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Evaluation of Enzymatic Activity during Growth of *Pleurotus* HK 37 on *Saba comorensis* Exocarp

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Abstract

Mushrooms degrade lignocellulosic biomass by releasing lignolytic and hydrolytic enzymes which convert lignocellulosic material into soluble and low molecular weight compounds which are then absorbed as nutrients. In the present study, enzymatic activities of *Pleurotus* HK 37 during growth on *Saba comorensis* exocarps were evaluated. It was observed that, *Pleurotus* HK 37 has ability to produce lignolytic enzyme (Laccase) and hydrolytic enzymes (Carboxymethyl cellulase, xylanase and filterpaperase). Maximum laccase activity of 3.33 ± 0 UmL^{-1} was observed during colonization period and the activity dropped during fruitification phase. Similar to hydrolytic enzymes, the activity was observed during colonization period and decreased during fruitification. However, higher filterpaperase activity of 0.93 ± 0.13 UmL^{-1} was observed compared to other hydrolytic enzymes (CMCase 0.78 ± 0.13 UmL^{-1}, and Xylanase 0.56 ± 0.07 UmL^{-1}). *Pleurotus* HK 37 showed ability to degrade *Saba comorensis* exocarps and to release enzymes which can be used in biotechnological industries.

Keywords: Mushroom, Lignolytic, Hydrolytic, Enzyme, *Saba comorensis*

Introduction

Pleurotus species produce oyster-shaped mushrooms (basidioma), hence they have been called oyster mushrooms (Patel et al. 2012). Oyster mushrooms usually grow on temperature range of 12–32 ℃ (Zadrazil 1980), thus their flexibility in temperature and environmental conditions make it easy for cultivation (Ragunathan et al. 1996). Cultivation of *Pleurotus* species has grown due to its nutritional values, medicinal properties, and other beneficial effects (Gregori et al. 2007). *Pleurotus* HK 37 is among oyster mushrooms mostly cultivated in Tanzania (Kivaisi 2007). This mushroom contains important nutritional components and has high antioxidant power (Muthangya et al. 2014). The *Pleurotus* species have ability to grow on different types of agricultural wastes as substrates (Ragunathan and Swaminathan 2003). There are varieties of agricultural wastes such as rice straws, banana leaves, maize cobs, and cotton seeds that can be used as substrates for mushroom cultivation (Mamiro and Mamiro 2011). Also, the use of agro industrial wastes such as sisal waste products can be used in mushroom cultivation (Kivaisi et al. 2010). Most of agricultural and agro-industrial wastes contain lignocellulose as structural element of plants.

Pleurotus species are known for their ability to degrade lignocellulosic waste. Mushrooms are adapted to grow and produce fruits on a wide variety of agro-industrial and agricultural lignocellulosic wastes due to

capability of synthesizing hydrolytic and lignolytic extracellular enzymes (Mikiashvili et al. 2006). Mushroom mycelia release enzymes that digest substrates by modifying the insoluble and low molecular weight compounds and subsequently absorb these compounds for their nourishment and growth (Singhania et al. 2009, Swamy 2015). Typical enzymes produced by mushrooms include; ligninolytic enzymes such as laccase, manganese peroxides and lignin peroxide which are involved in the degradation of lignin and their natural lignocellulosic materials. Likewise, hydrolytic enzymes such as cellulase, laminarinases, xylanase and carboxymethyl cellulase are also produced (Mata et al. 2016). Also, Raymond et al. (2015) reported that *Pleurotus* HK 37 has ability to degrade lignocellulosic materials by releasing lignolytic enzymes and hydrolytic enzymes.

Laccases are the lignolytic enzymes which are richly released by the fungi (Baldrian 2006). They are usually involved in lignin degradation (Ander and Eriksson 1976). Structurally, laccases contain four copper ions per molecule. Lacasses are known for their ability to carry out electron oxidation of phenolic and aromatic compounds (Gianfreda et al. 1999). Laccases play an important role in biotechnological industrial activities such as food processing, paper and pulp processing, textile processing, synthetic chemistry, cosmetics, and environmental bioremediation of organic pollutants (Couto and Herrera 2006).

