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# Production of oxidative and hydrolytic enzymes from *Pleurotus florida* and their implication in mushroom cultivation

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ABSTRACT

One of the most economically viable processes for the bioconversion of many types of lignocellulosic wastes is represented by edible mushroom cultivation. *Pleurotus florida* is one of the prominent economically cultivated mushrooms with varied abilities to utilize lignocellulose as a growth substrate. Thus, the profile of oxidative and hydrolytic enzymes was measured during the submerged and solid-state fermentation at Punjab Agricultural University, Ludhiana, Punjab during the winter (*rabi*) season of the year 2020–21. The intracellular activity of laccase (10.23 IU/mg), manganese peroxidase (9.98 IU/mg), cellobiohydrolase (5.46 IU/mg) was best obtained in *P. florida* on the 14$^{th}$ day. The extracellular activity of laccase (15.43 IU/ml), lignin peroxidase (11.21 IU/ml), endoxylanases (9.22 IU/ml) were found to be highest on the 14$^{th}$ day. *P. florida* had substantial levels of ligninolytic enzymes during substrate colonization but rapidly diminished during fruit body development. On the other hand, *P. florida* showed relatively low hydrolase activity during substrate colonization. The activity of these enzymes increased when primordia formed. The hydrolytic activities peaked at the mature fruiting body stage. When mushrooms switched to vegetative development, ligninolytic enzyme activity increased again, while hydrolase activity declined. The yield was found to be 58.78% after the enzymatic treatment of paddy straw. These findings suggest that fluctuations in oxidative and hydrolytic enzyme activities are linked to the growth of the fruiting body and the physiological state of mushroom cultivation.

**Keywords**: Hydrolytic enzymes, Oxidative enzymes, Paddy straw, *Pleurotus florida*, Solid state fermentation, Submerged fermentation

Various strategies such as chemical, physical, physico-chemical and biological have been modified to utilize massive quantities of lignocellulosic waste generated annually through the agricultural, forestry and food processing industries (Gill 2021). However, agro-industrial waste can potentially be converted into different high-value products, including biofuels, value-added fine chemicals and cheap energy sources for microbial fermentation and enzyme production (Saini *et al.* 2015).The development of edible mushrooms by solid-state fermentation is one of the most important strategies for obtaining a high-quality product from lignocellulosic waste (Sadh *et al.* 2018). Mushrooms have the unique ability to produce and secrete specialized oxidative and hydrolytic enzymes into the environment, allowing them to utilize a wide range of substrates as a nutrient source and energy to form fruiting bodies (Kumla *et al.* 2020). These are capable of breaking down the primary components of the substrate (cellulose, hemicellulose, and lignin) into low molecular weight molecules that the fungus may assimilate. As a result, it is critical to assess the activity of *P. florida* hydrolytic (endoxylanase, β-glucosidase, cellobiohydrolase, endoglucanase and total cellulase) and oxidative enzymes (aryl alcohol oxidase, laccase, manganese peroxidase, cellobiose dehydrogenase and lignin peroxidase) at various morphological stages of their cultivation because cellulose, hemicellulose and lignin all are related intra- and intermolecularly (Andlar *et al.* 2018). Previous studies using *Pleurotus ostreatus*, reported that production of ligninolytic enzymes increased with vegetative biomass production on solid growth substrates and it decreased during the fruiting stage (Ruhl *et al.* 2008). Similarly, Kaur *et al.* (2022) studied the production of lignocellulolytic enzymes isolated from *Calocybe indica* where the activity of ligninolytic enzymes decreased during the fruit body development while the activity of hydrolytic enzymes increased during the maturation stage of fruiting body. However, so far information on production of oxidative and hydrolytic enzymes by *P. florida* during submerged and solid state fermentation on lignocellulosic substrate is totally non-existent. Hence, the present paper deals with the production and extraction of oxidative and hydrolytic enzymes from *P. florida* under submerged and solid-state fermentation and their application for improving the mushroom yield and spawn run.

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## MATERIALS AND METHODS

The present study was carried out at the Department of Microbiology, Punjab Agricultural University, Ludhiana, Punjab during the winter (*rabi*) season in 2020–21 procuring *P. florida* strain. It was revived on potato dextrose agar media by incubating at 25±2ºC for 7 days followed by regular sub-culturing.

