Lignocellulolytic enzyme activity pattern of three white oyster mushroom (*Pleurotus ostreatus* (Jacq.) P. Kumm.) strains during mycelial growth and fruiting body development

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Abstract. *Pleurotus ostreatus* is one of the edible mushrooms that can utilize lignocellulose as substrate because of their ability to secrete the lignocellulolytic enzyme. The purpose of this research is to investigate and compare the lignocellulolytic enzymes production of *P. ostreatus* InaCC F209, F216 and LIPI on solid-state fermentation using sawdust during 70 days of mycelial growth (vegetative phase) and fruiting body development (reproductive phase). Supernatant of the extracted enzyme solutions were employed to investigate the reducing sugar, soluble protein, and enzyme activities. The results revealed that reducing sugar concentration of the three *P. ostreatus* strains subjected increased during growth and reached the maximum concentration on the reproductive phase, while the total protein content fluctuated during the growth but reached the maximum concentration on the reproductive phase. Laccase, LiP, and MnP activities of three *P. ostreatus* strains were higher on the vegetative phase, while the endoxylanase and endoglucanase activities were higher on the reproductive phase. β-glucosidase activity showed different variations between three *P. ostreatus* strains. *Pleurotus ostreatus* InaCC F209 produced the highest and most stable laccase, β-glucosidase, endoglucanase, and endoxylanase than two others.

Keywords: Lignocellulolytic enzymes, *Pleurotus ostreatus* InaCC F209, reproductive phase, vegetative phase

1. Introduction
Edible mushroom production in the world increased by 56 % for the last ten years (1997–2007). *Pleurotus ostreatus* is one of the edible fungi with the second largest production rate in the world after *Lentinula edodes* because it has high nutrient content and active compounds such as anticancer, antioxidant, antitumor, antidiabetic and antibacterial. These fungi belong to the Basidiomycota phylum and are classified as white rot fungi due to their potential to utilize lignocellulose as substrate [1, 2].

Lignocellulose itself is defined as the constituent of plants consisting of three major polymers, namely cellulose, hemicellulose, and lignin. Cellulose is the main component of lignocellulose and is composed of the cellobiose unit. Hemicellulose is a heteropolymer composed of xylan as its backbone and takes a part in the binding cellulose fibers to microfibrils. Lignin is composed of phenylpropanoid
units and takes a part in providing strength to the plant cell walls, as well as resistance to insects and pathogens. Lignocellulose is known to be difficult to degrade due to the crystallinity of cellulose, lignin hydrophobicity, and encapsulation of cellulose by the lignin-hemicellulose matrix [3, 4].

White rot fungi, including Pleurotus ostreatus, can degrade the lignocellulose because of its ability to secrete lignin oxidizing enzymes and also holocellulose hydrolyzing enzymes. Based on the degraded substrate, lignocellulolytic enzymes can be categorized into three groups, namely the ligninase, hemicellulase, and cellulase. Ligninase is a lignin oxidizing enzyme and consists of laccase enzymes, manganese peroxidase (MnP), and lignin peroxidase (LiP). Unlike ligninase, hemicellulase is a hemicellulose hydrolysis enzyme and can be divided based on its constituent polymers such as xylanase. Cellulase is a cellulose hydrolysis enzyme and based on chain-breaking ability, it can be divided into endoglucanase, exoglucanase, and ß-glucosidase [5].

The lignocellulolytic enzymes production by P. ostreatus is affected by several parameters, namely the growth and formation of fruiting bodies, as well as the differences in strains. Eriksson et al. [6] reported that ligninolytic enzyme (laccase and manganese peroxidase) activity of P. ostreatus during the growth period of mycelia is higher than that of the fruiting body. This is because lignin needs to be degraded first so that hyphae can get an access to break down cellulose and hemicellulose. In addition, differences between strains can also affect the production of lignocellulolytic enzymes [7]. Luz et al. [8] reported that two P. ostreatus strains (PLO2 and PLO6) produced different amount of lignocellulolytic enzymes where P. ostreatus PLO6 has a higher enzyme activity than P. ostreatus PLO2.

Pleurotus ostreatus InaCC F209 and F216 are isolates collected by Indonesian Culture Collection (InaCC) that have never been studied, while P. ostreatus LIPI is an isolate of P. ostreatus collected by Laboratory of Food Microbiology, Indonesian Institute of Science that is often to be cultivated. However, lignocellulolytic enzyme production of these three different strains has yet to be explored.

The purpose of this research is to investigate and compare lignocellulolytic enzymes, such as ß-glucosidase, endoglucanase, endoxylanase, laccase, lignin peroxidase (LiP), and manganese peroxidase (MnP), production of P. ostreatus InaCC F209, F216 and LIPI during mycelial growth (vegetative phase) and fruiting body development (reproductive phase).

