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LIGNINOLYTIC ENZYMES ACTIVITIES OF PLEUROTUS SAPIDUS P969 DURING VEGETATIVE GROWTH AND FRUIT DEVELOPMENT ON SUGARCANE RESIDUES-BASED SUBSTRATES

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ABSTRACT

Ligninolytic enzyme activities of extracts obtained from sugarcane residues-based substrates during solid-state fermentation (SSF) by Pleurotus sapidus P969 at mycelia colonization; primordial formation; harvesting and postharvest stages were investigated. SFF was performed in plastic bags using substrates formulated by mixing various proportions known weights of sugarcane bagasse (SB) and sugarcane tops (ST) on dry weight basis. Laccase, manganese peroxidase (MnP) and lignin peroxidase (LiP) activities in extracts of the substrates were determined using standard methods. The highest laccase activities reached were 43.94 U g-1 wet spent mushroom substrate (SMS) in SB/ST (60:40) mixed substrate on the first pinhead appearance (1st P.A). The highest manganese peroxidase activities observed were 11.90 U g-1 wet SMS in SB/ST (20:80) mixed substrate on the 1st P.A. The highest lignin peroxidase activities observed were 8.71 U g-1 wet SMS in SB/ST (20:80) mixed substrate after full substrate colonization (A.C). Thus, the results indicate enormous potential of P.sapidus for industrial production of ligninolytic enzymes on very low cost substrates through SSF. The study further demonstrates the possibility of production of specific ligninolytic enzymes by oyster mushrooms through tailor making the substrates using sugar cane-based residues.

Keywords: Ligninolytic enzymes, Pleurotus sapidus, Solid-state fermentation, Sugarcane based substrate.

1. INTRODUCTION

Ligninolytic enzymes are extracellular oxidative enzymes that are secondary metabolic products, differing in chemical compositions and are often species specific (Mtui and Nakamura, 2004 ; Dhouib *et al*., 2005). Production of ligninolytic enzymes by solid-state cultivation of white-rot fungi on lignocellulosic substrates is essential in the emerging field of biotechnology. During the growth and the development of mushroom mycelia, secretions of these enzymes cause biochemical changes in the substrate (Kurt and Buyukalaca, 2010). Such biochemical changes modify the insoluble and large components of lignocellulosic materials into the soluble and low-molecular-weight compounds which afterward are taken as source of nutrients and energy

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necessary for the fruiting bodies enlargement (Kurt and Buyukalaca, 2010). Therefore, these enzymes have a significant role in mushroom growth and development (Kuforiji and Fasidi, 2008). These enzymes are also important in various industrial processes in the production of chemicals, fuel, food, pulp and paper, textile, laundry, and animal feed (Elisashvili *et al*., 2006).

To develop commercially significant and low cost technologies for ligninolytic enzyme production, two approaches can be applied. The first one involves search for powerful producers of enzymes and the second one is the utilization ability of lignocellulosic residues. Many studies indicate that the structure of lignocellulosic materials, the type of mushrooms species as well as mushroom cultivation techniques play a vital role in the enzyme production potential by white rot fungi during SSF (Reddy *et al*., 2003; Elisashvili *et al*., 2006; Elisashvili *et al*., 2008; Kurt and Buyukalaca, 2010). It has been well documented that composition of the substrate influences the pattern of enzyme production by white rot fungi (Papinutti *et al*., 2003; Lechner and Papinutti, 2006). Thus, the mushrooms strains which are able to produce larger quantities of these enzymes are better suited to colonize the substrates and to produce higher and healthier fruiting bodies yields (Ruiz-Rodríguez *et al*., 2011). Ligninolytic enzymes have also repeatedly been linked to fruiting body production but species appear to differ at timings of high enzyme production and there is no clear-cut picture on the functional relevance of the enzymes in fruiting stage (Kües and Liu, 2000; Rühl *et al*., 2008). Previous studies using oyster mushroom, *Pleurotus ostreatus*, reported that production of ligninolytic enzymes increases with vegetative biomass production on solid growth substrates and that it drops during the sexual fruiting stage (Mata and Savoie, 1998; Mata *et al*., 2007; Savoie *et al*., 2007; Elisashvili *et al*., 2008; Rühl *et al*., 2008). However as far as we are informed, the entire pattern of enzyme activities of the oyster mushroom at various stages of its growth cycle including harvesting and post-harvest stages of mushrooms has never been reported.

