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Production of ligninolytic enzymes by white rot fungi on lignocellulosic wastes using novel pretreatments

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Abstract

Production of extracellular ligninolytic enzymes (laccase and polyphenol oxidase) secreted by three species of white rot fungi (*Pleurotus florida, P. flabellatus* and *P. sajor-caju*) under *in vivo* condition was studied on two lignocellulosic substrates i.e., paddy straw and wheat straw. These lignocellulosic substrates were treated with neem (*Azadirachta indica*) oil and ashoka (*Saraca indica*) leaves extract. Between the two lignocellulosic substrates, paddy straw pretreated with neem oil supported maximum activity of laccase and polyphenol oxidase (PPO). The activities of both the enzymes were low on the 5th day of cultivation which increased on the 10th day and reached at peak on the 15th day. Thereafter, there was continuous decrease in the enzymatic activity. Among the three species, *P. flabellatus* (P3) showed maximum ligninolytic enzymatic activity followed by *P. florida* (P2)and *P. sajor-caju* (P1).

Key words: Ligninolytic enzymes, White rot fungi, *Pleurotus* spp.

Introduction

A wide variety of lignocelluloses including agricultural and agro- industrial wastes such as wheat straw, paddy straw, peapod shell, cauliflower leaves, radish leaves, brassica straw, sugarcane bagasse, sugarcane dried leaves, mentha stalks, corncobs, used tea leaves, apple pomace, banana pseudostem, wood logs, sawdust, coconut waste, cotton stalks etc. can be degraded efficiently by extracellular enzymes secreted by white rot fungi (1, 2). Lignin being an important constituent of lignocelluloses is a phenolic polymer and serves as a protective barrier against microbial attack. Lignin is a three-dimensional network made of dimethoxylated (syringyl, S),monomethoxylated (guaiacyl, G) and non-methoxylated (*p*-hydroxyphenyl, H) phenylpropanoid units, derived from the corresponding *p*-hydroxycinnamyl alcohols, which give rise to a variety of subunits including different ether and C—C bonds (3). After cellulose, it is the most abundant renewable carbon source on Earth. The highest concentration of this recalcitrant polymer is found in the middle lamella, where it acts as a cement between wood fibres, it is also present in the layers of the cell wall (especially the secondary cell-wall), forming, together with hemicelluloses, an amorphous matrix in which the cellulose fibrils are embedded and protected against biodegradation (4).

Due to harmful effects of chemicals, research efforts have been initiated to exploit the fungicidal property of botanicals to replace chemicals for disease management in mushroom culture. Recently, Neem (*Azadirachta indica*) has received substantial attention particularly in terms of its potential for insect pest control. Antifungal, antiviral and nematicidal property of this plant has been exploited in rural India since time immemorial. It has been age old practice in rural India to mix dried neem leaves with stored grains or to place them among warm clothes to repel insects (5).

Oyster mushrooms (*Pleurotus* spp.) are wood inhabiting white rot Basidiomycetes with important biotechnological and environmental applications(6, 5, 7). They are highly adaptable to grow and fruit on a wide variety of forest and agro-industrial lignocellulosic substrates, because of their ability to synthesize the relevant hydrolytic and oxidative enzymes that convert the individual component of the substrate (cellulose, hemicellulose and lignin) into low molecular weight compounds, which can be assimilated for fungi nutrition (8). In present investigation we have mainly focused on laccase and polyphenol oxidase production under *in vivo* condition because of their role in effective biodegradation as well as bioremediation of recalcitrant and hazardous substances.

Materials and methods

The cultures and their maintenance

The pure culture of *P. florida, P. flabellatus* and *P. sajor-caju* used in the present investigation were procured from Indian Agricultural Research Institute(IARI), New Delhi. Throughout the study the cultures were maintained on malt extract agar (MEA) medium at 23-25^{0}C and was sub-cultured at the regular interval of three weeks.

Cultivation

Spawn preparation

Spawn is referred to as the vegetative mycelium of the fungus, which is grown on cereal grains i.e., grains of wheat. The preparation of spawn involved soaking of wheat grains in water followed by mixing of buffers, sterilization and inoculation with pure culture of

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appropriate *Pleurotus* species under aseptic conditions. The spawn was prepared in 500 ml of dextrose bottles or in polypropylene bags. After 3-4 days of inoculation fungal mycelium started spreading on the grains. The mycelium was white net web like in appearance. The bottles or bags were nearly half filled in 10-12 days and in 18-21 days these were completely filled with white mycelial growth.

Preparation of substrates

Paddy straw and wheat straw were used as substrates for the cultivation of *Pleurotus* spp. These substrates were treated and sterilized by plant extracts.