2. Method

2.1. Fungal strain and culture condition

This research used three strains of Pleurotus ostreatus that are the collection of Indonesian Culture Collection (InaCC) (P. ostreatus InaCC F209 and F216) and the collection of Laboratory of Food Microbiology, Research Center for Biology, Indonesian Institute of Sciences.

2.2. Cultivation of P. ostreatus

The cultivation process was done on a substrate supplemented by sawdust (83 % ; w/w), calcium carbonate (2 % ; w/w), calcium sulfate (2 % ; w/w), cracked corn (2 % ; w/w), rice bran (11 % ; w/w), and water (2 % ; v/w). The substrate was weighed for 1 kg and packed in polypropylene bags. Each bag was sterilized, inoculated with mother spawn of P. ostreatus InaCC F209, F216 and LIPI, then incubated for 28 days before moved to a fruiting room for induction of fruiting body development.

2.3. Measurement of reducing sugar, protein and enzyme activity

Crude extract enzyme was obtained by adding 50 mL of aquadest supplemented with Tween 80 (0.1 % ; v/v) and NaCl (1 % , v/v) to 10 g of substrate. The mixture was shaken at 150 rpm for 10 min, filtered, and centrifuged at 9,500 rpm for 20 min at 4 °C. The supernatant was stored at 4 °C and used to measure the reducing sugar concentration, total protein content, and enzyme activities. The measurement was done every seven days for 70 days. Reducing sugar was measured using the DNS method, while the dissolved protein in the samples was measured using the Bradford method. The method of measuring enzyme activities (table 1).
Table 1. Substrates, environmental conditions, and reagents profile for measuring the activity of the lignocellulolytic enzymes.

| Enzyme     | Substrate | Reagent | pH     | Incubation period | References |
|------------|-----------|---------|--------|-------------------|------------|
| Laccase    | ABTS      | -       | pH 5.0 | 30 minutes        | [9]        |
| LiP        | Veratryl alcohol | -       | pH 3.0 | 1 minute          | [10]       |
| MnP        | Guaiacol  | -       | pH 4.5 | 1 minute          | [11]       |
| Endoxylanase | Xylan   | DNS     | pH 5.3 | 10 minutes        | [12]       |
| Endoglucanase | CMC    | DNS     | pH 4.8 | 50 minutes        | [13]       |
| β-glucosidase | pNPG   | -       | pH 5.0 | 15 minutes        | [14]       |

3. Results and discussion

Three strains of *Pleurotus ostreatus* were successfully cultivated on sawdust as the lignocellulosic source. The mycelia of each strains filled the entire bag after 28 days, thus the induction of fruiting body development began. The formation of fruiting body *P. ostreatus* InaCC F209 could be observed 49 days after inoculation and isolate *P. ostreatus* LIPI could be observed 54 days after inoculation, while *P. ostreatus* InaCC F216 was unable to form any fruiting body during 70 days of growth.

The concentration of reducing sugars and soluble protein for 70 days of growth can be seen in figure 1. Reducing sugar was measured in order to see the fungal ability to use lignocellulose as substrate. It is shown that the concentration of reducing sugars of each strain increased during the mycelial growth period and reached the maximum value during the fruiting body development period. The increase of glucose could be related to the increase of glucose consumption which is due to fungi’s needs of larger amounts of energy to form fruiting body [15].

Total protein content was measured to examine the growth of *P. ostreatus* based on soluble protein in the media. The increase of total protein content during growth could be caused by biotransformation of carbohydrates into protein, enhancement of fungal biomass, and the ability of fungi to secrete the enzymes [16]. In general, microorganisms use carbohydrates as a carbon source and convert them into proteins through an intermediate metabolism. The reduction of carbohydrates and lipids after the cultivation process shows that carbon is needed for amino acid synthesis [17].

Ligninolytic enzymes, namely laccase, LiP, and MnP, activity patterns of *Pleurotus ostreatus* InaCC F209, F216, and LIPI during 70 days of growth are shown in figure 2. The activity between each strain showed a similar pattern. These enzymes were produced in a larger amount during the mycelial colonization period and declined after the fruiting body development. Widiastuti et al. [18] reported similar patterns on ligninolytic activity of *P. ostreatus*. Ligninolytic enzymes have a role in delignification process so the hydrolyzing enzymes could degrade polysaccharides that are protected by lignin [19]. Lignin and its derivatives such as para-phenolic benzoic acids, para-phenolic cinnamic acids, and para-phenolic phenylpropionic acids have a high toxicity. It is reported that the degradation of lignin by ligninolytic enzymes could enhance the mycelial growth [20]. In addition, ligninolytic enzymes are known to take a part in maintaining the fungi from oxidative stress. Oxidative stress is a condition when the number of reactive oxygen species (ROS) was bigger than the availability of antioxidants, which could damage the fungal cell structure. Several studies have shown that MnP and laccase production are increasing in response to excess ROS production so that they can form a fungal defense against oxidative stress [21, 20].