*Production and extraction of oxidative and hydrolytic enzymes*

Submerged fermentation (SmF): The 7 mm culture discs from the actively growing fungal culture *P. florida* were inoculated into the mushroom minimal medium (Singh *et al.* 2020). At 25±2°C, cultures were incubated at 120–160 rpm for days (7, 14, 21, 28). The broth with mycelial proliferation was filtered and was crushed in 50 mM phosphate buffer (*p*H 7.0), centrifuged at 10,000 rpm (10 min, 4°C) and the lignocellulolytic intracellular enzyme activities were assessed using crude enzyme extract. The broth left after the separation of mycelium was centrifuged and supernatant obtained was utilized as the crude enzyme extract, and used for extracellular enzyme activities assay.

Solid state fermentation (SSF): The substrate paddy straw was taken from different growth phases of *P. florida* mushroom (mycelial run, pinhead, first flush, second flush, third flush and terminated flush), rinsed with deionized water, homogenized in the extraction buffer, centrifuged at 10,000 rpm (10 min, 4ºC). The clear supernatants obtained were regarded as crude enzymes and later used for enzyme activity assay.

*Determination of oxidative and hydrolytic enzyme activities:* The activity of aryl alcohol oxidase was determined by measuring the amount of veratraldehyde produced following the oxidation of veratryl alcohol using the method described by (Okamoto *et al.* 2002). The laccase activity was determined spectrophotometrically by evaluating the absorbance change of the reaction mixture using guaiacol as a substrate at 470 nm (Singh *et al.* 1988). The buffered guaiacol solution was used to examine Mn-peroxidase activity, and the change in light absorbance was quantified at 465 nm as stated by Paszczynski *et al.* 1988. The enzyme assay for cellobiose dehydrogenase was performed using DCPIP (2,6-Dichlorophenolindophenol) as the substrate (Sadana and Patil 1985). The lignin-peroxidase activity assay was investigated by using veratryl alcohol as a substrate at 310 nm as predicted by Tien and Kirk (1988). Endoxylanase activity was measured using birchwood xylan (1% w/v) in 50 mM citrate buffer at 40°C for 10 min (Bailey *et al.*1992). The activity of the β-glucosidase enzyme was measured using the substrate pNPG (p-Nitrophenol-α-D-Glucopyranoside) and reaction mixture was stopped by adding $Na_2CO_3$ at 410 nm (Saha and Bothast 1996). The cellobiohydrolase activity was evaluated using cellobiose as a substrate, as described by Toyama and Ogawa (1977). Using the Mandels *et al.* (1976), endoglucanase activity was evaluated by calculating the reducing sugars produced following incubation of the substrate with enzyme extract. The reducing sugars were assessed using the DNS reagent (Miller 1959). Total cellulase activity (Filter paper activity, FPA) was measured using filter paper as the substrate (Ghose 1987).

*Cultivation of* Pleurotus florida*:* The cultivation studies were conducted in the Mushroom Research Complex, Department of Microbiology, Punjab Agricultural University, Ludhiana, Punjab. Cultivation of *P. florida* was carried out in polypropylene bags during *rabi* by using paddy straw as substrate. The paddy straw was wetted with water for 16–20 h until it reached a 70–75% moisture level. The bag was filled with alternating layers of around 1–2 wetted straw. The wheat grain-based spawn was used at a rate of 10% of the dry weight of the straw. The upper layer of the bag was secured with gunny thread after spawning, and the bag's lower sides were cut to drain the gathered water. Small mushroom spores appear after 2–3 weeks of spawning. The bag was opened and gentle watering was administered regularly to keep them hydrated. The fruit bodies were generally picked as soon as their margins started enrolling.

*Enzymatic pre-treatment of paddy straw for mushroom cultivation:* The fresh paddy straw was treated with ligninolytic crude enzyme. The crude enzyme (8 ml) was dissolved in citrate phosphate buffer (*p*H 6.0) to make a volume of 30 ml and sprayed on paddy straw for 48 h in the form of loose heap to achieve 70–75% moisture content. The numerous parameters were assessed such as the number of spawn run days, first flush harvesting, yield, the number of fruiting bodies harvested and an average weight of fruit bodies after the enzymatic cultivation of *P. florida* mushroom.

