

FUNGAL PRETREATMENT OF OIL PALM EMPTY FRUIT BUNCH: EFFECT OF MANGANESE AND NITROGEN

SRI HARMINI, MIKAEL DJATI HUTOMO, LUTFI BAHRUDDIN, RIA MILLATI,
M. NUR CAHYANTO, CLAES NIKLASON* and MOHAMMAD J. TAHERZADEH**

Faculty of Agricultural Technology, University Gadjah Mada, Yogyakarta 55281, Indonesia

**Department of Chemical Engineering, Chalmers University of Technology, Gothenburg, Sweden*

***School of Engineering, University of Borås, Borås, Sweden*

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Oil palm empty fruit bunch (OPEFB) was biologically pretreated using *Pleurotus floridanus* LIPIMC996 supplemented with various concentrations of manganese and nitrogen and incubated for 35 days at 30°C, and enzyme activities of manganese peroxidase (MnP) and laccase were examined. When OPEFB was supplemented with manganese, the highest lignin reduction was achieved up to 25.0±5.6% at the addition of 200 µg Mn²⁺/g OPEFB. In addition, *Pleurotus floridanus* LIPIMC996 grew best on OPEFB supplemented with 800 µg Mn²⁺/g OPEFB. When OPEFB was supplemented with nitrogen, the highest lignin reduction was achieved up to 27.2± 3.5% at the addition of 20 mM nitrogen. The best growth of *Pleurotus floridanus* was also achieved with the addition of 20 mM nitrogen. The addition of nitrogen and manganese on OPEFB did not significantly affect the activity of MnP and laccase.

Keywords: oil palm empty fruit bunch, pretreatment, ligninolytic enzymes, glucosamine, fungal growth, *Pleurotus floridanus*

INTRODUCTION

Oil palm (*Elaeis guineensis*) is the largest plantation commodity product in Indonesia. The plantation areas of the palm trees have been expanded each year, having reached 7.8 million ha in 2010, and producing 18.09 million tons of palm oil.¹ In 2011, 22.5 million tons (dry base) OPEFB were obtained.² Large amounts of OPEFB remain on oil palm industry sites as residues. The conventional way of handling waste is no longer applicable, since OPEFB is stacked in open areas, which could attract pests. In addition, the incineration of OPEFB has technical challenges due to the bunch size, and would cause air pollution. Ways to overcome this problem by creating valuable products, such as ethanol and biogas, from OPEFB have raised interest.

The main obstacle in the bioconversion processes is the morphological complexity and

crystallinity of lignocellulosic biomass. A pretreatment prior to bioconversion is needed in order to reduce the crystallinity and the hindrance of e.g. lignin, and make the carbohydrates accessible to the enzymes and microorganisms.^{3,4,5} Accordingly, an appropriate pretreatment is essential to break down the lignin complex, which hinders the enzyme attack.⁶ Several pretreatment methods have been developed for lignocellulosic materials. Generally, most of them are high energy-consuming, such as milling, irradiation, pyrolysis, high pressure steaming, alkaline and acid hydrolysis, gas treatment (chlorine dioxide, nitrogen dioxide, sulfur dioxide, ozone), organosolv treatment, hydrothermal treatment (water, elevated temperature), steam explosion, and wet oxidation.^{7,8}

Biological pretreatment offers advantages over these methods as it requires low energy, mild environmental conditions, low chemical need, introduces less polluted material to the environment, and might be more environmentally friendly.

From the class of Basidiomycetes, white-rot fungi are the most effective microorganisms for biological pretreatment of lignocellulosic materials.⁹ These fungi are remarkable in their ability to degrade lignin. In general, this ability is thought to be dependent on peroxidases secreted by the fungi. The fungi secrete one or more of three extracellular enzymes that are essential for lignin degradation, i.e. lignin peroxidase (EC 1.11.1.14), manganese-dependent peroxidase (EC 1.11.1.13), and laccase (EC 1.10.3.2).¹⁰ By exerting peroxidase enzymes, the lignin-hemicellulose sheath is altered, thus facilitating the accessibility of cellulolytic enzymes to cellulose.⁵

