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Evaluation of Enzymatic Activity during Vegetative Growth and Fruiting of *Pleurotus* HK 37 on *Agave sisalana* Saline Solid Waste

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Abstract: Mushroom cultivation is an effective method for the production of nutritional food in addition to offering a holistic approach to agro waste management by utilization of the abundant lignocellulosic waste including sisal leaf decortications waste (SLDW). Production of 1 kg of mushrooms generates 5 kg of a co-product called spent mushroom substrate (SMS). Alternative means of disposal of the resulting SMS is production of extracellular enzymes generated by mushrooms during their growth and development. In this study, *Pleurotus* HK 37 was studied for its ability to produce laccase manganese peroxidase (MnP), lignin peroxidase (LiP) and xylanase on SLDW under solid-state fermentation. Laccase activities reached the highest values of 27.3 U/ml when the substrate was fully colonised. The activity then declined with each subsequent harvest to 15.0 U/ml. MnP assay had two peaks of 8.9 U/ml and 8.0 U/ml on full colonisation and during the 3rd flush, while LiP and xylanase activities had highest recorded activities of 0.34 U/ml and 0.28 U/ml, respectively. The results of this study demonstrate the potential utilization of sisal leaf decortications waste as raw materials for simultaneous production of edible mushrooms and extracellular enzymes from a commercial fungus, *Pleurotus* HK 37, which is a potential biotechnological application.

Keywords Spent mushroom substrate, *Pleurotus* HK 37, Laccase, Manganese peroxidase, Ligninolytic enzymes

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INTRODUCTION

Three quarters of Kenya's population suffer from malnutrition[1] mainly due to lack of protein, vitamins and minerals. Most staple foods are mainly starch, and protein foods like fish, meat and milk, which are rare and expensive especially to the rural population[2]. That has resulted in the need to explore alternatives for low cost production of unconventional protein-rich food and one of the options is edible fungi or mushrooms belonging to the Basidiomycetes[3]. Globally according to Li[4], China has a current production of more than 20 metric tons, accounting for over 80 % of the world mushroom production, which is estimated at 25 metric tons. In Kenya however, mushrooms were introduced in the last two decades, with the Oyster mushroom being the species popular with small scale farmers owing to its relatively robust production techniques, relatively good yields, wide fruiting temperature range, superior flavour, high nutritional content, and low capital investment[5].

Kenya generates huge quantities of organic agro-industrial waste annually from agricultural, forest and food processing industries. These wastes are conceived as a negative factor in both the industrial and agricultural settings, since they generate adverse environmental effects related to their disposal such as GHG emissions. Oyster mushroom can be grown on various substrates[6]. They can utilize almost all agricultural wastes such as wood logs; saw dust, straw and cotton waste and sisal waste as substrates[7]. The utilization of the insoluble lignocellulosic substrates by edible mushrooms depends upon the production of a wide array of lignocellulolytic enzymes by the fungal mycelium which is a crucial part of the colonization process and is an important determinant of mushroom yield[8]. The enzyme systems are capable of utilizing complex organic compounds, which occurs in organic matter residues[9].

Spent mushroom substrate (SMS) is the left over substrate (compost in case of button mushroom) generated from mushroom production, after which production becomes unremunerative. The quantity of spent mushroom substrate generated varied from 3-5 times of mushroom produced in different mushrooms. It is 5 times in button mushroom, 3 times in oyster and milky mushrooms, and 3-8 times in paddy straw mushroom. SMS from button mushroom and paddy straw mushroom can be recycled to prepare manure out of it. It can also be used in crop disease management, reclamation of abandoned site and in preparation of briquettes for their use in boilers. Similarly, the SMS from oyster mushrooms can be used as feed for animals and as feeding material for vermicomposting and biogas production. The SMS of button, oyster, paddy straw and shiitake mushrooms can be used for bioremediation of chemically polluted sites[10]. Production of 1 kg of mushrooms, generate 5 kg of spent residual material called spent mushroom substrate (SMS) [11]. Similarly in Kenya, production of 1kg of oyster mushroom on saline solid sisal waste at 40% biological efficiency will lead to generation of 5.88 kgs of SMS[11].