In the context of mushroom cultivation, hydrolytic enzymes (cellulases and hemicellulases) are known and are responsible for polysaccharide degradation (Kumla et al. 2020). Cellulases are involved in degradation of cellulose; classes of cellulase enzymes involved in degradation of cellulose include; carboxymethyl cellulase (endoglucanase), exoglucanase and glucosidase. CMCase convert cellulose into oligosaccharides then exoglucanase separate cellobiose into glucose units (Iqbal et al. 2010). Filterpaperase enzyme (FPA) is mainly used to determine total cellulase activity (Kumla et al. 2020). Hemicellulases include xylanases, α-arabinofuranosidase and α-glucuronidase (Farani de Souza et al. 2006). Xylanases involve degradation of xylan into xylose unit where xylan is the major component of hemicellulose (Van Gool et al. 2011).

Those enzymes are produced in different phases of mushroom growth. Raymond et al. (2015) reported that lignolytic enzymes are released at somatic stage and decrease during fruiting phase. Singh et al. (2011) reported that laccase degrades lignin so that more cellulose and hemicellulose are made available for cellulase to act on.

Different studies have evaluated various lignolytic and hydrolytic enzymes produced by *Pleurotus* species on different lignocellulosic wastes, but there is little information on *Saba comorensis* exocarp used to explore different hydrolytic and lignolytic enzymes. The aim of this study was to evaluate hydrolytic enzymes and lignolytic enzymes produced by *Pleurotus* HK 37 on *Saba comorensis* exocarp.

Materials and Methods

Saba comorensis fruits (Figure 1) were collected in various local markets in Dar es Salaam between December 2020 and March 2021. The fruits were brought to the laboratory for processing to obtain the exocarps.

Figure 1: *Saba comorensis* fruit.

Sample preparation

The pulp and seeds were removed to remain with only the exocarps which were chopped into small pieces of 1–3 cm. Exocarp pieces were dried in shade for seven days and were divided into two sets. One set (regarded as unsoaked) was soaked into water

for 5 minutes to moisturize the substrate. Another set (regarded as soaked) was soaked into water for 5 days whereby on each day, water was changed to avoid fermentation of substrate. On the fifth day, the mixture on this set was filtered and the exocarp was used for mushroom cultivation.

Preparation of fungi mycelia

The fruit body of *Pleurotus* HK 37 was obtained from the University of Dar es Salaam, Department of Molecular Biology and Biotechnology. Tissue culture was maintained on malt extract agar (MEA). Spawn was prepared by using sorghum grains obtained from local markets in Dar es Salaam. The sorghum grains were washed into water and were then boiled until soften, and then grains were filtered prior to being sun dried for 20 to 30 minutes. The dried sorghum grains were put in wide mouth bottles, and were then autoclaved at 0.15 MPa and 121 ℃ for 15 minutes. Colonized mycelia were chopped into pieces of 1 cm long and inoculated into bottles containing sorghum grains and then incubated for 8 days for colonization to take place.

Saba comorensis exocarp weighing 20 g of both soaked and unsoaked were put into 150 mL conical flask. Used conical flasks with *Saba comorensis* exocarp were autoclaved at 0.15 MPa and 121 ℃ for 15 min and left to cool. Sorghum grains covered with 70% mycelium of 5–8 g were inoculated in 15 conical flasks with sterilized unsoaked exocarp and other 15 conical flasks with sterilized soaked exocarp then both incubated in dark. The samples for enzyme analysis were taken at the interval of 7, 14, 21, 28 and 35 days during the mushroom growth and were arranged in triplicates for each day.

Enzyme extraction

Phosphate buffer (pH 7, 0.01 M) was used for extraction to obtain crude enzyme. About 50 mL of buffer was added in conical flask containing substrates colonized with mycelia, then was shaken using a mechanical shaker (Edmund Buhler, 7400 Tubingen, SM 25) at 125 rpm for 30 min. Soon after shaking, the crude enzymes were filtered using clean cotton cloth. Obtained crude enzyme was centrifuged (Hettich Zentrifugen, Universal 320 R) at 4000 rpm, 4 ℃ for 10 min, supernatant (crude enzyme) obtained was used for enzyme analysis according to Risdianto et al. (2010).