Moreover, most studies utilized cereal straw based- substrates to investigate ligninolytic activities trend in some growth stages during solid state cultivation of *Pleurotus species* such as *Pleurotus ostreatus* (Elisashvili *et al*., 2008; Rühl *et al*., 2008; Isikhuemhen and Mikiashvilli, 2009; Sherief *et al*., 2010). This work therefore, examined the ligninolytic activity profiles of *P. sapidus* during vegetative growth, at primordial formation and post-harvest stage in two flushes of fruiting body production under solid-state cultivation on sugarcane residues-based substrate.

2. MATERIALS AND METHODS

2.1. Culture Maintenance

Pleurotus sapidus P969 was obtained from China. It was maintained at 4 °C on 2 % malt extract agar (MEA) plates and sub-cultured every 2-3 months.

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2.2. Spawn Production

Spawn of *P. sapidus* were prepared using sorghum grains. The grains were washed thoroughly, and 1 kg was boiled in 2 litres of water until they became semi-soft. They were then decanted over a sieve to remove excess water and cooled to room temperature. Hundred grams wet weight grains were packed in 250 ml glass bottles, and then they were covered with aluminium foil and were sterilized at 121 ºC for 15 minutes. Thereafter, each cooled bottle of sterilized grains was aseptically inoculated with three mycelia agar fragment (1 cm^2 each) taken from 5- day-old cultures of *P. sapidus*. The grains were shaken to evenly distribute the mycelia, and then incubated at 25±2 ºC until when fully colonized by mycelia.

2.3. Substrate Preparation and Experimental Design

Two types of sugarcane residues namely sugarcane tops and bagasse were used as substrates in this study. Fresh sugarcane tops were harvested manually from a sugarcane estate at Tanganyika Planting Company (TPC), Kilimanjaro region, Tanzania. They were shredded into 1-2 cm long bits and sun-dried for 7 days. Fresh sugarcane bagasse residues were collected from TPC factory after juice extraction and were submerged in water and left for 14 days to allow fermentation of free sugars which could later attract the growth of microbial contaminants during mushroom mycelia colonization. The bags containing such residues were then hanged to drain-off excess water before were sun dried for 7 days and kept until when needed.

In order to search for the best substrate combination which will lead to highest enzyme and mushroom yield, the following proportions of sugarcane bagasse (SB) and sugarcane tops (ST), (w/w on dry basis) were mixed: SB/ST (100:0); (80:20); (60:40); (50:50); (40:60); (20:80) and (0:100). The fractions were then moisturized with tap water using hand sprayer and mixed to reach homogenous moisture content ranging from 45.6 to 72.23 %. The substrates were packed in polypropylene bags, 6 x 24 cm long (100 g wet weight per bag), steamed in an autoclave for 2 hours at 100^{o}C and then left at room temperature to cool.

2.4. Substrate Inoculation, Spawn Running, Pinhead Formation and Harvesting

After cooling, all substrates were inoculated with fully colonized 14-day old spawn at 5 % (wet weight spawn/wet weight substrate) under aseptic conditions. Spawn grains were placed on the substrate surfaces through the opening at the top and at the bottom of the bag and were homogeneously distributed. After inoculation, both openings of the bags were tied with a sisal string and the inoculated substrates were then incubated at 27 ±1 ºC and at a relative humidity of 75–78 %. The spawning room was kept humid by pouring 15 litres of tap water per day on the floor that was laid with mattress to retain water for long period. Vegetative growth was monitored by observing the inoculated substrates until they were fully colonized. Fruiting body formation was triggered by shifting environmental variables namely moisture, air exchange, temperature and light in the cropping room. When mycelia colonized the whole substrate mass,

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random holes were made on the bags to initiate pinheads or primordia formation by lowering carbon dioxide concentration in the substrate. Curtains on the windows were removed to allow ventilation and more light. Relative humidity in the room was increased to (82 ± 2) % and temperature decreased to (25 ± 2) °C by pouring 20 litres of ice-cold water twice a day on the floor. The bags were also sprayed with ice cold water twice a day to keep them moist. After primordial formation, large holes were made direct on the pinheads to allow development of mature fruit bodies.