According to previous report, an average farm in Malaysia discards 24 tonnes of SMS per month[12], while the annual production of SMS in Ireland and Netherlands is estimated at 21,000 and 67,000 tones, respectively. Currently, the Kenya National farmers information service (NAFIS) estimates overall demand of mushroom in Kenya at 1200 tons, whose production will result in generation of 6,960 tones of SMS calculated according to Lau *et al.*[11]. The disposal of SMS pose a major challenge to farmers as experienced in other developed countries, besides being a menace to the environment it can be employed in value addition for production/extraction of already secreted extracellular enzymes. SMS principally consists of mycelia, extracellular enzymes produced by the fungus during its growth and unutilized lignocellulosic substrate[12]. *Pleurotus* species have been reported to synthesize and release cellulolytic, lignolytic and xylanolytic enzymes[13]. Earlier studies indicate that SMS of *P. sajor-caju* is an excellent

source of ligninolytic and cellulolytic enzymes[14]. These enzymes have potential in the decolourisation of different dyes[15], biological pre-treatment of biomass to produce biofuels, treatment of effluents in the textile, paper and pulp industries, clarification of wines and juices[16], pig production[17]and in biomedical uses such as cancer biotherapy[18], Antioxidant properties[19], antigenotoxic and bio-antimutagenic activity[20], antiproliferative activity[21] and other biomedical activities[22,23].These enzymes are the group of hydrolytic enzymes that are capable of degrading all types of lignocellulosic materials[24-27].

Facchini *et al*. [28] noted that utilization of agricultural wastes for production of hydrolytic enzymes offers an attractive option due to the fact that it would stimulate the natural environment. Fungi have been reported to secrete higher hydrolytic enzymes extracellulary[29] hence offering a more attractive option for their production. However, so far information on production of these enzymes by *Pleurotus* HK 37 during solid state cultivation on SLDW is totally non-existent. Therefore, the aim of this study was to evaluate *Pleurotus* HK 37 extracellular and hydrolytic enzymatic activities during its substrate colonisation and growth over three flushes of fruiting body production (cultivation) on SLDW.

MATERIALS AND METHODS

Source of commercial strains of *Pleurotus* HK 37 and sisal waste: *Pleurotus* HK 37 used in this study was obtained from the strain bank of the Department of Molecular Biology and Biotechnology, University of Dar es Salaam in Tanzania. The oyster mushroom *Pleurotus* HK-37 strain originated from South Africa and is among the oyster mushrooms grown in Tanzania[30]. The fungus was raised on PDA and stored in a refrigerator at 4 $^{\circ}$C for subsequent use. The sisal leaf decortications waste (SLDW) used in this study was obtained from Kilifi Plantation limited, in Kilifi County, Kenya.

Spawn and substrate preparation, pinhead formation and harvesting: Mushroom spawn was prepared using sorghum grains (*Sorghum bicolor* (L.) Moench) as described by Stamets[31]. The fibrous sisal leaf decortications wastes were chopped into 2-3 cm length pieces and pre-treated with cold water, according to Muthangya *et al*.[32], while the dried reference substrate, grass-*Panicum colorotum* L. (henceforth referred to as control) was obtained from Pwani University, Kilifi, Kenya. The control was chopped into 2-3 cm length and soaked in cold water for 1 day at 1:5 (water: grass) ratio. The substrates were drained off excess water to reduce the moisture content to 65-70% which was determined by the hand squeeze. The substrates were packed into 1kg bags and steam pasteurizedfor 4-5 hours at 80 ºC in a fabricated in metallic firewood-heated drum and cooled for 24 hours in the spawning room. The substrates were all spawned at 5 % (wet weight spawn/wet weight substrate) with a two weeks old spawn. The bags were place on shelves in the dark room where a temperature of 24-28°C was maintained during the incubation and monitored until they were fully colonized. After full colonization, the bags were transferred to the cropping room, where the environment was illuminated by sunlight and maintained at a temperature and humidity of 28°C and 75-85%, respectively. The mushroom fruiting bodies were harvested according to Mshandete and Cuff[33].