One of the important factors affecting the activity of ligninolytic enzymes is the presence of metal ions in the substrate.¹¹ Metal ions comprise an important group of ligninolytic enzyme activity modulators and are present in the environment either naturally (Cu, Mn) or as a result of human activities (Cd, Hg, Pb).^{12,13} The importance of manganese for the lignin degradation process by *Pleurotus sp.* has also been demonstrated.¹⁴ Manganese is both an active mediator for manganese peroxidase (MnP) and a regulator for MnP and laccase production.¹⁵ Increasing manganese levels were reported to enhance ligninolytic enzyme levels as well as lignin degradation by some white-rot fungi.¹⁶

Oil palm empty fruit bunch has a low nitrogen content, which is demonstrated by the content of C:N ratio of 53.5:1.0.¹⁷ Several authors reported that an intermediate C:N ratio results in the best yield in terms of fungal growth. Shah *et al.*¹⁸ achieved maximum yields in a 35:1 C:N medium. Similarly, Jackson and Schisler¹⁹ observed a maximum spore yield in a 30:1 C:N medium, but not at higher or lower C:N ratios, therefore nitrogen should be supplemented before biological pretreatment. Nitrogen supplementation is necessary to improve mycelial growth of the fungus, e.g. *Pleurotus sp.*, and to increase final biomass.²⁰

The aim of this work was to investigate the effects of the addition of nitrogen and manganese in the pretreatment of OPEFB on the growth of

Pleurotus floridanus LIPIMC996 and the activity of ligninolytic enzymes.

EXPERIMENTAL

Oil palm empty fruit bunch

Oil palm empty fruit bunch was obtained from an oil palm mill in Medan, Indonesia. It was sun-dried until the water content was 10% or less. It was shredded, ground, sieved through 40 mesh, and stored in plastic bags at room temperature.

Fungal strains

Pleurotus floridanus strain LIPIMC996 (Laboratory of Microbial Systematic and LIPI Microbial Collection, Lembaga Ilmu Pengetahuan, Cibinong, Indonesia) was used in this experiment. Prior to being used as inoculum, *Pleurotus floridanus* LIPIMC996 was maintained on lignocellulosic medium at room temperature. A small amount of mycelia was cut from the fungal culture on the lignocellulosic medium and inoculated on potato dextrose agar.

Pretreatment

Biological pretreatment by the fungus was carried out in a jar bottle containing 25 g of ground OPEFB with a moisture content of 70-75%, and 8.3 mL basal medium.³² The concentrations of manganese added to the OPEFB were 0, 200, 400, 600, 800 $\mu\text{gMn}^{2+}/\text{g}$ OPEFB. The concentrations of nitrogen added to the OPEFB were 0, 5, 10, 20, 40 mM. OPEFB containing basal medium was sterilized by heating at 121°C for 20 min. The jar was then inoculated with a mycelium of the fungus and closed with aluminum foil sealed with a rubber band, and incubated at 30°C for 35 days. Samples were taken once a week for analysis of lignin, glucosamine and ligninolytic enzyme.

Glucosamine analysis

The glucosamine analysis method was modified starting from a laboratory analytical procedure developed by The National Renewable Energy Laboratory (NREL) for lignocellulosic materials. A sample of pretreated OPEFB (0.1 g) was mixed with 0.3 mL 72% H₂SO₄, stirred and supplemented with 8.4 mL Milli-Q water. The mixture was then autoclaved at 121°C for 1 h. An autoclaved sample (1 mL) was taken and divided equally into two different tubes (A and B), and supplemented with 0.5 mL of NaNO₂ and 0.5 mL Milli-Q water, respectively. The caps were opened after 6 h and kept at room temperature overnight, followed by the addition of 0.5 mL ammonium sulfate (12% w/w). Samples (1 mL) were taken and put into other tubes, which were then supplemented with 8 mL of Milli-Q water and 0.5 mL of 0.5% (3-methyl-2-benzothiozolone-hydrazone-hydrochloride) MBTH. The mixture was then incubated for 1 h. After incubation, 0.5 mL of 0.5% FeCl₃ was added to the tubes and incubated for 1 h.

One mL of mixture was then taken out and placed in another tube to which 4 mL of Milli-Q water was added. The absorbance of the mixture was then analyzed using a UV-spectrophotometer at 650 nm. The absorbance values were then calculated using the following equation:

Glucosamine absorbance = absorbance from tube A – absorbance from tube B

The glucosamine absorbance was converted to glucosamine yield (g glucosamine/g OPEFB).