*Statistical analysis:* All the enzyme activities were performed in triplicates, and data were analyzed statistically using one-way Anova IBM SPSS Statistics version 16.0 and presented in graphs and the data presented in Table 1,2 was performed using CPCS1 software.

## RESULTS AND DISCUSSION

*Effect of incubation period on intra-extracellular oxidative enzyme production under submerged fermentation: Pleurotus florida* intra-extra enzyme activities based on mycelium and the liquid broth were represented in Fig 1 (A,B). The *P. florida* showed the highest intracellular laccase (10.23 IU/mg), manganese peroxidase (9.98 IU/mg) and aryl alcohol oxidase (4.21 IU/mg) activity on 14$^{th}$ day. The decrease in activity with storage period was due to the oxidative enzymes produced by the basidiomycete fungus during secondary metabolism, which may be induced by nutritional deprivation (Sousa *et al.* 2019). Moreover, high ligninase activities might be initiated only at the third–fourth day of incubation but not during the exponential growth phase (Kaur *et al.* 2022). The highest extracellular enzyme activity of laccase (15.43 IU/ml), lignin peroxidase (11.21 IU/ml), manganese peroxidase (5.24 IU/ml), cellobiose dehydrogenase (2.25 IU/ml) and aryl alcohol oxidase (1.32 IU/ml) was found to be maximum on the 14$^{th}$ day. The intracellular lignin peroxidase enzyme activity was highest