Extraction of crude enzymes from spent substrate of *Pleurotus* HK-37: Samples of about 5 g (± 1 g) were taken at different intervals from the 21st day (full colonization, after 1st, 2nd and 3rd flush) from the cultivation bags. The samples with the submerged mycelium were weighted and mixed with 50 mL of the subsequent extraction buffer and homogenized. The mixture was shaken in 250 mL Erlenmeyer flasks for 15 minutes at 125 rpm in an orbital shaker. The solids were removed by filtration through a cheese cloth and the filtrates were centrifuged at 11000 *g* for 5 min. The supernatant containing the fungal enzymes was henceforth referred to as crude enzyme extract.

Laccase activity assay: Laccase activity in the crude enzyme extract was measured by monitoring the oxidation of 2, 2-azino-bis (3-ethylbenzthiazoline-6 sulphonic acid) (ABTS) in an assay mixture (1.5 ml) containing 0.5 mM ABTS, 0.1 M sodium acetate buffer (pH 5.0, and 50 µl ml crude enzyme. The crude enzyme was added after incubating the reaction mixture at 30 °C for 5 minutes. Oxidation of ABTS was then monitored spectrophotometrically by determining the increase in absorbance at 420 nm, (A_{420nm}) with a molar extinction coefficient, ε_{420} = 36000 $M^{-1}cm^{-1}$, using Jenway UV-Visible spectrophotometer, (Bibby Scientific Ltd, United Kingdom). One unit (1 U) of laccase activity is defined as the amount of enzyme oxidizing 1 µmole of ABTS per minute at pH 5.0 and 30 °C.

Lignin Peroxidase activity assay: Lignin peroxidase activity was determined at 30 °C using the method of Sugiura*et al.*[34]in a reaction mixtures (1.5 ml) containing 20 mM citrate buffer (pH 3.0), 10 mMveratryl alcohol (= 3,4-dimethoxybenzyl alcohol) and 0.1 ml crude enzyme extract. The reaction was started by adding 10 mM H_2O_2 in the assay mixture after incubation at 30 °C for 5 minutes, and the increase in absorbance was followed spectrophotometrically using Jenway UV-Visible spectrophotometer, (Bibby Scientific Ltd, United Kingdom) at 310 nm (extinction coefficient, ε_{310} = 9300 $M^{-1}cm^{-1}$,) due to oxidation of veratryl alcohol to veratraldehyde (= 3,4-dimethoxybenzaldehyde). One unit (1 U) of LiP activity was defined as the amount of enzyme that oxidized 1.0 µmole of veratryl alcohol per minute at pH 3 and 30°C.

Manganese Peroxidase activity assay: Manganese peroxidase activity was determined by monitoring the oxidation of guaiacol (= 2-methoxyphenol) as the substrate[35] at 465 nm with extinction coefficient, ε_{465} = 12100 $M^{-1}cm^{-1}$, using Jenway UV-Visible spectrophotometer (Bibby Scientific Ltd, United Kingdom). The 1.5 ml reaction mixture was composed of 50 mM sodium succinate buffer (pH 4.5, 30 °C), 5 mMguaiacol, 2 mM $MnSO_4$, 100 µl crude enzyme and 50 mM H_2O_2, after which hydrogen peroxide was added to initiate the reaction. The mixture was incubated at 30 °C for 5 minutes. One unit (1 U) of MnP activity was defined as the amount of enzyme that oxidized 1.0 µmole of 2-methoxyphenol per minute at pH 4.5 and 30°C.

Xylanase activity assay: The xylanase activity was measured by the dinitrosalicylic acid (DNS method) described by Bailey *et al.*[36] using the DNS reagentdescribed by Miller[37]. The activity was determined by mixing 0.5ml crude enzyme extract with 0.5ml of Xylan from oat spelts (Sigma, Germany) (0.5% w/v) in 50mM citrate buffer (pH 6.0) and incubated at 50°C for 10min**.** Eight different concentrations (0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, and 1.6 mg/mL) of xylose were prepared (standard) in distilled water and treated the same way as the enzyme tubes. After incubation, 3ml DNSA reagent was immediately added to all the tubes and enzyme of 0.5 ml was added to the enzyme blank tube. The tubes were placed n boiling water bath for 10 minutes after which 5 mL of distilled water were added in all tubes and all the tubes were cooled to room temperature in water. The reducing sugars produced were measured at 540 nm on a Jenway UV-Visible spectrophotometer (Bibby Scientific Ltd, United Kingdom). One unit of enzyme activity was defined as the amount of enzyme releasing 1µmole of xylose equivalent per ml per min.