Enzyme extraction and analysis

The method of enzyme extraction was adapted from Gassara *et al.*²¹ Laccase activity was measured using 2,2-azino bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) in a 0.5 M acetic buffer at pH 5. One unit of enzyme activity was defined as the amount of enzyme to oxidize 1 μmol ABTS per minute.²¹

The determination of manganese peroxidase was performed according to Gassara *et al.*²¹ and Buswell *et al.*²² One unit of MnP enzyme activity was defined as the amount of enzyme, which transforms Mn²⁺ to Mn³⁺. Lignin peroxidase activity was determined by monitoring the conversion of veratryl alcohol to veratryl aldehyde at 25°C by hydrogen peroxide at 310 nm. One unit of enzyme is defined as the amount of enzyme required for 1 μmol of veratryl aldehyde per minute.

Lignin analysis

The analysis was based on LAP “Determination of Acid-Insoluble Lignin in Biomass” and LAP “Determination of acid-soluble lignin in Biomass” NREL method (2008).²⁴ The total lignin is the sum of acid-soluble and acid-insoluble lignin. Insoluble lignin was determined by the gravimetric method and soluble lignin was determined by the colorimetric method.

RESULTS AND DISCUSSION

This study aimed to determine the effects of nutrient supplementation to fungal pretreatment of OPEFB. The added nutrients were manganese in concentrations of 0, 200, 400, 800 μg Mn²⁺/g OPEFB and nitrogen at concentrations of 0, 5, 10, 20, 40 mM.

Effect of manganese on the activities of ligninolytic enzymes

The enzyme activity of MnP and laccase was measured and the results showed that the addition of manganese had little influence on the enzyme activity (Fig. 1). All concentrations of Mn added showed similar trends and the enzyme activity showed a narrow range. The activity of MnP obtained from *Pleurotus floridanus* LIPIMC996 grown with the highest manganese concentration

(800 μg Mn²⁺/g OPEFB) showed no significant differences, compared to the experiment without manganese addition. In fact, the highest enzyme activity was obtained when the fungus was grown without manganese addition, which was reached on day 35 of the pretreatment. During the observation, manganese peroxidase activity continued to increase with the incubation time.

Similar to that of MnP, the activity of laccase showed that there was no significant difference among the fungal pretreatments. The highest activity of laccase was obtained from *Pleurotus floridanus* LIPIMC996 grown in the presence of 400 μg Mn²⁺/g OPEFB. As shown in Fig. 1, in all Mn concentrations, laccase activity was always higher than manganese peroxidase activity, which is probably a property of the fungus used in this study. However, it is noted that laccase activity increased at the beginning of incubation time on day 7 (for 0 and 800 μg Mn²⁺/g OPEFB used) and on day 14 (400 μg Mn²⁺/g OPEFB), and then continually decreased. This result is in agreement with the results from a previous study by Dinis *et al.*²⁵

Pleurotus floridanus LIPIMC996 is a fungus, which specifically degrades lignin by exerting extracellular enzymes. The enzymes used in lignin degradation are laccase and peroxidases. Meanwhile, metal ion addition enhanced the enzyme activity. The activity of ligninolytic enzymes was slightly affected by the addition of manganese. OPEFB contains ions, such as copper, calcium, manganese, iron and sodium.²⁶ It probably indicates that the content of Mn in the OPEFB was sufficient for the enzyme to be activated.

The investigation was also carried out for lignin peroxidase (LiP) enzyme that might be produced by *Pleurotus floridanus* LIPIMC996. The result showed that LiP was produced in considerably smaller amounts, compared to laccase and MnP.

Effect of manganese and nitrogen on lignin

The effect of the addition of manganese and nitrogen to the pretreatment can be observed from the results of lignin degradation (Fig. 2). As one of the main objectives of the pretreatment is to delignify the substrate, the lignin content was measured and the results are presented in Fig. 2. In general, total lignin was decreased during the incubation period. The highest lignin removal was obtained after 28 days and 35 days of pretreatment

from the samples supplemented with manganese

and nitrogen, respectively.