2.5. Sampling and Extraction of Enzymes

Samples of substrate were taken on day 7 after inoculation (7d-A.I), day 10 after inoculation (10d-A.I), after substrate colonization (A.C), day 7 after substrate colonization (7d-A.C), day 14 after substrate colonization (14d-A.C), day 21 after substrate colonization (21d-A.C), on first pinheads appearance (1st P.A), after first flush mushrooms harvest (1st F.H), day 7 after first flush mushrooms harvest (7d-1st F.H), day 14 after first flush mushrooms harvest (14d-1st F.H), on the second pinheads appearance (2nd P.A), and after second flush mushrooms harvest (2nd F.H). One bag of substrate was taken from each substrate combination design and homogenized manually. The samples were harvested for determination of enzyme profiles and chemical compositions.

The crude enzyme filtrate was obtained by soaking 50 g of spent mushroom substrate (SMS) in 50 ml of 100 mM sodium acetate buffer (pH 5.0) in 250 ml Erlenmeyer flasks, and then they were subjected to a rotary shaker (Edmund Buhler, 7400 Tubingen shaker-SM 25, Germany) at 150 rpm for 2 hours. During this step, all flasks containing the SMS-buffer mixture were kept on ice throughout the entire shaking period. The SMS-buffer mixtures were then filtered through double layered cheesecloth followed by centrifugation at 6000 rpm for 10 minutes (Mikro 22R centrifuge, Hettich, England) at 4 °C to remove residual particles. The clear filtrates obtained were regarded as crude enzyme filtrates which were later used for ligninolytic enzyme activity assays.

2.6. Laccase Activity

Laccase activity was determined according to Bourbonnais *et al.* (1995). The reaction assay mixture (1.5 ml) contained 0.5 mM of 2,2'-azino-bis (3-ethylbenzthiazoline)-6-sulfonate (ABTS) in 100 mM sodium acetate buffer (pH 5.0) and 50 µl of the enzyme filtrate (Patrick *et al.*, 2009). Oxidation of ABTS was observed by the formation of intense blue-green colour which was monitored by measuring the increase in absorbance at 420 nm, with a molar extinction coefficient, ε_{420} = 36000 $M^{-1}cm^{-1}$, using UV-Visible spectrophotometer (Jenway Genova, Bibby Scientific Ltd, England). Laccase activity was expressed in units (U) defined as follows: 1 U = 1 µmol of ABTS oxidized per min.

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2.7. Manganese Peroxidase Activity

Manganese peroxidase (MnP) activity was assayed as described by Kofujita *et al.* (1992) with some modifications, using a reaction mixture containing 5 mM guaiacol and 2 mM $MnSO_4$ in 50 mM sodium succinate buffer (pH 4.5), 10 mM H_2O_2 and 100 µl enzyme filtrate in a total volume of 1.5 ml. Hydrogen peroxide was added lastly to initiate the reaction after incubating the mixture at 30 °C for 5 minutes. The increase in absorbance was monitored by the oxidation of guaiacol (2-methoxyphenol) as the substrate at 465 nm with extinction coefficient, ε_{465} = 12100 $M^{-1}cm^{-1}$, using UV-Visible spectrophotometer. One unit of MnP activity was defined as the amount of enzyme that oxidized 1.0 µmole of 2- methoxyphenol per minute.

2.8. Lignin Peroxidase Activity

Lignin peroxidase (LiP) activity was determined according to the method of Sugiura *et al.* (2003) with some modifications. The assay mixture (1.5 ml) contained 10 mM veratryl alcohol in 20 mM citrate buffer (pH 3.0), 10 mM H_2O_2 and 100 µl enzyme filtrate. H_2O_2 (300 µl, 50 mM) was added last to initiate the reaction after incubating the substrate and enzyme at 30 °C for 5 minutes. The increase in absorbance was followed spectrophotometrically at 310 nm (extinction coefficient, ε_{310} = 9300 $M^{-1}cm^{-1}$,) where the oxidation of veratryl alcohol to veratraldehyde (3,4-dimethoxybenzaldehyde) took place. One unit of LiP was defined as the amount of enzyme that oxidizes 1.0 µmole of veratryl alcohol per minute at pH 3.0 and 30°C.