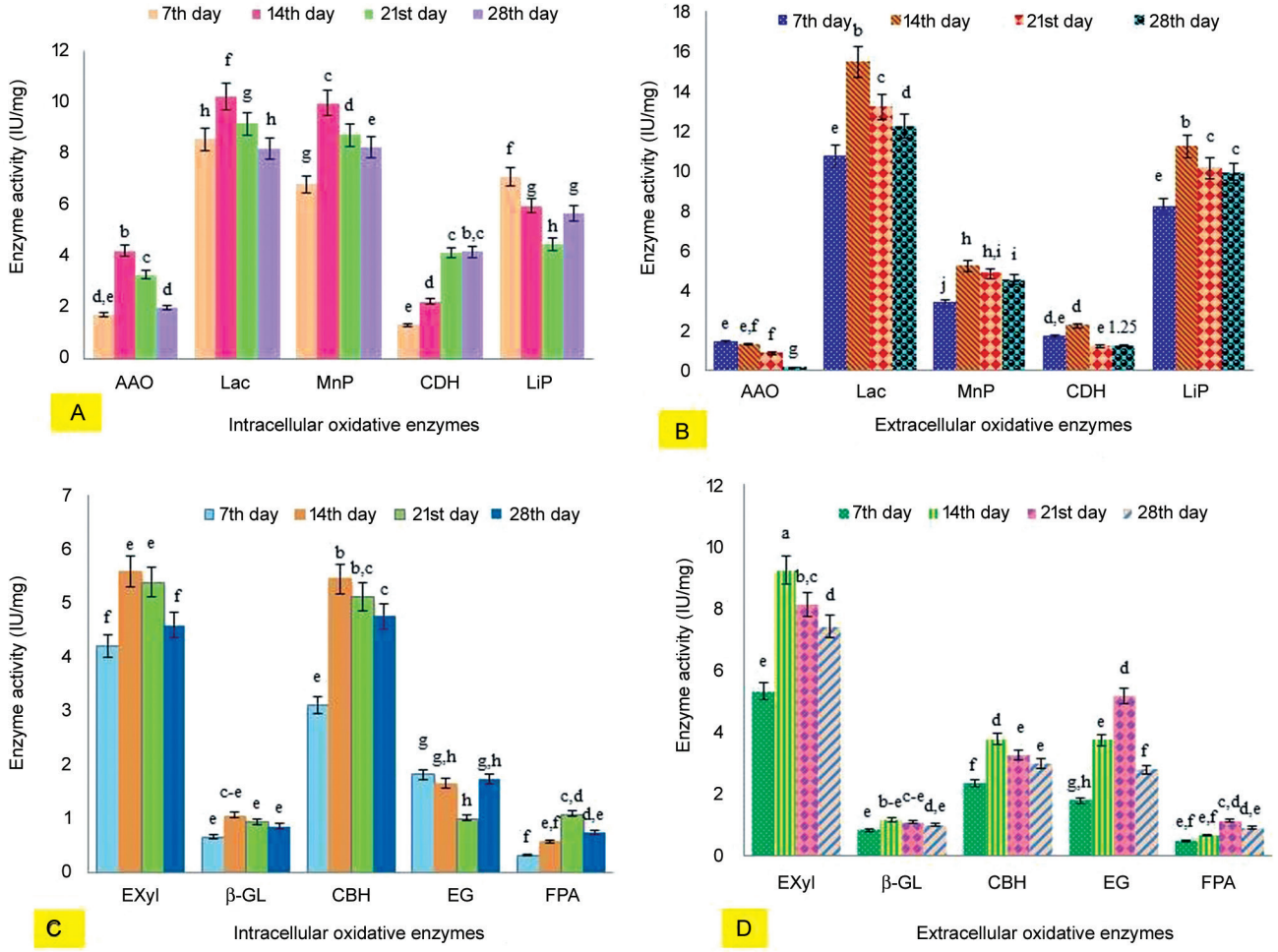


Fig 1 Intra-extracellular oxidative (A, B) and hydrolytic (C,D) enzyme activities of *Pleurotus florida* under submerged fermentation. AAO, Aryl alcohol oxidase; Lac, Laccase; MnP, Manganese peroxidase; CDH, Cellobiose dehydrogenase; LiP, Lignin peroxidase; EXyl, Endoxylanase; β-GL, β-glucosidase; CBH, Cellobiohydrolase; EG, Endoglucanase; FPA, Total cellulase. The alphabetical letters were significantly different within each group at P≤0.05 using Tukey's post hoc test for intracellular and extracellular oxidative and hydrolytic enzymes.

on the 7th day (7.13 IU/mg) and activity was found to be at par on 14th and 28th day of incubation. This fluctuation might be due to the organic polymers which induce higher peroxidase activity (Sharma *et al.* 2020). During the vegetative phase, the cellobiose dehydrogenase activity was found to be maximum on the 28th day (4.18 IU/mg). This might be due to the metabolically most active phase of *P. florida* as the extracellular activity of oxidative enzymes directly related to the fungal culture entering the log phase (Kaur *et al.* 2022). The above results agreed with Akpinar and Urek (2014) who reported that the *P. eryngii* produces laccase, aryl alcohol oxidase, lignin peroxidase, and manganese peroxidase on the 12th and 17th day of cultivation using apricot as agro-industrial waste. Similarly, Rahmadiyanti *et al.* (2020) observed that the highest laccase activity was produced on the 10th day (15.1 IU/ml) from *Marasmius* sp.

*Quantitative oxidative enzyme activities during solid-state fermentation of paddy straw by* Pleurotus florida*:* The oxidative enzymes laccase (18.08 IU/mg), lignin peroxidase (13.86 IU/mg), manganese peroxidase (13.21 IU/mg), cellobiose dehydrogenase (6.78 IU/mg) and aryl alcohol oxidase (6.20 IU/mg) showed maximum enzyme activity during the pinhead stage as shown in Fig 2(A) Higher levels of ligninolytic activities during vegetative development and the mycelia regenerative stage indicates that enzymes are responsible for degradation of physical barrier of lignin so that more cellulosic and hemicellulosic content is exposed for further degradation and thus linked to the energetic demands for initiating fructification (Raymond *et al.* 2015a). Table 1 showed maximum positive correlation (r=0.988) for cellobiose dehydrogenase and aryl alcohol oxidase amongst all oxidative enzymes, thus indicating a direct relationship between enzymes. The crystal structures of aryl alcohol oxidase and cellobiose dehydrogenase shared a highly conserved catalytic site, suggesting a similar oxidation mechanism (Hernandez-Ortega *et al.* 2012). Similarly, positive correlation between laccase and manganese peroxidase (r=0.911) were observed. Moreover, laccase and lignin peroxidase also showed significant positive correlation (r=0.904). The linear positive