RESULTS

Spawn run, pinhead formation and fructification interval: The mycelia vegetative growth (spawn running) took an average of 21±3 days for *Pleurotus* HK 37 on saline solid sisal leaf decortications waste (SLDW) substrate while on *Panicum colorotum* L. the spawn run took 19±1 days. The primordial appeared on SLDW and *Panicum colorotum* L. after 6±2 and 3±2, while the flush intervals were 7 and 4 days respectively (Table 1).

Table -1: Days for completion of spawn running, pinhead and fruiting bodies formation

Substrate	Spawn Run (days)	Pinhead formation(days)	Flush Interval (days)
SLDW	21±3	6±2	7±3
Panicumcolorotum L.	19±1	5±1	6±1

Laccase activities during solid-state cultivation of *Pleurotus* HK 37 on SLDW: Laccase activity profile in this study was followed during solid state cultivation of *Pleurotus* HK 37 cultivation on sisal leaf decortications waste (SLDW). The enzyme activities in the sisal substrate as well as a control substrate (grass; *Panicum colorotum* L.) displayed a similar pattern with those in the control substrate having high activities throughout the period (**Figure 1)**. After 21 days of incubation (after full colonization of the substrates), the highest laccase enzyme activities were recorded at 27.3 U/mL in the sisal substrate and 37.1 U/mL in the control. In the subsequent analysis there was an increase in laccase activities during the subsequent primordial development followed by a decrease during the fruiting body development. The activities declined to 26.1 U/ml and 34.0 U/ml in the experimental and control respectively, when the first primordials were detected 84 hours after the substrate was moved to the fructification room. The activities during the first flush which was 7 days after full colonisation and 28 days from the day the substrates were spawned were 23.0 U/mL SLDW and 25.9 U/ml in the control. After the 3rd flush when the fruiting body production has declined, the activity had decreased to 15.0 and 26.4 U/ml in the SLDW and control experiments, respectively.

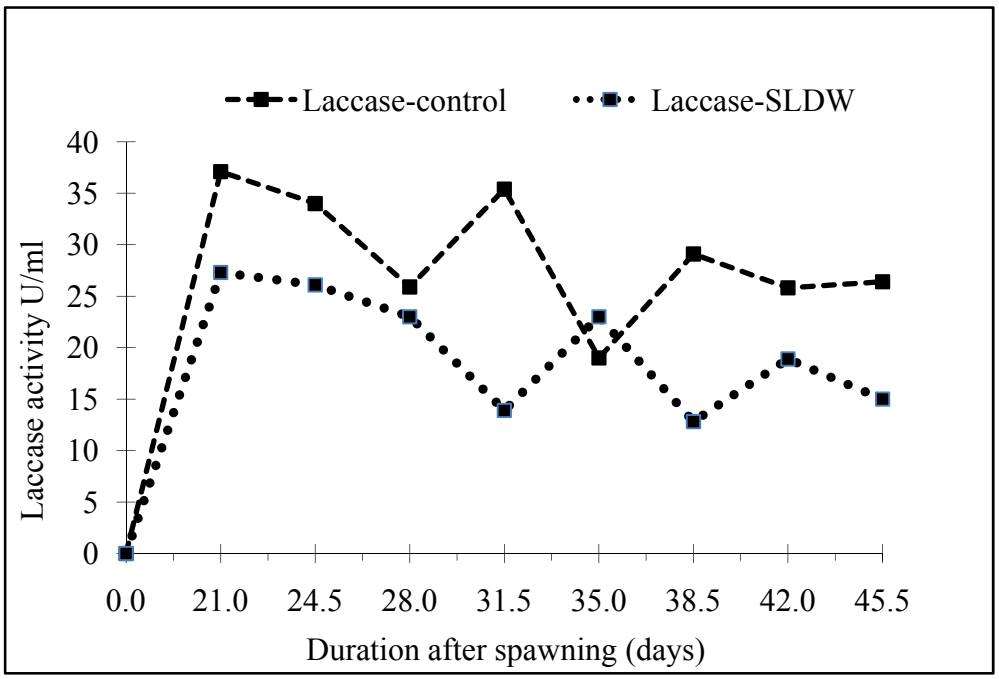

Figure 1: Laccase activity profiles by *Pleurotus* HK 37 at different stages of vegetative growth and fruit-bodies development during solid-state cultivation on sisal leaf decortications waste