However, it is shown that there was an enhancement of laccase activity of *P. ostreatus* InaCC F209 and LIPI after fruiting body production. It is believed that *P. ostreatus* shifted back to mycelial growth phase after the maturation of the fruiting body so that laccase activity was gradually increased [22].
LiP activity of *P. ostreatus* InaCC F216 and MnP activity of three *P. ostreatus* strains showed two maximum activities around 7th and 21st days. The fluctuation of LiP and MnP is suspected due to the ability of *P. ostreatus* to form isoenzymes. A study showed that *P. ostreatus* cultivated using sawdust could synthesize MnP isozyme, such as MnP2 and MnP3 [23]. Kamitsuji et al. [24] reported that MnP isozymes production of *P. ostreatus* could be influenced by the availability of nitrogen and manganese while on *P. chrysosporium*, the production is affected by heat shock, the availability of H2O2, and other chemical stress.

It is shown that maximum LiP and MnP activity of isolate *P. ostreatus* LIPI is the highest among other strains while maximum laccase activity of *P. ostreatus* InaCC F216 is the highest. However, the production of ligninolytic enzymes of *P. ostreatus* InaCC F209 is the most stable and in larger amounts during 70 days of growth compared to others.

Hydrolyzing enzymes, such as endoxylanase, endoglucanase, and β-glucosidase, of *Pleurotus ostreatus* InaCC F209, F216 and LIPI, activity patterns during 70 days of growth are shown in figure 2. The patterns were also similar between three strains. The increase of hydrolyzing enzyme activity means the rate of degradation of xylan and cellulose are high. It is due to xylan and cellulose’s role as the carbon source for metabolism [25].
Figure 2. Laccase (A), MnP (B), LiP (C), endoxylanase (D), endoglucanase (E), and \( \beta \)-glucosidase (F) activities pattern during 70 days of mycelial growth and fruiting body development of *P. ostreatus* InaCC F209 (■), F216 (○), and LIPI (▲).

Endoxylanase activity was low at the early stage of cultivation, increased and reached maximum activity during fruiting body development, and decreased after harvesting. Kabel et al. [26] reported a similar endoxylanase activity pattern on *Agaricus bisporus*. Xiao et al. [20] reported that the degradation
of hemicellulose by *P. ostreatus* in solid media is known to be selective because xylan is a constituent of hemicellulose which can be used as a carbon source and known to provide benefits to the growth of *P. ostreatus*. The presence of xylan could stimulate the accumulation of mycelial biomass and activate the expression of genes that play a role in xylose metabolism pathways such as xylose reductase and xylulose kinase, so that xylan can be used by fungi as a carbon source [20].

Both endoglucanase and β-glucosidase activities of three strains were high during both mycelial growth and fruiting body development. It is because *P. ostreatus* needs to degrade other alternative substrates as an energy source to support lignin degradation because lignin degradation could not produce high energy [25]. In addition, glucose can be used by fungi to stimulate the production of ligninolytic enzymes. The oxidation process of glucose produces endogenous hydrogen peroxide which plays a role in reducing heme-peroxidase enzyme so it can oxidize lignin [27].

*Pleurotus ostreatus* InaCC F209 has the highest maximum activity of endoxylanase while *P. ostreatus* InaCC F216 has the highest maximum endoglucanase activity and isolate *P. ostreatus* LIPI has the highest maximum β-glucosidase activity. Similar to the production of ligninolytic enzyme, hydrolyzing enzyme production of *P. ostreatus* InaCC F209 are the most stable and in larger amounts during 70 days of growth compared to others.

*Pleurotus ostreatus* InaCC F209 consistently produced endoglucanase and β-glucosidase in larger amounts during 70 days of growth compared to others while *P. ostreatus* InaCC F216 produced endoglucanase and β-glucosidase in a smaller amount. We suspect it is the reason for *P. ostreatus* InaCC F209 to form fruiting body in a shorter period while *P. ostreatus* InaCC F216 did not form any fruiting body. However, the correlation between lignocellulolytic enzyme production and age of harvest has yet to be known.

4. Conclusion
Laccase, LiP, and MnP activities of three *P. ostreatus* strains were higher on the vegetative phase while endoxylanase and endoglucanase activities were higher on the reproductive phase. *Pleurotus ostreatus* InaCC F209 produced the highest and most stable on laccase, β-glucosidase, endoglucanase, and endoxylanase than two other strains during growth.

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