Enzyme analysis

One lignolytic enzyme (laccase) and three hydrolytic enzymes (carboxymethyl cellulase, filterpaperase and xylanase) were analysed. Laccase was determined by adding 100 µL of crude enzyme and a 100 µL of 1 mM 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS) to 100 µL 0.05 M sodium acetate buffer (pH 5.5), then the mixture was incubated for 25 minutes at 30 ℃. The absorbance was measured using spectrophotometer at 420 nm. One unit of laccase activity was defined as the amount of enzyme required to oxidize 1 µmol of ABTS per min at 30 ℃. Enzyme activity was calculated by the following formula:

$$U\ mL^{-1} = \frac{\Delta A}{t * \varepsilon * d}$$

Where: U = enzyme unity, ΔA = change in absorbance, t = time, D = path length 1 cm and ε = molar extinction coefficient.

Carboxymethyl cellulase (CMCase) was determined by 3,5-dinitrosalicylic acid (DNSA) method according to Miller (1959). The amount of reducing sugar was measured by adding 0.5 mL of crude enzyme with 0.5 mL of carboxymethyl cellulose and 0.5 mL 0.1 M citrate buffer (pH 5) then incubated for 60 min at 37 ℃ in water bath. Immediately after removal from the water bath, DNSA was added to stop the reaction and was then incubated at boiling water for 5 min followed by addition of 10 mL of distilled water to all test tubes. Change in colour from yellow to orange red was observed at 540 nm. One unit of CMCase activity was defined as the amount of enzyme required to reduce 1 µmol of carboxymethyl cellulose per min at 37 ℃.

Filterpaperase was determined by 3,5-dinitrosalicylic acid (DNSA) method according to Miller (1959). The amount of reducing sugar was measured by mixing 0.5 mL of crude enzyme with 1 x 6 cm Whatman

filter paper no 1 and 0.5 mL 0.1 M citrate buffer (pH 5). This mixture was then incubated for 60 min at 37 ℃ in a water bath. Immediately after removal from the water bath, DNSA was added to stop the reaction, and the mixture was then incubated at boiling water for 5 min followed by addition of 10 mL of distilled water to all tubes. Change in colour from yellow to orange red was observed at 540 nm. One unit of Filterpaperase activity was defined as the amount of enzyme required to reduce 1 µmol of filter paper per minute at 37 ℃.

Xylanase was determined by 3,5-dinitrosalicylic acid (DNSA) method according to Miller (1959). The amount of reducing sugar was measured by adding 0.5 mL of crude enzyme with 0.5 mL of xylan and 0.5 mL 0.1 M citrate buffer (pH 5) then incubated for 60 min at 37 ℃ in water bath. Immediately after removal from the water bath, DNSA was added to stop the reaction and mixture was then incubated at boiling water for 5 min. Thereafter, 10 mL of distilled water was added to all tubes. Change in colour from yellow to orange red was observed at 540 nm. One unit of xylanase activity was defined as the amount of enzyme required to reduce 1 µmol of xylan per min at 37 ℃. Hydrolytic enzyme activity was calculated by using the following formula:

$$U/ml = \frac{Vg}{t * v} * DF$$

Where: U = enzyme unity, Vg = µmoles glucose liberated, t = time used, v = volume of enzyme (mL) and DF = dilution factor.

Results

Saba comorensis exocarps waste for the first time was used in the determination of enzymatic activities during growth of *Pleurotus* HK 37 mushrooms. After thirty five days of incubation of exocarps with mycelia, there was a difference in rates of colonization between the soaked and unsoaked exocarps. In soaked exocarps, mycelia colonized the whole exocarps quicker than in unsoaked exocarps where there was delay on mycelia colonization. Enzymes were analysed during colonization stage from first day of incubation to 25th day and fruitification stage from 25th day to 35th day of incubation.