3. RESULTS

3.1. Vegetative Growth Phase, Pinhead Formation and Fruiting Bodies Development

Table 1 illustrates the time (days) in which various growth stages of *P. sapidus* took during SSF on SB/ST mixed substrates. All substrate formulations were fully colonized by mycelia after 14 days of incubation (Table 1). Pinheads initiation was induced by subjecting all substrates to cold shock for 1 hour. The first pinheads appearance (1st P.A) in all substrate formulations occurred after 17-19 days from the day after complete substrate colonization. The second pinheads appearance (2nd P.A) occurred after 26–28 days from the day of first mushroom flush harvest (1st F.H) in all substrate formulations. Fruit bodies developed to maturity and were harvested 2-3 days later after pinhead formation in both mushroom flushes (1st and 2nd Flush harvest) and in all substrate formulations.

3.2. Laccase Activities

Results on laccase activities profile during solid-state cultivation of *P sapidus* on various mixed proportions of SB and ST substrate are presented in Figure 1. Laccase activity levels (Figure 1) were high in the vegetative growth phase i.e. on the 7th (7d-A.I) and 10th day (10d-A.I) after substrate inoculation in all substrates formulations with the SB/ST (0:100) substrate displaying the highest activity peak of 40.56 U g^{-1} at this stage. However, activities in all

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substrates declined sharply on the stage of full substrate colonization (A.C), 7th day after substrate colonization (7d-A.C) and on the 14th day after substrate colonization (14d-A.C). Activities increased again to variable sharp peaks in all substrates designs on the first pinheads appearance (1st P.A). The SB/ST (60:40) formulation displayed the highest activity peak of 43.94 U g^{-1} at 1st P.A and this marked the highest activity overall in all substrate formulations. Again laccase activities displayed a steep decrease pattern in all substrates after first flush mushrooms harvest (1st F.H), then showed a gradual increase on the 7th day after first flush mushroom harvest (7d-1st F.H), thereafter, slightly leveled off through the stage of second flush mushroom harvest (2nd F.H).

3.3. Manganese Peroxidase (MnP) Activities

MnP activities profile during solid-state cultivation of *P sapidus* on various mixed proportions of SB and ST substrate is presented in Figure 2. MnP activities in all substrates formulations (Figure 2) were peaked at 7d-A.I stage. The highest activity of 7.44 U g^{-1} was observed in SB/ST (0:100) substrate combination at this stage. Activities declined steadily on the 10d-A.I stage through 7d-A.C, then rose gradually to peak at 1st P.A stage with SB/ST (80:20) substrate mixture displaying the highest MnP activity of 11.90 U g^{-1} at this stage, and overall for all substrate formulations. Nevertheless, a decrease-increase activity patterns were observed in all substrates formulations from 1st F.H to 2nd F.H.

3.4. Lignin Peroxidase (LiP) Activities

Figures 3 presents the LiP activities profile during solid-state cultivation of *P sapidus* on various mixed proportions of SB and ST substrate. LiP activities for all formulations (Figure 3) increased gradually from 7d-A.I to the A.C stage, and thereafter dropped rapidly at 7d-A.C stage. Activity again increased progressively from 7d-A.C to some peaks on the 14d-A.C and 1st P.A stages. It then declined progressively until on the 7d-1st F.H when suddenly increased again and peaked at 14d-1st F.H and 2nd P.A stages. However, a decline pattern of activity was again observed in almost all substrate formulations on the 2nd F.H stage. The uppermost LiP activity of 8.71 U g^{-1} was observed in the SB/ST (20:80) substrate at the A.C stage.