Manganese peroxidase (MnP) activities during solid-state cultivation of *Pleurotus* HK 37 on SLDW
The evaluations of MnP activities **(Figure 2)** showed similar pattern in the two substrates with greater activity on the 21st day on full colonisation of the substrates and before the sexual stage of vegetative formation had began. A MnP activity of 8.9 U/ml and 14.4 U/ml in the experimental SLDW and control,

respectively were recorded. On the 84th hour after initiation of fruitification the first primordial/pinheads appeared and the enzyme activities decline to 2.2 U/mL and 8.1 U/mL in the SLDW and control, respectively, after the first flush. MnP enzymes activities increased during the vegetative stage of growth, immediately following the first flush to 8.0 U/ml in SLDW and 10.2 U/ml in the controls. This was followed by a decline in activities at the 3rd flush and 84 hours thereafter.

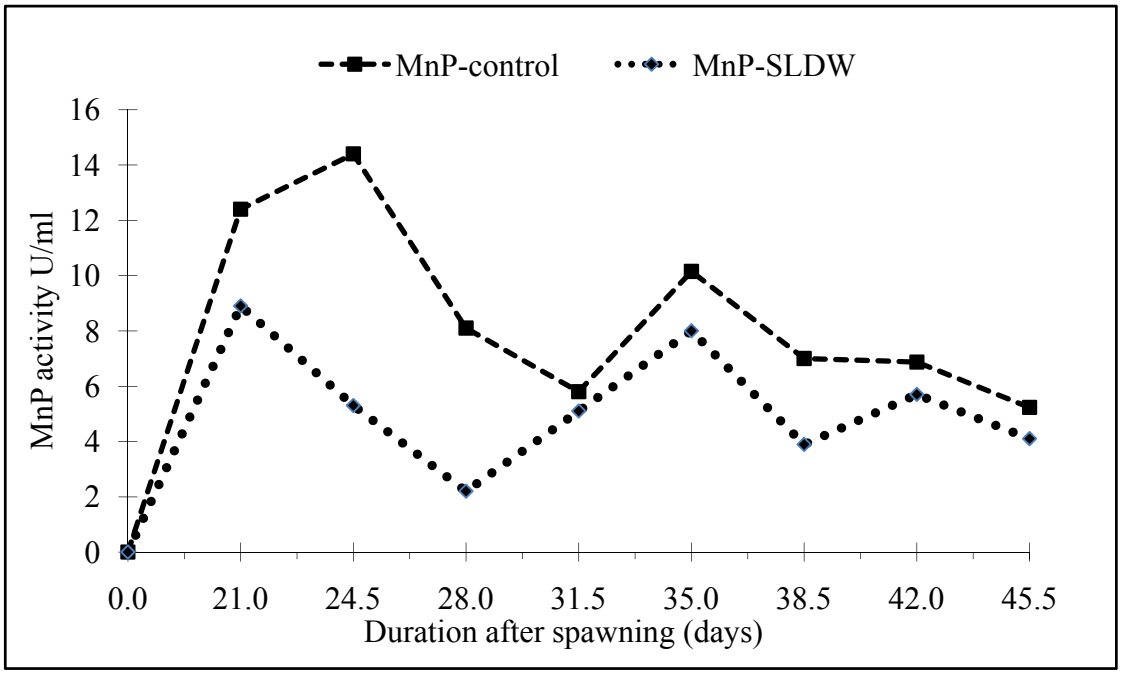

Figure 2: Manganese peroxidase activity profiles by *Pleurotus* HK 37 at different stages of vegetative growth and fruit-bodies development during solid-state cultivation on sisal leaf decortications waste.