Plant extracts treatment

During present investigation, the lignocellulosic wastes were treated with two plant extracts *i.e.* aqueous extract of ashoka leaves (A) and neem oil (N). Twenty ml of these extracts were mixed in 1 litre of water at the time of soaking of substrates. After 24 hrs of treatments excess water was drained out. The substrates were evenly spread on the clean platform for 30 minutes to further remove free water. These aqueous plant extracts treated substrates were ready for spawning.

Spawning

Spawning is the process of mixing spawn in the sterilized substrate. Three percent wet weight basis spawn grain was mixed with the substrate and filled into polypropylene bags. The mouth of each bag was tied with rubber band and 12 holes of about 1cm diameter were made, two at each corner at the base, four each on the broader area and one each on the narrow, rectangular side to drain out extra water and for proper aeration. Sixty bags of each of the treatment were filled and kept in mushroom house on the iron racks on the bricks.

Sample collection and extraction of extracellular enzyme

After every five days interval three bags for each treatment were removed for enzyme assay. The contents of a set of three bags were mixed uniformly. Ten gram sample was homogenized in 100 ml of 50 mM sodium phosphate buffer (pH 6.0) for laccase and PPO assay. Homogenized samples were filtered through Whatman no.1 filter paper and filtrate was used for enzymatic studies.

Enzyme assay

Laccase (EC 1.10.3.2)

Laccase was assayed following Dhaliwal *et al.* (9) using a reaction mixture consisting of 1ml of enzyme filtrate and 3 ml of guaiacol substrate prepared in 0.1 M sodium phosphate buffer (pH 6.0). Change in absorbance was observed at 495 nm by UV-visible spectrophotometer (Elico SL164), per minute.

Polyphenol oxidase (EC 1.10.3.1)

Polyphenol oxidase (PPO) was assayed using methodology of Rai and Saxena (10). One ml of enzyme extract mixed with 3 ml of catechol prepared in 0.1 M sodium phosphate buffer (pH 6.0). Change in absorbance was observed at 495 nm by UV-visible spectrophotometer (Elico SL 164), per minute. Unit of enzyme for laccase and PPO were calculated as change in absorbance by 0.001 per min per ml of culture filtrate.

Results

Activity of ligninolytic enzymes under in vivo condition

Laccase (EC 1.10.3.2)

Figure 1 shows the laccase activity of three species of *Pleurotus* on aqueous extracts of ashoka leaves and neem oil treated substrates. Laccase activity by *P. sajor-caju* on paddy and wheat straw on the 5th day was recorded as 2.18 and 1.98 unit/ml/minute on ashoka leaves extracts treated straw and 2.41 and 2.24unit/ml/minute on neem oil treated straws in given order. Maximum laccase activity was observed on the 15th day on the both substrates, paddy as well as wheat straw. The enzyme activity on the 15th day on ashoka leaves treated paddy straw and wheat straw was found as 3.47 and 3.20 unit/ml/minute and on neem oil treated paddy straw and wheat straw it was 3.79 and 3.44 unit/ml/minute, respectively.

The laccase activity of *P. florida* on the 5th day on ashoka leaves treated paddy straw and wheat straw was observed as 3.10 and 2.90 unit/ml/minute and on neem oil treated paddy straw and wheat straw 3.27 and