4. DISCUSSION

4.1. Vegetative Growth Phase, Pinhead Formation and Fruiting Bodies Development

The change from the vegetative state of mycelia growth to the generative one of primordia formation is called pinning, pin setting, pinhead initiation or fructification. Primordia or pinheads are knots of mycelium that precede development into small mushrooms. All species require a set of environmental conditions for pinning that are quite different from the conditions for mycelia growth. Oyster mushrooms grow very well on various lignocellulosic substrates due to secretion of extracellular oxidative enzymes capable of degrading lignin which is a barrier to the available

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carbon sources in those substrates (Tisdale *et al*., 2006; Mane *et al*., 2007). However, the duration for different growth stages of *Pleurotus* species during solid-state cultivation on lignocellulosic substrates depends on the type of substrate and substrate formulation used; the type of species and/or the strain employed; the spawn type and amount of spawn used; and the prevailing cultivation conditions (Royse *et al*., 2004; Shah *et al*., 2004; Tisdale *et al*., 2006; Vetayasuporn, 2006; Mshandete and Cuff, 2008).

Under this study, *P. sapidus* took 14 days to completely colonize all substrate formulations used (Table 1). When SB/ST (60:40) substrate formulation was supplemented with various fractions of sugarcane filter cake, it took between 11 to 12 days for complete substrate colonization (data not shown, unpublished work). Pinhead appearance took 17 days after substrate colonization (Table 1), but when various fractions of sugarcane filter cake was added in SB/ST (60:40) substrate formulation, pinheads appearance took 18 – 22 days after complete substrate colonization (data not shown, unpublished work). Previous studies have reported a range of 12 to 41 days for complete mycelia colonization in various *Pleurotus* species cultivated on various lignocellulosic substrates (Royse *et al*., 2004; Shah *et al*., 2004; Tisdale *et al*., 2006; Vetayasuporn, 2006; 2007; Mane *et al*., 2007; Mshandete and Cuff, 2008). Mshandete and Cuff (2008) reported pinhead appearance of 2 days later after complete substrate colonization by *P. flabellatus* grown on both composted and non-composted sisal decortications residues. Also, Rühl *et al*. (2008) reported pinhead appearance of 4 days later after complete substrate colonization by *Pleurotus ostreatus* on wheat straw.

These variations in time for vegetative growth and pinhead development could be attributed to the substrate and supplement constituents, prevailing cultivation conditions and the type of *Pleurotus* species cultivated. This finding agrees with previous work whereby correlation studies between the constituents of the substrates and the number of days to primordium formation revealed a significant positive relationship with cellulose content and total sugar indicating that cellulose and sugar content in each substrate were directly proportional to the spawning duration and the primordium formation (Gaitán-Hernández *et al*., 2011).

4.2. Quantitative Ligninolytic Activities during SSF of Sugarcane Residues by *P. Sapidus* P969

From the results in Figures 1, 2 and 3 laccase, MnP and LiP activities were detected in all SB/ST substrate formulations all over the cultivation periods. The level of enzyme activities seemed to be strongly regulated during the life cycle of *P. sapidus* in all substrate formulations with distinctive variations. Nevertheless, despite the quantitative discrepancies, each substrate formulation displayed similar patterns of enzymatic profiles along the cultivation period.

Generally, laccase, manganese peroxidase (MnP) and lignin peroxidase (LiP) activities were high in vegetative growth phases before and after substrate colonization but declined upon completion of substrates colonization by fungal mycelia. Activities of these enzymes sharply

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increased again when pinheads/primordia appeared as a prelude to initiation of fruiting body formation, and there was a drastic decline in activity for all three ligninolytic enzymes just after mushroom harvest at all observed flushes. When mushroom life cycle shifted to mycelia regenerative stage (postharvest stages), the activity of ligninolytic enzymes again gradually increased in the mushroom spent substrate. However, the quantities of these enzymes recovered from SMS at different mushroom life cycle stages during cultivation differ from one substrate formulation to another. The increase in enzyme activities during vegetative growth and during mycelia regenerative stage is possibly associated with the energetic requirements for initiating fructification whereby enzymes are secreted to digest the substrate to provide carbon and other nutrients (Kurt and Buyukalaca, 2010; Gaitán-Hernández *et al*., 2011). Mushrooms need easily assimilated sources of carbon. These accelerate mushroom growth and the degradation of the medium, and also reduce fructification time since the mycelium will convert these carbohydrates into reserves for fruiting (Gaitán-Hernández *et al*., 2011).