Lignin peroxidase (LiP) activities during solid-state cultivation of *Pleurotus* HK 37 on SLDW: LiP activities at different stages of vegetative growth and fruit-bodies development during solid-state cultivation on sisal leaf decortications waste and a control experiment utilizing grass (*Panicum coloratum* L.) as control are illustrated in **Figure 3** below. The LiP activities were high in both substrates during the early stages of cultivation, and declined progressively with the progression. The highest activities were observed at the initial vegetative growth phase with 0.34 U/ml being recorded when the substrate was fully colonised after 21 days of incubation in the SLDW, while in the control experiment the LiP activity at 21 day of incubation was 0.88 U/mL. On appearance of the first primordial, the activities had declined to 0.11 U/mL and 0.10 U/mL in the SLDW and Control, respectively. There was an increase in the activity on the 28th day when the fruiting bodies had fully developed which continued to increase till the appearance of primordial's when the highest activity of LiP was recorded at 0.38 U/ml in the SLDW, while in the control there was a decline in activity to 0.43 U/mL. The LiP activity declined to 0.10 U/ml and 0.21 U/ml at the end of the 3rd flush on SLDW and the control substrate respectively.

Xylanase activities profile during solid-state cultivation of *Pleurotus* HK 37 on SLDW:*Figure 4* shows Xylanase activity profiles by *Pleurotus* HK 37 at different stages of vegetative growth and fruit-bodies development during solid-state cultivation on sisal leaf decortications waste as well in a control grass. The activities in both SLDW as well as grass showed an increase in activities from the start of the incubation of the substrates. The highest recorded activities were 0.045 U/ml and 0.028 U/ml for the Xylanase control and SLDW substrates respectively, at the 3rd flush for the control and immediately after the 3rd flush in the SLDW. The increase from the 1st day of incubation reached a steady state in the two

substrates when the activities for the enzyme in the control and SLDW were 0.041U/ml and 0.17U/ml, respectively on the 3rd day following the 2nd flush.

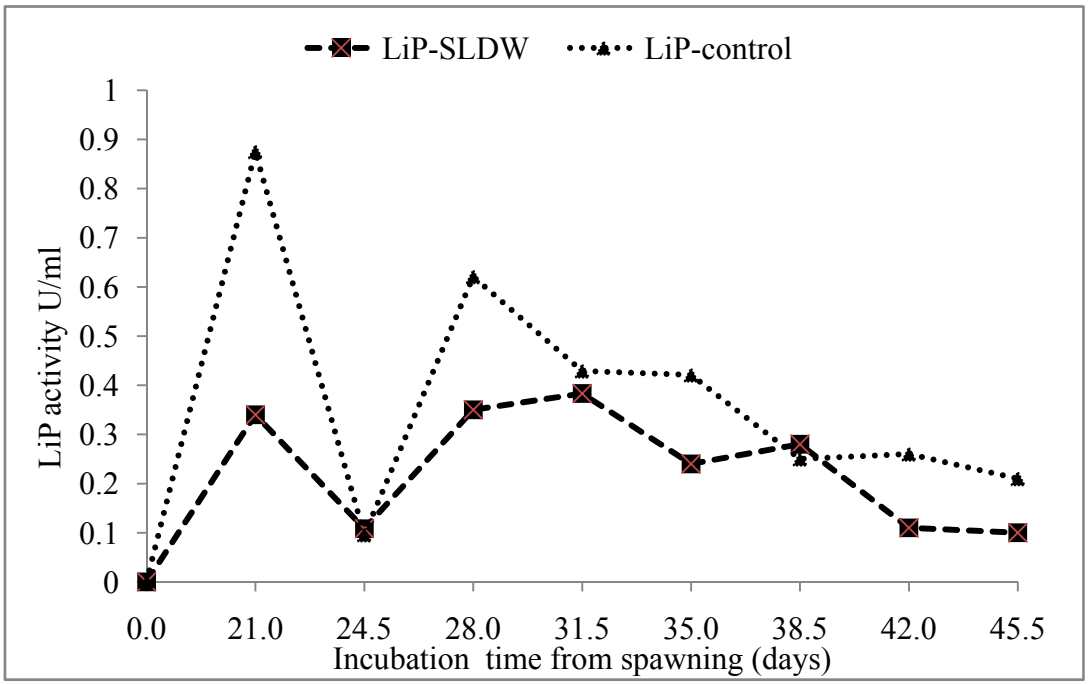

Figure 3: Lignin peroxidase activity profiles by *Pleurotus* HK 37 at different stages of vegetative growth and fruit-bodies development during solid-state cultivation on sisal leaf decortications waste.