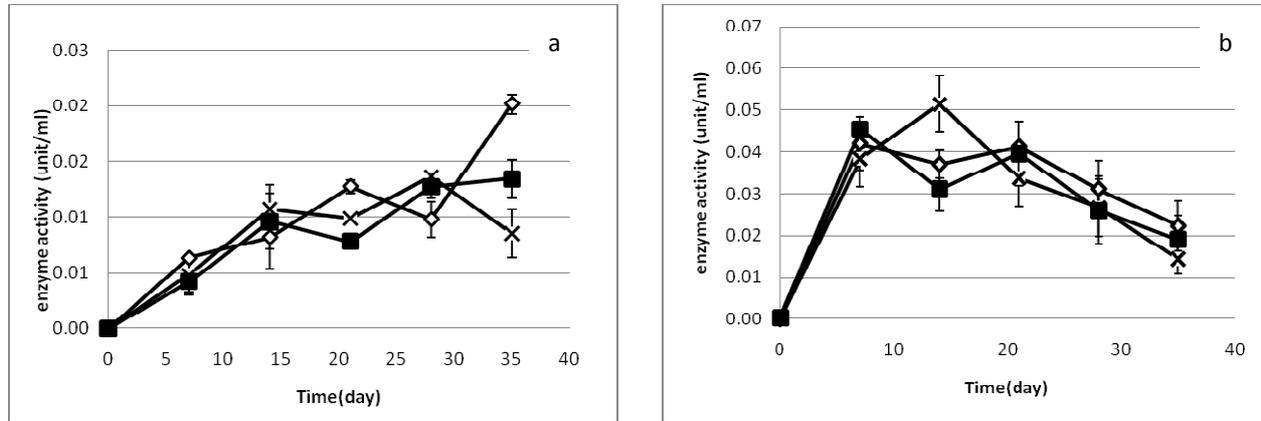


Figure 1: Effect of manganese addition on the activity of (a)Manganese peroxidase and (b)Laccase during pretreatment of OPEFB using *Pleurotus floridanus* LIPIMC996 (◇: 0 µg Mn²⁺/g OPEFB; X: 400 µg Mn²⁺/g OPEFB; ■: 800 µg Mn²⁺/g OPEFB)