Laccase activities in soaked exocarps and unsoaked exocarps showed different trends as shown in Figure 2. Maximum enzyme activity was on the 21st day where soaked exocarps released higher activity of 3.33 ± 0.0 U mL^{-1} than unsoaked as shown in Figure 2 during colonization. During fruitification, the enzyme activity dropped. But for unsoaked exocarps on the 35th day, laccase activity shoot to 2.95 ± 0.08 UmL^{-1}, and this may have been caused by the start of second growth phase. On soaked exocarps, laccase activity on day 7 was 1.7 ± 0.22 UmL^{-1}, while on unsoaked exocarps there was low laccase activity of 0.08 ± 0.12 UmL^{-1}. Laccase activity for the soaked exocarps was increasing form day 7 to 21st day. The enzyme activity was the highest during colonization and the activity started to decrease on 28th day from 1.5 ± 0.34 UmL^{-1} to 1.36 ± 0.37 UmL^{-1} on the 35th day during fruitification.

Hydrolytic enzymes showed similar trends in soaked and also in unsoaked exocarps. In soaked exocarps, hydrolytic enzymes were low compared to unsoaked exocarps as shown in Figures 3, 4 and 5. The trend of CMCase activity for unsoaked exocarps was decreasing from 7th to 35th days of incubation, thus during colonization there was higher CMCase activity than during fruitification stage. CMCase activity was maximum on 7th day of inoculation with 0.78 ± 0.13 UmL^{-1} for unsoaked exocarps and minimum CMCase activity was shown on 35th day 0.03 ± 0.003 UmL^{-1}. Maximum CMCase activity was 0.06 ± 0.3 UmL^{-1} on 7th day and minimum CMCase activity was 0 UmL^{-1} on 14th day for the soaked exocarps.

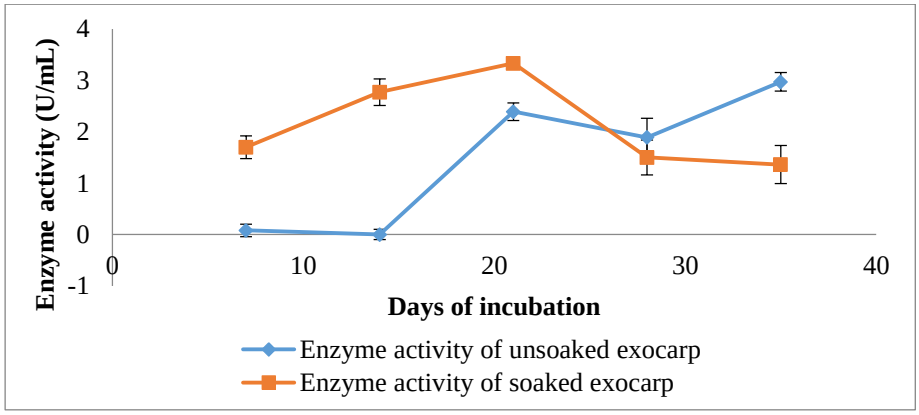

Figure 2: Laccase activities of unsoaked and soaked exocarps. The amount of laccase increased and reached maximum at twenty-one days for soaked exocarps. Unsoaked exocarps showed maximum increase during 35 days. Error bars show standard deviations.

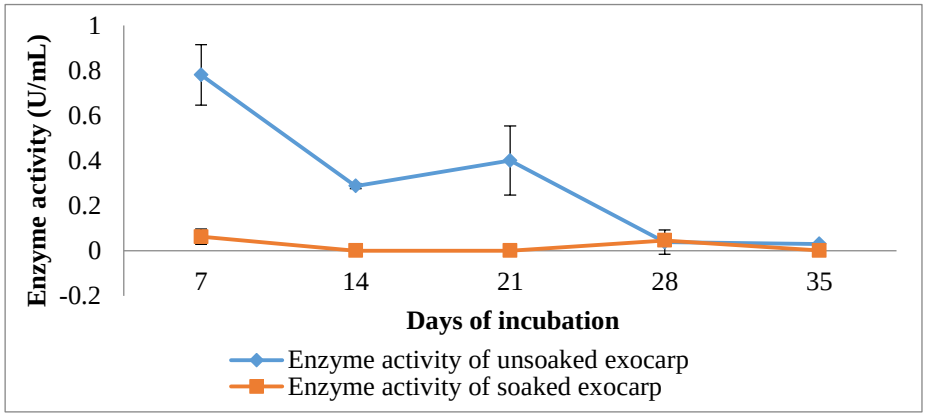

Figure 3: CMCase activities of unsoaked and soaked exocarp. Higher CMCase activity was observed on unsoaked exocarps from day seven and decrease as day of incubation increase. Low CMCase activity observed on soaked exocarps. Error bars represent standard deviations.