High ligninolytic enzyme activities by Pleurotus species have been reported during growth phases in various lignocellulosic substrates, with drastic reductions during the period of fruiting body formation (Mata *et al*., 2007; Elisashvili *et al*., 2008; Rühl *et al*., 2008; Kurt and Buyukalaca, 2010); *Lentinula edodes* (Mata and Savoie, 1998; Ohga and Royse, 2001); *Lentinus tigrinus* (Lechner and Papinutti, 2006); and *Grifola frondosa* (Montoya *et al*., 2012). Mata *et al*. (2007) reported ligninolytic activities of *Pleurotus djamor*, *Pleurotus ostreatus*, and *Pleurotus pulmonarius* grown on sugarcane bagasse to be high during vegetative growth phase, low during fruiting stage and increased again during the postharvest stages. Elisashvili *et al*. (2008) investigated ligninolytic enzymes of various strains of *Pleurotus ostreatus* during solid state cultivation on wheat straw and tree leaves substrates independently and found that each strain had similar pattern of enzymes profiles in the substrates. The study reported that ligninolytic activities were high during substrate colonization; declined rapidly during fruiting body development; and again increased gradually when mushroom life cycle shifted to mycelia regenerative phase.

Another study by Rühl *et al*. (2008) followed up ligninolytic activities within the wheat straw based-substrate over the cultivation periods of two strains of *Pleurotus ostreatus* and found high laccase, MnP and LiP activities during vegetative growth phases and dropped upon initiation of fruiting body formation to be hardly detectable at the harvest of the mature mushrooms, and then directly after harvest at all observed flushes, there was a sharp increase in all three enzymatic activities in the substrate for both strains. Also Kurt and Buyukalaca (2010) followed up laccase activities of *Pleurotus ostreatus and Pleurotus sajor-caju* during solid state cultivation on wheat straw, paddy straw, sesame straw, sawdust, and viticulture wastes as either single substrate or mixed with wheat bran at a ratio of 2:1 on a dry weight basis. The study reported highest laccase activities values on the 10th day of mycelial growth phase for both species but amounts of enzymes differed in each species and for each substrate formulation used.

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Furthermore, Lechner and Papinutti (2006) investigated the growth and production of lignocellulosic enzymes from *Lentinus tigrinus* during solid state fermentation using wheat straw as substrate and found that enzyme activities were high during colonization, and then declined drastically or were almost constant during fruit body formation. Thereafter, all enzyme activities increased rapidly to highest levels after harvesting the last flush, with the exception of laccase that showed highest activity around day 20. Montoya *et al.* (2012) investigated enzyme activity profiles from *Grifola frondosa* during different stages of the growth cycle on oak sawdust mixed with corn bran and oak sawdust plus coffee spent-ground substrates. The study reported that all ligninolytic enzymes from all substrates, displayed a similar pattern along the cultivation time in which activities increased during the colonization phase, reaching a peak when the substrate was fully colonized at days 20–30. They gradually declined afterwards, but decreased markedly during fruiting body formation. Thus, for these species, it is very likely that the enzymes are produced to digest lignocellulosic substrates in order to provide nutrients for the growing organism.

Results obtained in the present study are in good agreement with the previous reported findings. However, the quantities of ligninolytic enzymes recovered from various spent mushroom substrates at different mushroom life cycle stages from the previous findings differ markedly within themselves and from the present study. In this work, it was found that the levels of enzyme production were also different at each mushroom life cycle stage for each substrate formulations used. The differences found in enzyme production might be due to the relative composition of polysaccharides, the size of the wastes used, and probably the presence of natural inducers such as aromatic compounds (Papinutti and Forchiassin, 2007). Many aromatic compounds present in wood and other lignocellulosic materials are potent inducers of ligninolytic enzymes. For example, ferulic acid, ubiquitously present in plant cell walls, induce laccase activity in white-rot fungi (Herpoël *et al.*, 2000; Patrick *et al.*, 2010; 2011). Therefore, specifically this study succeeded to undertake solid-state fermentation of sugarcane residues-based substrate, and consequently offers a viable alternative use for these abundant agricultural residues with a triple benefit: cultivation of valuable mushroom, production of industrially important enzymes and a reduction in environmental impact.