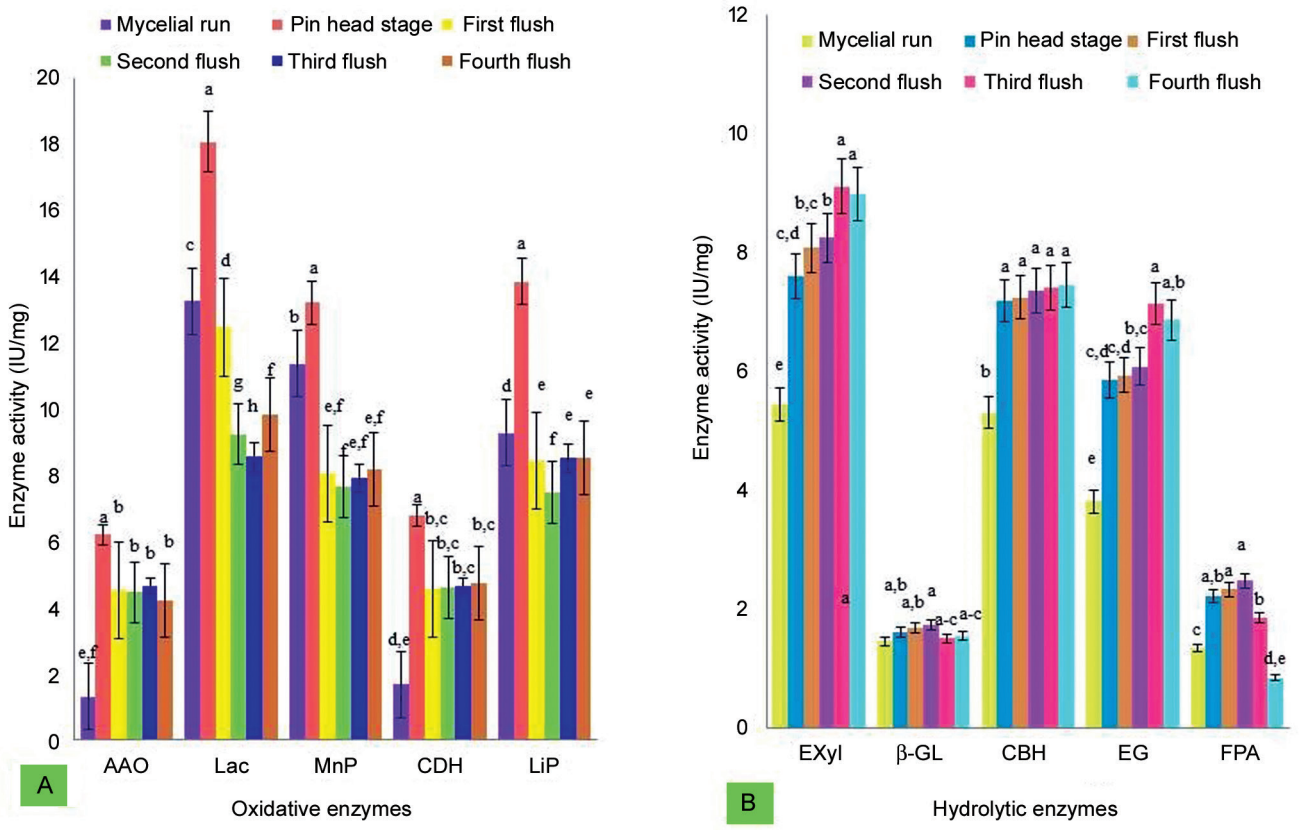


Fig 2 Oxidative (A) and hydrolytic (B) enzyme activities during reproductive stages of *P. florida.* AAO, Aryl alcohol oxidase; Lac, Laccase; MnP, Manganese peroxidase; CDH, Cellobiose dehydrogenase; LiP, Lignin peroxidase; EXyl, Endoxylanase; β-GL, β-glucosidase; CBH, Cellobiohydrolase; EG, Endoglucanase; FPA, Total cellulase. The alphabetical letters were significantly different within each group at P≤0.05 using Tukey's post hoc test for oxidative and hydrolytic enzymes during different mushroom cultivation stages.