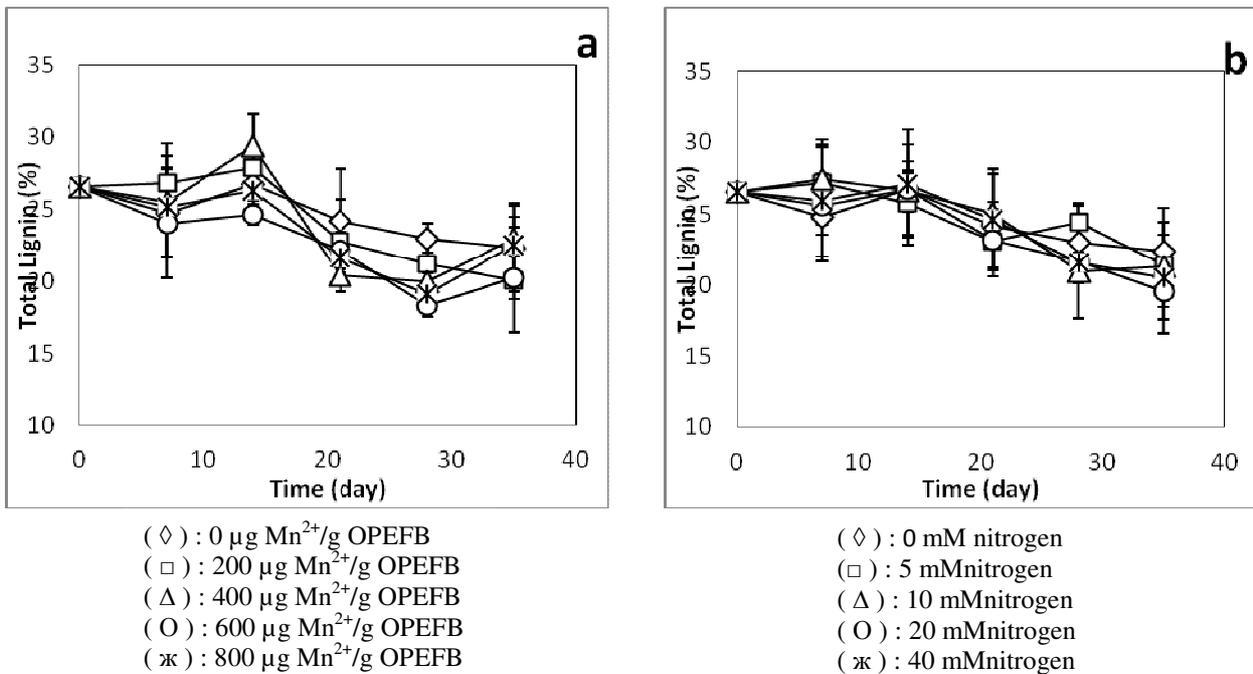


Figure 2: Effect of manganese and nitrogen addition on lignin content in fungal pretreatment using *Pleurotus floridanus* LIPIMC 996 (a) Percentage of total lignin with manganese addition; (b) Percentage of total lignin with nitrogen addition

The highest lignin reduction of 25.0±5.6% was obtained when OPEFB was supplemented with Mn at 200 µg, which occurred on day 28 of the pretreatment. Biological pretreatment of OPEFB with the addition of 20 mM nitrogen resulted in the highest lignin removal up to 27.2±3.5%, compared to the untreated OPEFB.

This was obtained on day 35 of the pretreatment. The results are summarized in Table 1.

In comparison with other previous studies, this result is in accordance with those obtained for lignin removal of OPEFB pretreated by *Pleurotus ostreatus*, in which 25.70% of lignin could be

removed.²⁷ Gupte *et al.*²⁸ studied the fungal pretreatment on wheat straw using *Pleurotus ostretatus* and obtained lignin degradation up to 40%. Another study showed that the addition of 600 μg manganese/g cotton stalk in the fungal pretreatment using *Pleurotus sp.* resulted in a 50% lignin loss.²⁹

Effect of manganese and nitrogen on the fungal growth in the pretreatment of OPEFB

Several methods have been developed to determine fungal growth, for example, a physical method, which determines solid-state fungal growth by Fourier Transform Infrared-Photoacoustic spectroscopy³⁸ and a method using chitin analysis, which involves indirect

determination to measure the glucosamine in chitin as a fungal growth indicator.³⁰ In this work, the growth of *Pleurotus florida* LIPIMC996 was determined by measuring the glucosamine content. Glucosamine is the major component in fungal cell walls. When the fungus grows, its cell wall thickens causing a rise in the internal glucosamine content. Therefore, glucosamine content can be used as an indicator of fungal growth. The growth of the fungus was calculated by changes of glucosamine content from the start (day 0) up to incubation day (day 35). The amount of glucosamine was then used to calibrate to the amount of fungal biomass.

Table 1
Effect of adding manganese and nitrogen on lignin removal after 35 days of fungal pretreatment using *Pleurotus florida* LIPIMC 996

Manganese concentration ($\mu\text{g Mn}^{2+}/\text{g OPEFB}$)	Lignin removal (%)	Nitrogen concentration (mM)	Lignin removal (%)
0	16.6 \pm 8.6	0	16.6 \pm 8.6
200	25.0 \pm 5.6	5	20.0 \pm 6.4
400	14.2 \pm 7.4	10	14.8 \pm 7.8
600	24.5 \pm 2.7	20	27.2 \pm 3.5
800	16.0 \pm 3.4	40	6.1 \pm 6.6