5. CONCLUSIONS

This study showed that *P. sapidus* P969 can be successfully cultivated on the sugarcane residues-based substrates and has ability to produce enzymes which are essential for lignocelluloses degradation. In addition, experimental results provided an insight into the patterns of laccase, MnP and LiP production at different growth stages during the degradation of the substrates by *P. sapidus*. The findings of this work underline the importance of applying cheap substrates for production of not only industrially important enzymes but also valuable edible mushrooms.

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Table-1. Time (days) for the growth stages from the first day of substrate inoculation during SSF of *Pleurotus sapidus* P969 on sugarcane residues-based substrates.

SB/ST substrate ratio	7th day after substrate inoculation (7d-A.I)	10th day after substrate inoculation (10d-A.I)	After colonization (A.C)	7th day after colonization (7d-A.C)	14th day after colonization (14d-A.C)	At 1st pinhead appearance (1st P.A)	After 1st flush harvest (1st F.H)	7th day after 1st flush harvest (7d-1st F.H)	14th day after 1st flush harvest (14d-1st F.H)	At 2nd pinhead appearance (2nd P.A)	After 2nd flush harvest (2nd F.H)
SB/ST (100:0)	7	10	14	21	28	33 (**19**)*	36	43	50	64 (**28**)*	67
SB/ST (80:20)	7	10	14	21	28	32 (**18**)*	35	42	49	63 (**28**)*	65
SB/ST (60:40)	7	10	14	21	28	31 (**17**)*	34	41	48	62 (**28**)*	64
SB/ST (50:50)	7	10	14	21	28	31 (**17**)*	34	41	48	61 (**27**)*	63
SB/ST (40:60)	7	10	14	21	28	32 (**18**)*	34	41	48	60 (**26**)*	62
SB/ST (20:80)	7	10	14	21	28	31 (**17**)*	34	41	48	61 (**27**)*	63
SB/ST (0:100)	7	10	14	21	28	32 (**18**)*	35	42	49	63 (**28**)*	65

*Numbers in the brackets indicate days taken from colonization stage (A.C) to first pinhead appearance in the 1st P.A column; and from first flush harvest (1st F.H) to second pinheads appearance in the 2nd P.A column

Figure-1. Laccase activity profiles of *Pleurotus sapidus* at different stages of vegetative growth and fruit-bodies development during solid-state cultivation on different mixed proportions of sugarcane bagasse (SB) and sugarcane tops (ST). Values are means of triplicates measurements; Error bars denote standard error.