Xylanase activity was 0.56 ± 0.07 UmL^{-1} on 7th day of inoculation and minimum was 0.07 ± 0.11 UmL^{-1} by 28th day as shown in Figure 4 for unsoaked exocarps. Similarly, for soaked exocarps there was low amount of xylanase enzyme as it was on CMCase activity. Maximum xylanase activity was 0.09 ± 0.04 UmL^{-1} on 21st day and minimum was 0.006 ± 0 UmL^{-1} by 35th day for soaked exocarps. Also, this shows that during colonization, higher amounts of enzyme are released to degrade lignocellulosic waste.

Filterpaperase showed maximum activity of 0.93 ± 0.13 UmL^{-1} on 14th day of inoculation and minimum activity of 0.02 ± 0.001 UmL^{-1} by 35th day for unsoaked exocarps. Filterpaperase activity increased from 0.574 ± 0.10 UmL^{-1} on 7th day to 0.93 ± 0.13 UmL^{-1} on 14th day, after that there was a sharp decrease on filterpaperase activity up to the 35th day (Figure 5). Maximum enzyme activity was slightly higher than for other hydrolytic enzymes. Also, the filterpaperase showed the trend as for other hydrolytic enzymes, which was higher during colonization and low during fructification.

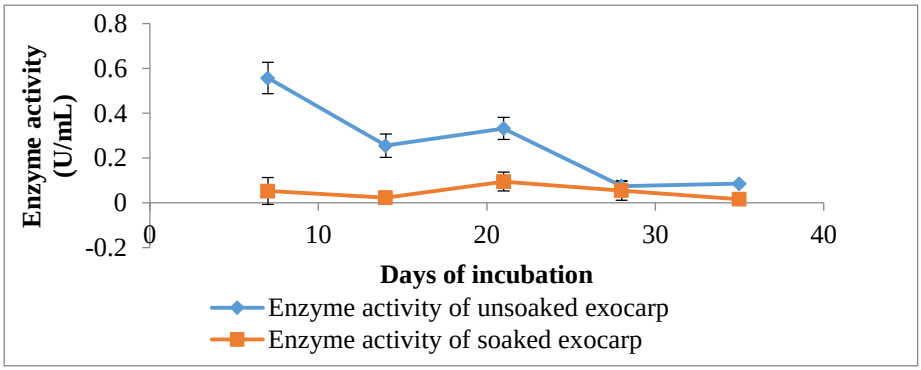

Figure 4: Xylanase activities of unsoaked and soaked exocarp. Xylanase activity was low on soaked exocarps. Decrease of xylanase activity as days of incubation increase was observed. Error bars represent standard deviations.

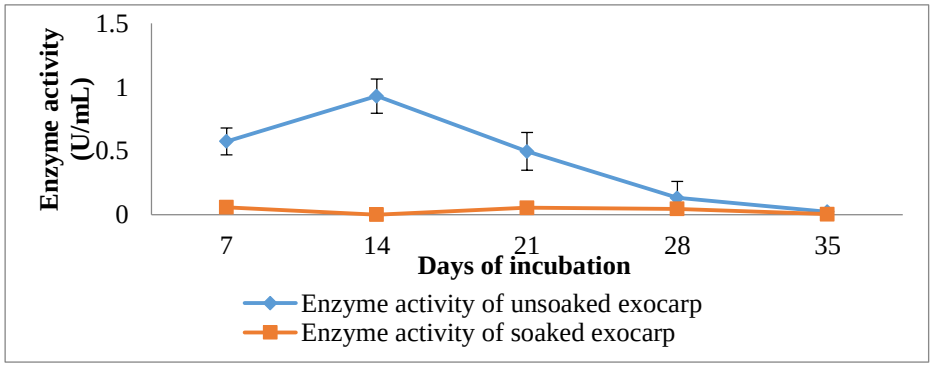

Figure 5: Fpase activities of soaked and unsoaked exocarp. Maximum Fpase activity was on 14th day and the activity was decreasing as days of incubation increased for unsoaked exocarps. Soaked exocarps showed low Fpase activities. Error bar represent standard deviations.