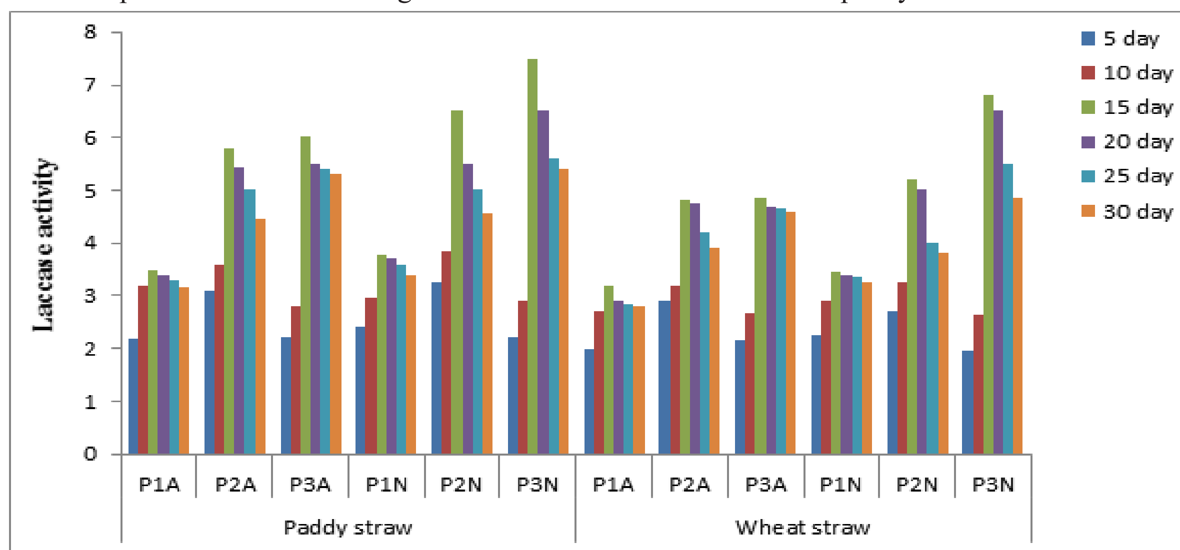

Figure 1. *In vivo*Laccase (unit/ml/minute) activity of *Pleurotus* species on plant extract treated substrates.

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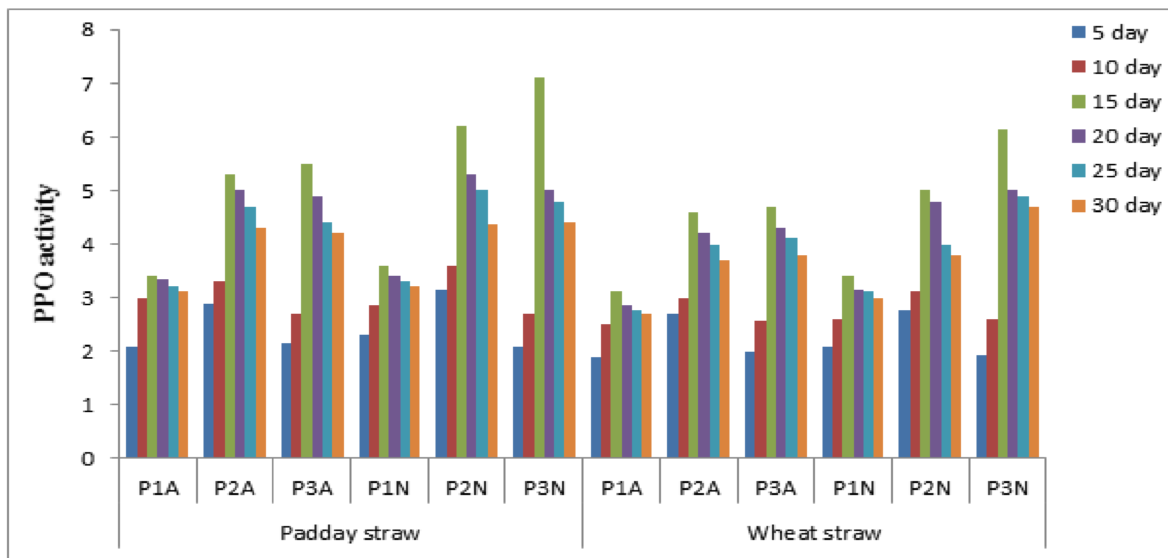

Figure 2. *In vivo*PPO (unit/ml/minute) activity of *Pleurotus* species on plant extract treated substrates.
A= ashoka leaves extract, N= neem oil, P1= *P. sajor-caju,* P2=*P. florida*and, P3= *P. flabellatus.*

2.70 unit/ml/minute, respectively. The enzyme activity increased gradually after 5th day on these two substrates and exhibited a peak on the 15th day. On 15th day laccase activity on ashoka leaves treated paddy straw and wheat straw was found as 5.8 and 4.81 unit/ml/minute and on neem oil treated paddy straw and wheat straw it was 6.50 and 5.21 unit/ml/minute, respectively.

In case of *P. flabellatus* on the 5th day the laccase activity was noted as 2.21 (on paddy straw) and 2.14 unit/ml/minute (on wheat straw) on ashoka leaves extracts treated substrates and 2.23 (on paddy straw) and 1.94 unit/ml/minute (on wheat straw) on neem oil treated substrates. Maximum laccase activity was observed on the 15th day on paddy as well as wheat straw. The enzyme activity on the 15th day on ashoka leaves treated paddy straw and wheat straw was found as 6.01 and 4.85 unit/ml/minute, respectively and on neem oil treated substrates it was 7.50 unit/ml/minute on paddy straw and 6.80unit/ml/minuteon wheat straw. The enzyme activity decreased after 15th day on paddy straw and wheat straw till the end of experiments.

Maximum laccase activity was observed on paddy straw followed by wheat straw. Neem oil treatment supported maximum production of the enzymes followed by ashoka leaves extract. Among the three species of *Pleurotus*, *P. flabellatus* showed maximum laccase activity followed by *P. florida* and *P. sajor-caju*.