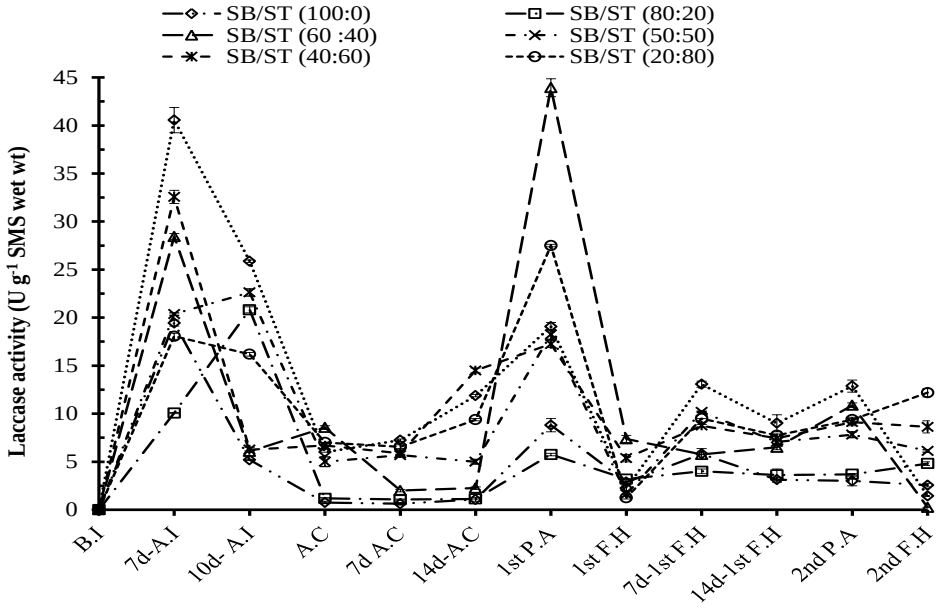

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Figure-2. MnP activity profiles of *Pleurotus sapidus* at different stages of vegetative growth and fruit-bodies development during solid-state cultivation on different mixed proportions of SB and ST. Values are means of triplicates measurements; Error bars denote standard error.

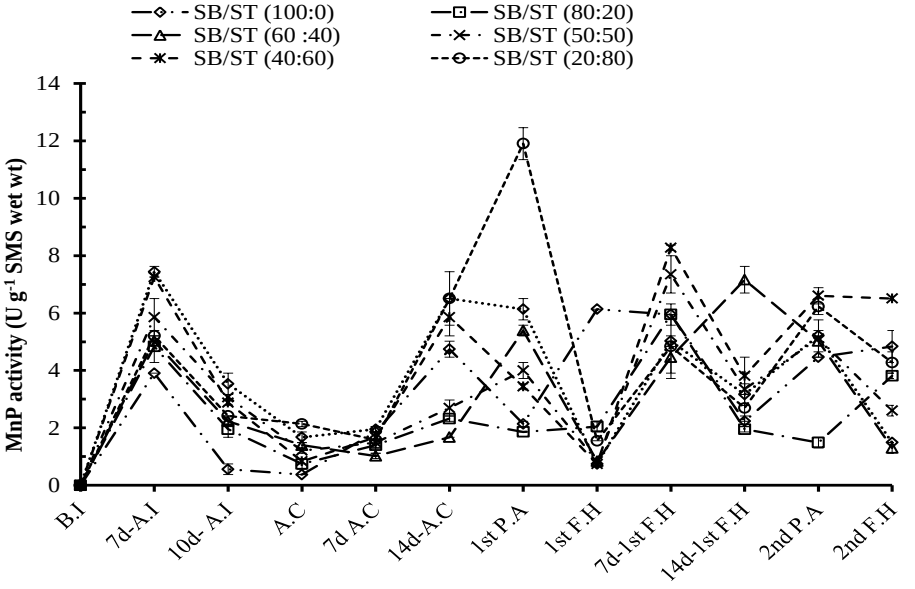
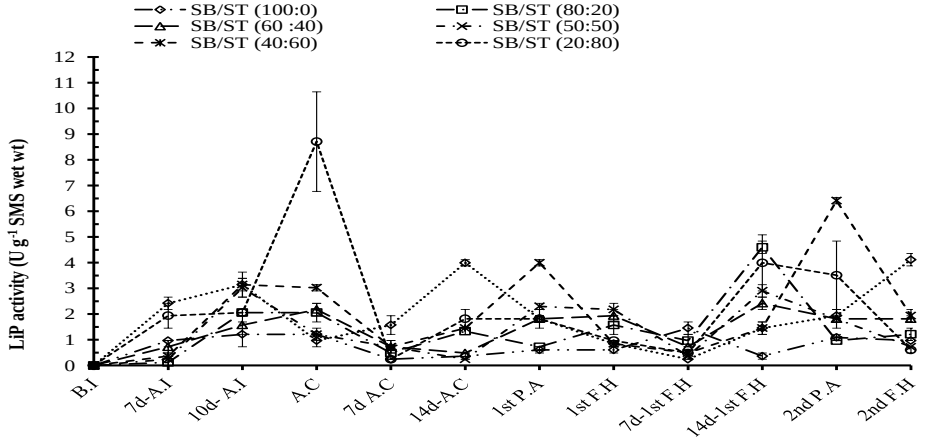


Figure-3. LiP activity profiles by *Pleurotus sapidus* at different stages of vegetative growth and fruit-bodies development during solid-state cultivation on different mixed proportions of sugarcane bagasse (SB) and sugarcane tops (ST). Values are mean of triplicates measurements; Error bars denote standard error.



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