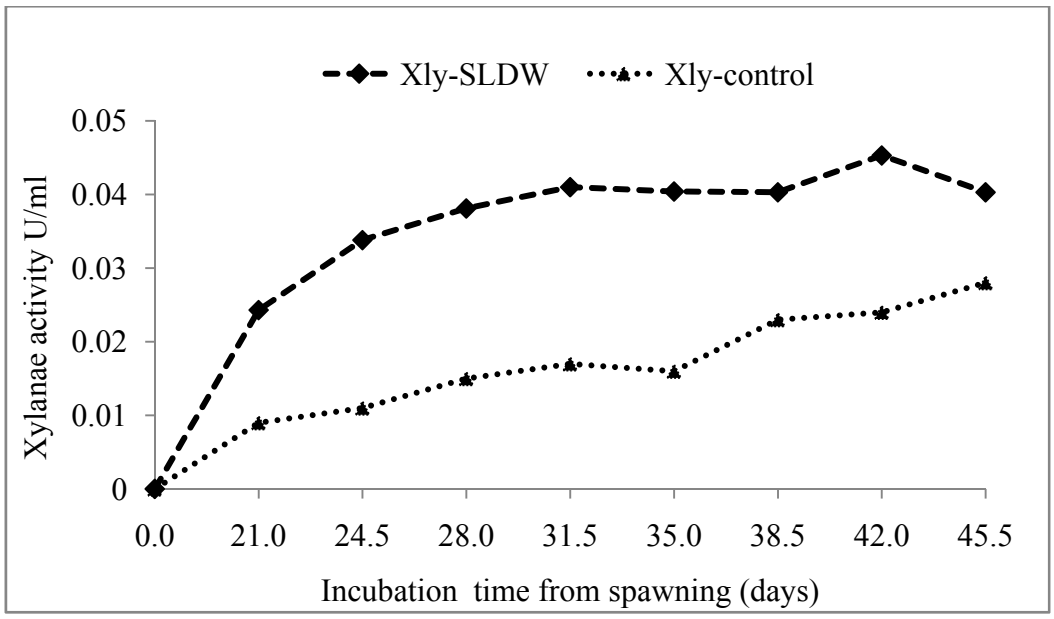

Figure 4: Xylanase activity profiles by *Pleurotus* HK 37 at different stages of vegetative growth and fruit-bodies development during solid-state cultivation on sisal leaf decortications waste.

DISCUSSION

Spawn run, pinhead formation and fructification interval: Substrate is one of the important parameter in mushroom cultivation as mushrooms depend on substrates for nutrition to support mycelia growth and development into mushroom fruiting bodies. For the growth and penetration of the mycelium into basal substrates, which ultimately influences fruiting of mushrooms, the structure and porosity levels of substrate are important factors to be considered. From the results obtained in this study, Table 1, mycelial run ranged between 18-24 days which was more or less similar 16-25 days of *Pleurotus ostreatus* reported by Shah *et al*.[38], working on wheat straw, leaves and saw dust. On the other hand, Baysal*et al*.[39] found the fastest spawn running (15.8 days) in waste paper as substrate. Tripathy *et al*.[40] and Mshandete[41], observed that the duration of different growth stages of cultivated mushrooms are affected by several factors which would include, but not limited to, type of substrates and supplements used, the type of species and/or the strain employed, spawn type and the rate of inocula/spawn applied, spawning method, spawning /cropping containers as well as on the prevailing mushroom growing conditions.

After completion of the spawn run the bags were moved to the cultivation room where there was higher light intensity. Primordial/pinhead formation was observed after 6±2 days in the substrate, the pinheads developed to mature fruiting bodies in 7±3 days, the subsequent 2nd and 3rd flushes followed 7±5 days thereafter. In general the first flush appeared in all the substrates after 26 days of inoculation with the longest 1st flush taking 42 days, similar results on *Pleurotus sp.* have been reported by Shah *et al*.[38].