Polyphenol oxidase (EC 1.10.3.1)

Figure 2 shows the PPO activity of three species of *Pleurotus* on aqueous extracts of ashoka leaves and neem oil treated substrates. The enzyme activity by *P. sajor-caju* on the 5th day on ashoka leaves extracts treated paddy straw and wheat straw was found as 2.10 and 1.90 unit/ml/minute and on neem oil treated paddy straw and wheat straw 2.30 and 2.10 unit/ml/minute, respectively. Maximum PPO activity was observed on the 15th day on paddy as well as wheat straw. The enzyme activity on the 15th day on ashoka leaves treated paddy straw and wheat straw was observed as 3.40 and 3.10 unit/ml/minute and on neem oil treated paddy straw and wheat straw it was 3.59 and 3.39 unit/ml/minute, respectively. The enzyme activity decreased after 15th day on paddy straw and wheat straw till the end of experiments.

The enzyme activity by *P. florida* on the 5th day on ashoka leaves treated paddy straw and wheat straw was recorded as 2.90 and 2.70 unit/ml/minute and on neem oil treated paddy straw and wheat straw, it was 3.15 and 2.75 unit/ml/minute, respectively. Maximum PPO activity was observed on the 15th day on paddy as well as wheat straw. The enzyme activity on the 15th day on ashoka leaves treated paddy straw and wheat straw was found as 5.30 and 4.60 unit/ml/minute and on neem oil treated paddy straw and wheat straw it was 6.20 and 5.00 unit/ml/minute, respectively.

PPO activity by *P. flabellatus* on the 5th day on ashoka leaves extracts treated paddy and wheat straw was observed as 2.15 and 2.00 unit/ml/minute and on neem oil treated paddy straw and wheat straw 2.10 and 1.92 unit/ml/minute, respectively. Maximum PPO activity was observed on the 15th day on paddy as well as wheat straw. The enzyme activity on the 15th day on ashoka leaves treated paddy straw and wheat straw was found as 5.50 and 4.70 unit/ml/minute and on neem oil treated paddy straw and wheat straw it was 7.10 and 6.15 unit/ml/minute, respectively.

The aforesaid observations indicate that paddy straw supported maximum production of PPO followed by wheat straw. Neem oil treatment facilitated better enzyme activity than ashoka leaves extract. Among the three species of *Pleurotus, P. flabellatus* showed maximum PPO activity followed by *P. florida* and *P. sajor-caju*

Discussion

Under *in vivo* conditions all the three species of *Pleurotus* showed maximum laccase and PPO activities on paddy as well as wheat straw on 15th day in the present investigation. Among the three species *P. flabellatus* showed maximum laccase and PPO activities on paddy straw followed by *P.florida* and *P. sajor-caju*. Among all the treatments,neem oil treatment supported maximum production of laccase and PPO followed by aqueous extracts of ashoka leaves extract treated substrates by all species.

Singh (11)reported that the laccase concentration under *in vivo* condition increased during the mycelial

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growth of *Pleurotus* species grown on paddy straw, wheat straw and common grass used as substrates. Sugimoto *et al*. (12) and Ellisahvilli *et al*. (8) observed that laccase activity of *Pleurotus* was high during the colonization stage and declined during first primordial formation and fruiting stage. Similar result was reported by Malarczyk and Widenska(13) in *P. cystidiosus* where laccase was active during mycelial growth on solid saw dust. Elisashvilli *et al*. (8) observed high laccase and manganese peroxidase activity during the colonization stage and declined activity during the first primordial formation in *P. ostreatus*. However, Chen *et al*. (14, 15) observed that cellulase and xylanase activity was crucial during the vegetative growth of mycelium while, laccase is important for sporophore development in the mushroom *Volvariella vovacea*.

The probable reason for the better production of enzymes on plant extracts (neem oil and ashoka leaves extract)treated substrate could be that the plant extract containedsome elements or substances which when taken up by thegrowing mycelium induced better enzyme activities. Thus,lignocellulosic materials which are available in plenty canbe utilized as resource for production of enzymes (5). Periasamy and Natrajan (16) observed that during the cultivation of *P. djamor* the changes in extracellular enzyme activities of laccase are directly correlated with growth and fruit body formation. Ahlawat*et al.* (17) observed laccase and PPO activity were more on 13th day grown on paddy straw during the cultivation of *Volvariella vovacea* and found laccase was important for sporophore development. Mahfooz *et al*. (18) observed that Mn- peroxidase and laccase enzyme were found to be positively correlated with the yielding abilities of single spore progenies indicating the use of indirect selection for high yielding genotypes in *A. bisporus*.

Other articles in this theme issue include references (19-34).

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