correlation for manganese peroxidase and lignin peroxidase (r=0.896) was recorded. For cellobiose dehydrogenase and lignin peroxidase, a significant positive correlation at $p \leq 0.05$ (r=0.533) was observed. The findings are in proximation with Elisashvili *et al.* (2015) during cultivation of *Lentinus edodes* on wheat straw reported that laccase and manganese peroxidase activities were higher during substrate colonization and declined during primordial appearance and fruit body development. Raymond *et al.* (2015a) observed that during the cultivation of *Pleurotus* HK-37 laccase and lignin peroxidase activity was found to be maximum during vegetative growth phase and mycelial colonization and declined during the fruiting phase.

*Effect of incubation period on intra-extracellular hydrolytic enzyme activities under submerged fermentation:* The time course for *P. florida* intra-extracellular hydrolytic enzyme production is shown in Fig 1 (C,D). The intracellular activity of endoxylanase (5.6 IU/mg), cellobiohydrolase (5.46 IU/mg), β-glucosidase (1.06 IU/mg) and extracellular enzyme activity of endoxylanase (9.22 IU/ml), β-glucosidase (1.15 IU/ml), cellobiohydrolase (3.76 IU/ml) was found to be maximum on the 14$^{th}$ day. The intracellular endoglucanase enzyme (1.83 IU/mg) activity was found to be highest on the 7th day (1.83 IU/mg). However, an abrupt increase in endoglucanase enzyme activity was observed on 28$^{th}$ day of incubation which might be due to the products released

Table 1 Pearson correlation coefficients between oxidative enzymes of *Pleurotus florida*

| | Aryl alcohol oxidase | Laccase | Manganese peroxidase | Cellobiose dehydrogenase | Lignin peroxidase |
|---|---|---|---|---|---|
| Aryl alcohol oxidase | 1 | 0.224 | 0.021 | 0.988** | 0.442 |
| Laccase | 0.224 | 1 | 0.911** | 0.308 | 0.904** |
| Manganese peroxidase | 0.021 | 0.911** | 1 | 0.124 | 0.896** |
| Cellobiose dehydrogenase | 0.988** | 0.308 | 0.124 | 1 | 0.533* |
| Lignin peroxidase | 0.442 | 0.904** | 0.896** | 0.533* | 1 |

** Correlation is significant at the 0.01 level (2-tailed). *Correlation is significant at the 0.05 level (2-tailed).

during utilization of media supplements that act as inducers and thus stimulate the physiological conditions (Chellapandi and Jani 2008). However, the intracellular total cellulases (FPA) (1.09 IU/mg) and extracellular endoglucanase (5.16 IU/ml), total cellulases (1.12 IU/ml) activities were found to be the utmost on the 21st day. Mycelium growth rates have been found to decelerate after the third colonization week, resulting in a drop in enzyme activity due to a lack of usable nutrients and carbon sources (de Lima *et al.* 2020). Similarly, Das *et al.* (2015) stated that *Pleurotus pulmonarius* produced maximum cellulase activity on the 9th day of submerged fermentation. Megersa and Gure (2018) stated that *Phellinus tremulae* displayed the highest total cellulase activity (7.83 IU/ml) on the 12th day of incubation.

*Quantitative hydrolytic enzyme activities during solid state fermentation of paddy straw by* Pleurotus florida: The highest endoxylanase (9.12 IU/mg) and cellobiohydrolase activity (8.99 IU/mg) was observed at the terminated stage in *P. florida*. The β-glucosidase activity was observed maximum during the second flush stage (1.74 IU/mg). The highest activity of cellobiohydrolase (7.47 IU/mg) was found to be at the terminated stage. The utmost endoglucanase activity was found to be highest at the respective third flush stage (7.14 IU/mg) as endoglucanase enzymes decrease the polymer chain and provide the energy source to meet the demand of biomass production. The total cellulase activity was uttermost in the second flush (2.23 IU/mg) as shown in Fig 2(B). The increase in hydrolytic activities beyond full mycelium colonization is an indication that the enzymes were active in hydrolyzing hemicellulose during this stage and provide energy to the mycelium till the development of fruiting body (Raymond *et al.* 2015a). Table 2 represented the maximum positive correlation between endoxylanase and endoglucanase (r=0.980). Similarly, positive correlation was recorded between endoxylanase and cellobiohydrolase (r=0.934) indicating a direct relationship between the hydrolytic enzymes. The significant positive correlation at p≤0.01 was observed between endoglucanase and cellobiohydrolase (r=0.918). The results agreed with Kaur *et al.* (2022) who observed highest cellobiohydrolase activity (5.60 IU/mg) in *Calocybe indica* during the terminated stage. Raymond *et al.* (2015b) observed that xylanase activity (1.23 IU/mg) in *Coprinus cinereus* (Schaeff.) was higher during the fruiting phase.