Enzymatic activity during vegetative growth and fruiting of *Pleurotus* HK 37 on SLDW :The extracellular lignocellulolytic enzyme production from the genus *Pleurotus* has been reported in literature both in submerged cultivation on synthetic media[42] and in solid-state fermentation of lignocellulosic substrates[41]. The enzymes have been linked to fruiting body production in higher basidiomycetes but species appear to differ at timings of high enzyme production and there is no clear-cut picture on functional relevance of the enzymes in fruiting. The results obtained in this study show that the fruiting bodies grown on SLDW biodegrade the lignocellulose using extracellular degrading enzymes, which were assayed in the substrate. In this study, the activities of laccase (Figure 1), MnP (Figure 2) and LiP (Figure 3) were evidently high during the vegetative stage of growth of the mushrooms especially during the optimal colonization stage and decreased during the sexual stage (fructification) indicating that these enzymes play a major role in biodegrading the external nutrients in the lignocellulosic waste to be utilized for fruiting body formation in the asexual stage, where the laccase activity was low. Kalmis and, Sargi[43] working on *Pleurotus ostreatus* reported a similar pattern, and envisaged that, there existed an inverted correlation between laccase and manganese peroxidase activities and fruiting body maturation, with the minimum of activity occurring when the mushrooms were mature. Similarly, Elisashvili *et al*.[44] reported that, the production of ligninolytic enzymes increases with vegetative biomass production on solid growth substrates and that it drops during the sexual fruiting stage an observation that is in agreement with the results of this study.

Theses enzymes are extracellular which are excreted into the substrate for the fungi to utilize the lignocellulosic substrates. Lignocellolytic enzymes in *P. ostreatus,* is postulated in literature to be produced for vegetative hyphal growth but not for fruiting body development. Lechner*et al*.[45] observed increased enzymatic activites in *Lentinus tigrinus* during the growth phase and drastically low enzyme activities during fruiting body formation period an observation reported as well by Xing *et al*.[46] working with *Grifola frondosa*. These species, likely depend on the enzymes produced for lignocellulosic substrate degradation in order to provide nutrients for the growing organism. Over two decades ago, Wood and Goodenough[47], reported that, in *Agaricus bisporus*, laccase and peroxidase activities increased in the substrate from vegetative growth to early stages of fruiting body development and drop drastically during fruiting body maturation. However, in the straw fungus *Volvariella volvacea,* it has been

observed that, the laccase activities increase immediately after vegetative growth at fruiting body initiation where the activities remain high although the fruiting are mature enough for harvest[48]. The biological function of laccases and peroxidases in fruiting body development has not been documented and still remain unclear[49].

In this study the results of xylanase production (Figure 4) showed a similar production pattern in both the experimental and the control using grass. The activities in SLDW, showed in gradual increase from incubation with a highest activities being recorded after 42 days. These results are in agreement with earlier previous reported findings. Elisashvili *et al*.[43] reported, *P. ostreatus* xylanase activities gradually increased during 49 days (second fruiting stage) of mushroom cultivation and then decreased. Sherief*et al*.[50] reported an early increase in xylanase production by *P. ostreatus* cultivatedon sawdust and rice straw with the highest activity being recorded after 20 and 40 days of incubation in the substrates, respectively. While earlier studies by Garzillo *et al*.[51], xylase activities of *P. ostreatus* in wheat straw were detected after 16 days. Matsumoto[52], working on *L. edodes* cultivated on eucalyptus sawdust observed xylanase activities increase during the development of the fruiting bodies, with highest levels during mushroom maturation. The increase in the enzyme activities during fructification has been reported to be due to the fungus's need to mobilize large amounts of carbon for mushroom formation[53]. The results of xylanase pattern in this study are correlated with the results obtained by Isikhuemhen and Mikiashvilli[54] and Sherief *et al*.[50], who observed higher xylanase productivity from *P. ostreatus* in fruiting than mycellial growth period when cultivated on solid wastes, moreover, different lignocellulosic portray varying enzyme activity pattern and their productivity which confirms supplementation of the substrate during *Pleurotus* HK 37 cultivation could increase the N source hence the enzyme activities.

CONCLUSION

The present study indicates that it is feasible to use sisal leaf decortications waste as raw materials for the production of fruit bodies and some enzymes by a commercial fungus *Pleurotus* HK 37. The results show the importance of further research on the ligninolytic enzymes to optimize both their production as well as their activities for potential application in biotechnological processes. Moreover, cultivation of the mushroom is an effective method for the production of nutritional food in addition to offering a holistic approach to agro waste management as well as additional revenue to the sisal producing countries.

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