Discussion

The change of substrates composition and mushroom species are influential in enzyme production (Kumla et al. 2020). *Saba comorensis* exocarps wastes have for the first time been investigated for their potential uses in enzyme production by mushrooms. Parallel to the current study, proximate analysis was conducted on the exocarps and exocarps, which were found to contain a carbon to nitrogen ratio (C/N ratio) of 50 and 15 for soaked and unsoaked exocarps (Molobele 2022). This C/N ratio for the soaked exocarps is good for the mushroom colonization and growth unlike for the unsoaked exocarps (Osunde et al. 2019, Ma et al. 2021).

Different rates of colonization were observed for soaked and unsoaked exocarps. In soaked exocarps, there was fast colonization; this was due to availability of nutrients compared to unsoaked exocarps on which there was a delayed colonization of mycelia. De Siqueira et al. (2012) reported that, soaking of substrates is important because it tends to reduce anti nutritional factors. Moreover, Elmaki et al. (2007) emphasized on the importance of substrate soaking as it enhances nutritive values. On the unsoaked exocarps, delayed mycelia colonization could have been brought by presence of some chemicals which inhibit its colonization. For example, it was observed by Salmones et al. (2005) that, complex

chemical compositions of coffee pulp impede its efficient conversion to mycelia.

The laccase activity found in the present study is similar to what was reported by Kurt and Buyukalaca (2010), where a laccase activity on 10th day after incubation for sesame straw with bran was found to be 3.85 Umg^{-1} and wheat straw with bran was 2.28 Umg^{-1} for *Pleurotus sajor-caju*. However, Reddy et al. (2003) reported maximum laccase activity on banana leaves at 1.7106 Umg^{-1} which is slightly low compared to that of the present study. Present results show that, during colonization there was increase in laccase production, and this enzyme declined during fruitification. Raymond et al. (2015) and Muthangya et al. (2013) also reported on the similar trend of enzyme production between colonization and fruitification phases. Studies by Risdianto et al. (2010) reported that, laccase enzyme activity starts to appear after 2–5 days of incubation and that, laccase production depends on carbon and nitrogen source and their concentrations in medium and therefore, nutrients limitations trigger laccase production (Stajić et al. 2006).

Hydrolytic enzymes tested in this study showed a similar trend. For the unsoaked exocarps, the maximum activity was observed during colonization and was shown to be minimal during fruitification stage. However, this is contrary to what was reported by Singh et al. (2011) and Mata et al. (2016) who observed hydrolytic enzyme activities to be low during vegetative phase and high during fruit body formation.

The production of lignocellulolytic enzymes depends on the amounts of lignin present in the substrates (Ullah et al. 2018). Enzyme profiles differ depending on mushroom species, size of waste used and nature of substrate. CMCase activity observed in present study is almost similar to what was reported by Buswell and Chang (1994). Also CMCase activity from the present study is not different to what was reported by Inácio et al. (2015) whose study was on orange peel waste. The decrease in CMCase activity during fruitification could be caused by the increase of glucose concentration, which may be regarded as an end product of complex sugar metabolism, thus inhibiting the CMCase activity by end product inhibition mechanism. Thus, the production of CMCase is stimulated by low glucose level, but is inhibited by presence of high glucose concentration in the substrate.

In this study, there was a record of maximum xylanase activity on the 7th day of inoculation similar to what was reported by Mata et al. (2016) who showed a maximum activity during the first seven days, and also by Raymond et al (2015), who reported highest xylanase activity during colonization stage to 30 days which declined during primordia and fruit body formation. Dolma et al. (2014) reported slightly higher xylanase activity than what was found in present study. Fpase compared to other hydrolytic enzymes, showed higher maximum activity similar to what was reported by Choudhary et al. (2009). The CMCase also cleaves β-1,4 linkage of cellulose just like the Fpase (Dolma et al. 2014).

Conclusion

This study has shown that *Pleurotus* HK 37 has an ability to degrade *Saba comorensis* exocarp and produce hydrolytic and lignolytic enzymes. However, further studies are recommended to show how these enzymes can be produced in large scale for industrial uses.

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