*Enzymatic mushroom production:* The paddy straw was treated with a lignocellulolytic crude enzyme, which stimulated faster mushroom growth and fructification, as shown in Table 3. The *P. florida* resulted in maximum yield (58.78%) when paddy straw was treated with crude enzyme as compared to untreated straw. Spawn run and substrate colonization by mushroom proceeded very quickly with the formation of the whitish mycelia on the surface. Moreover, it completely colonized after 20 days compared to the control. The average weight of fruit bodies and number of fruiting bodies were maximum (9.57 g and 6142 no./q dry straw) in enzyme-treated paddy straw that could be associated with improved digestibility of substrate. The efficiency of *P. florida* to colonize and utilize paddy straw substrate differed depending on the fungus ability to synthesize extracellular enzymes required to break down the main components of the lignocellulosic waste biomass. It indicated that fungus could digest polysaccharides, ensuring that mycelia had enough carbon and energy to colonize large substrate areas and produce fruit bodies (Kaur *et al.* 2022). As a result, the enzymes produced by *P. florida* interacted to aid efficient cell wall disintegration, resulting in maximum yield and fruit body growth.

*Pleurotus florida* produced oxidative and hydrolytic enzymes in submerged and solid-state fermentation on paddy straw. The different responses for the production of lignocellulolytic enzymes towards culture conditions indicate separate regulatory mechanisms. The findings highlight the significance of conducting additional research on oxidative and hydrolytic enzymes to enhance

Table 3 Cultivation of *Pleurotus florida* on enzymatic pretreated paddy straw

| *Pleurotus florida* | Treatment | | CD (P≤0.05) |
|---|---|---|---|
| | Control | Crude enzyme | |
| Spawn run (d) | 23±0.4 | 20±0.5 | |
| First harvest (d) | 35±0.6 | 31±0.3 | |
| Yield (kg/q dry straw) | 42.12±0.3 | 58.78±0.4 | 8.28 |
| NFB (no./q dry straw) | 4996±0.8 | 6142±0.6 | NS |
| Avg. wt. of a FB (g) | 8.43±0.2 | 9.57±0.1 | |

Data given is mean of three replicates. NFB, number of fruiting bodies; Wt, weight; FB, fruiting body.

Table 2 Pearson correlation coefficients between hydrolytic enzymes of *Pleurotus florida*

| | Endoxylanase | β-glucosidase | Cellobiohydrolase | Endoglucanase | Total cellulases (FPA) |
|---|---|---|---|---|---|
| Endoxylanase | 1 | 0.231 | 0.934** | 0.980** | 0.068 |
| β-glucosidase | 0.231 | 1 | 0.398 | 0.162 | 0.437 |
| Cellobiohydrolase | 0.934** | 0.398 | 1 | 0.918** | 0.300 |
| Endoglucanase | 0.980** | 0.162 | 0.918** | 1 | 0.069 |
| Total cellulases (FPA) | 0.068 | 0.437 | 0.300 | 0.069 | 1 |

** Correlation is significant at the 0.01 level (2-tailed).

their production and enzyme activity in biotechnological processes. These enzymes with high activity and cheap cost could be useful in industrial applications requiring large volumes of the enzyme (pulp and paper, textiles, and bioremediation of industrial pollutants). Furthermore, mushroom cultivation is an efficient strategy for producing nutritious food and offering a comprehensive approach to agricultural waste management.

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