1b: L1-Synthetic IAA (1mg/mL); L2, L3, L4-PO, TV, PC grown in JSC+BHM, respectively; L5,L6,L7-PO, TV, PC grown in JSC+D/W, respectively; L8, L9, L10-PO, TV, PC grown in Malt extract, respectively; L11-JSC control and L12-Malt extract control)

The culture supernatants of all the three fungi grown in different media showed a clear red spot at the $R_f$ value 0.33 corresponding to standard IAA on silica gel TLC
plates, upon spraying with Salkowskasi reagent. The TLC analysis (Fig. 5.1b) revealed that high concentration of IAA estimated by colorimetric assay in fermentation broth of PC using JSC+BSM and Malt extract medium was due to some other metabolite produced in the fermentation medium that interfered with the assay. But, when PC was grown in JSC+ D/W, only IAA was produced. In malt extract medium PO and TV did not produce IAA or the amount was negligible whereas in JSC+D/W medium both these cultures produced significant amount of IAA. Thus on the basis of results obtained by colorimetric assay and TLC analysis, PO was selected for further study, as it exhibited least production of interfering metabolites in comparison to other two white rot fungi and produced significant amounts of IAA on JSC.

5.3.2. Profile of IAA production by P. ostreatus under submerged fermentation

The time course of IAA production by PO grown in JSC medium containing 0.1% (w/v) L-tryptophan is shown in Fig. 5.2. The fungal strain, PO was able to grow and produce IAA from 2 d after incubation but during the early stages of fungal growth, it secreted extracellular phenol oxidase (laccase) which would have oxidized the newly formed IAA during this period. The relationship between production of IAA and peroxidases depending on the culture periods has been demonstrated for a white rot fungus-Trametes trogii (Unyayar et al 2001).

![Graph of IAA and laccase production](image)
Later when the level of these IAA degradative enzymes began to decrease after 8 d (Fig. 5.2), the level of IAA in the broth began to rise. PO was able to produce a maximum of IAA (362.53 ± 3.29 µg /mL) by 18 d of incubation after which it decreased sharply by 22nd d. This decrease in level of IAA might be due to ageing and death of the fungal biomass. Henceforth, 18 d was considered as optimum incubation period for IAA production by PO in subsequent experiments.

5.3.3. Effect of physico-chemical parameters for IAA production by P. ostreatus.

Microbial phytohormones are mostly extracellular and are greatly influenced by nutritional and physico-chemical factors, such as pH, L-tryptophan concentration, carbon-nitrogen source, agitation and dissolved oxygen concentration. PO produced maximum IAA (362.53±3.29 µg/mL) by 18 d of incubation at 30°C under shaking condition, in 0.1% (w/v) L-tryptophan supplemented JSC broth which was quiet higher than that reported by Unyayar and Unyayar (2000) for P. chrysosporium ME446 (55.91 µg IAA/mL) in basal mineral medium. Yurekli et al (1999) reported 111.76 µg/mL and 75.2 µg/mL IAA production employing olive oil mill waste as substrate by Funalia trogi ATCC 200800 and Trametes versicolor ATCC 200801, respectively.

The influence of varying concentrations of deoiled Jatropha seedcake on IAA production is shown in Fig. 5.3. IAA production increased in media with increasing
concentration of deoiled JSC from 0.5-2.0% (w/v). The IAA production depends on deoiled seedcake concentration because its protein and carbohydrate content act as feedstock for the growth of white rot fungi and thereby for IAA production. Further increase in seedcake concentration above 2.0% (w/v) did not result in higher yield of IAA. The lower yield at higher concentrations (above 2% (w/v) JSC) may be due to reduced mass transfer as a result of increase in viscosity or may be due to toxicity associated with substrate at that concentration (Schügerl 1981, Saetae and Suntornsuk 2010).

The IAA production was found to increase with an increase in L-tryptophan concentration from 0 to 1% (w/v) in fermentation medium (Fig. 5.4). At higher L-tryptophan concentration in medium, PO exhibited significantly lower yields of IAA.

![Figure 5.4 Effect of increasing concentration of L-tryptophan on IAA production by P. ostreatus](image)

The culture PO produced significantly higher amount of IAA in the pH range of 7.0 to 8.0, with maximum IAA in medium with initial pH 7.0 (Fig. 5.5). Our results are in agreement with the findings of Strzelczyk et al (1992) who reported that auxin biosynthesis was favoured in media over a pH range of 6.0 to 9.0 in mycorrhizal fungi.
The IAA production by PO was found to increase with incubation temperature with maximum production at 37°C (Fig. 5.6). Further increase in temperature retarded growth as well as IAA production, which reduced to negligible amount at 50°C when the fungal growth ceased. In contrast to IAA levels, maximum growth was observed at 30°C which gradually decreased with either increase or decrease in incubation temperature.
5.3.4. *Characterization of IAA produced by P. ostreatus*

The silica gel thin layer chromatogram of ethyl acetate extract from culture supernatant of PO exhibited clear red spot of IAA with $R_f$ value 0.33 corresponding to standard IAA when sprayed with Salkowskis’s reagent. The TLC findings (Fig. 5.7) are in good agreement with the report of Bayer (1969). In addition to IAA, other compounds were also detected on TLC, which might represent intermediates in biosynthetic or degradation pathways or may be degradation products or structural analogs of IAA that remains to be identified.

![HPTLC chromatogram of ethyl acetate fraction obtained from culture supernatant of P. ostreatus upon fermentation of 2% (w/v) JSC for 18 d](image)

The two major peaks with retention time of 12.34 and 19.57 min were detected in ethyl acetate extract of PO cell free fermentation broth, when analyzed by gas chromatography (Fig. 5.8). The mass spectrometric analysis (Fig. 5.9) of the compound eluted at a retention time of 19.57 min, suggested it to be IAA.

Upon fragmentation this compound yielded ions ($m/z$ 128, 129, 130, 131) corresponding to indole moiety. The mass spectrometry analysis of minor peak with 18.23 min retention time, suggested it to be of indole acetamide. The presence of indole acetamide in culture supernatant of PO indicates the biosynthesis of IAA through indole-3-acetamide pathway in this organism. The indole acetamide pathway was long believed to be restricted to bacteria (Costacurta and Vanderleyden 1995) until Robinson *et al* (1998) and Moar *et al* (2004), showed indole-3-acetamide as a major pathway utilized by a plant pathogenic fungus *Colletotrichum gloeosporioides* f. sp. *aeschynomene* to produce indole-3-acetic acid.
Figure 5.8 Gas chromatogram of crude ethyl acetate fraction obtained from culture supernatant of *P. ostreatus* upon fermentation of 2% (w/v) JSC for 18 d.

Figure 5.9 MS fragmentation pattern of putative IAA with the major fragment ion at *m/z* 130.
5.3.5. *Etiolated wheat coleoptile bioassay of IAA produced by P. ostreatus*

Wheat-coleoptile bioassay was performed to determine potency of fungal auxin in reference to the commercially available synthetic IAA at corresponding concentrations (Table 5.1). The 0.1 µg IAA/mL of synthetic and fungal origin caused 2.85 and 2.89 mm increase in coleoptile length, respectively. The higher IAA concentrations (5 and 2 µg/mL) were less effective in comparison to the lower concentrations. The yellowing of the coleoptiles and retarded growth observed at higher concentrations might be due to phytotoxic effect of IAA at supraoptimal concentration (Foster *et al* 1952, Badenoch-Jones *et al* 1982). A low activity was detected in control where the coleoptile length was 5.13 ± 0.093 mm. This increase in control coleoptile length might be due to the endogenous IAA content. Coleoptile lengths with synthetic and bacterial IAA were very close to each other, and the difference between the coleoptile lengths was not significant. This indicates that the standard synthetic-IAA and fungal-IAA possessed equivalent biological activities.

Table 5.1: Comparative data of wheat coleoptile bioassay for IAA

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<th>Treatments</th>
<th>IAA concentration (µg/mL)</th>
<th>Length before treatment (mm)</th>
<th>Length after treatment (mm)</th>
<th>Growth (mm) a</th>
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</thead>
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<tr>
<td>Control</td>
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<td>5.13 ± 0.093</td>
<td>1.13</td>
</tr>
<tr>
<td>Synthetic IAA</td>
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<td>0.69</td>
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<tr>
<td></td>
<td>2</td>
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<td>5.50 ± 0.093</td>
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<tr>
<td></td>
<td>10^{-4}</td>
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<tr>
<td>Fungal IAA</td>
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<tr>
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<td>1.67</td>
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<tr>
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| a Each value represents means ± S.E of replicates per treatment. |

5.4. Conclusion

Jatropha oil seedcake can be used as a raw material for production of IAA by white rot fungi. Amongst three cultures, *P. ostreatus* was found to synthesize maximum IAA under submerged fermentation using medium containing 2% (w/v) JSC, 0.1% (w/v) L-tryptophan, with initial pH 7.0 at 37°C upon 18 d of incubation. The
production of IAA by PO was confirmed by TLC and GC-MS analysis and on the basis of wheat coleoptile bioassay it was found to be biologically potent as synthetic IAA.
References


Manulis S, Haviv-Chesner A, Brandl MT, Lindow SE, Barash I (1998) Differential involvement of indole-3-acetic acid biosynthetic pathways in pathogenicity and