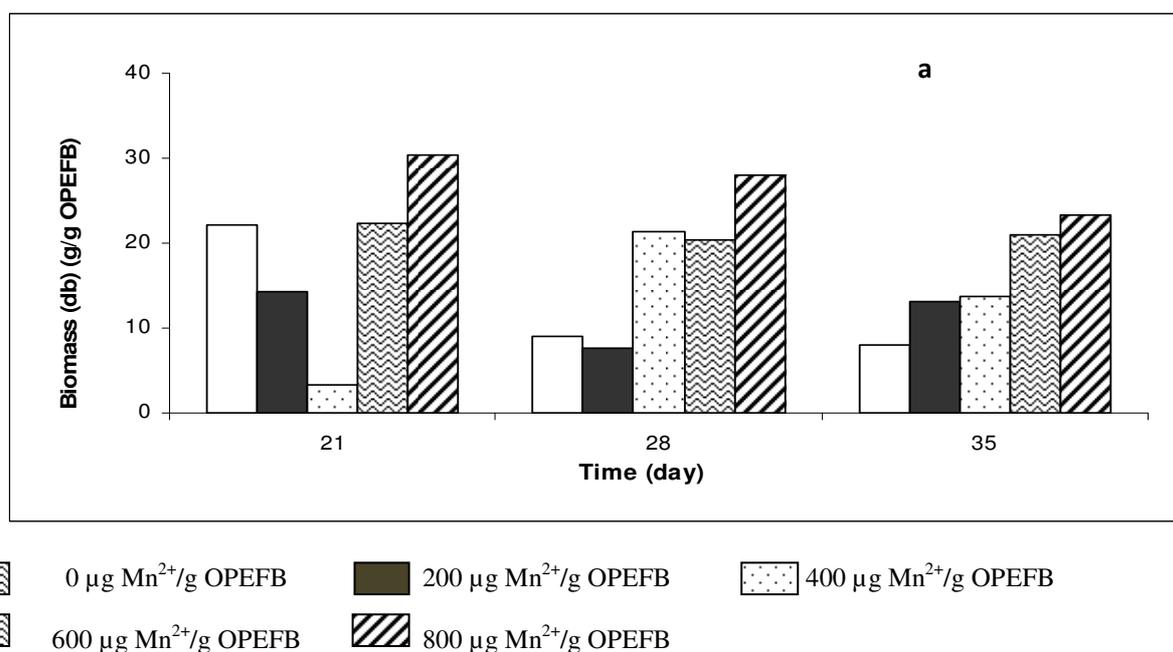


Figure 3a: Growth of *Pleurotus florida* LIPIMC996 during pretreatment of OPEFB with addition of different concentrations of Mn^{2+}

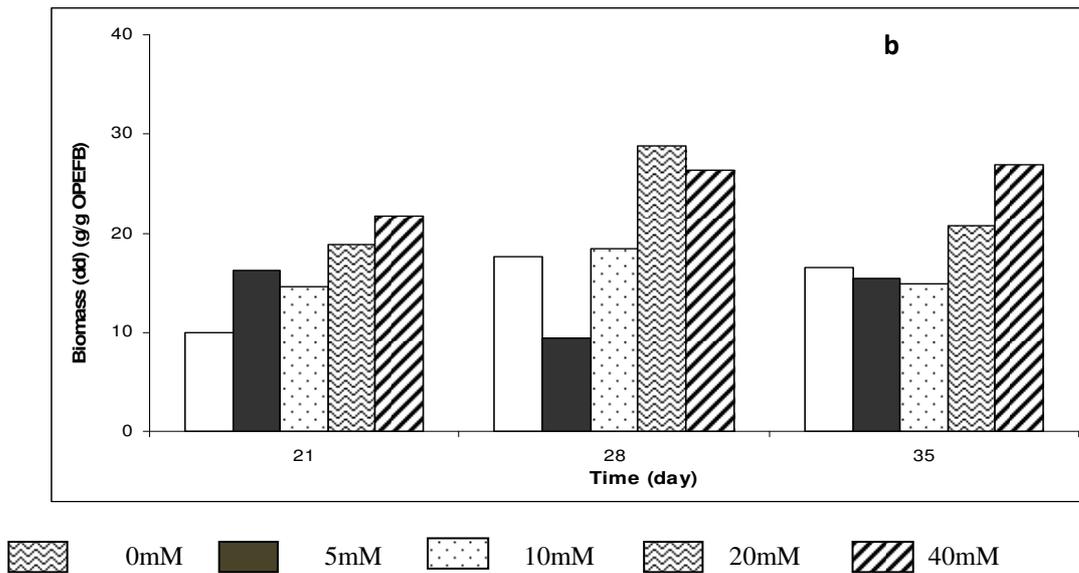


Figure 3b: Growth of *Pleurotus floridanus* LIPIMC996 during pretreatment of OPEFB with addition of different concentration of nitrogen

Fig. 3 shows the amount of fungal biomass (g/g OPEFB) during incubation from day 21 up to day 35. The results showed that the increase of fungal biomass is in line with the increase in the manganese and nitrogen concentrations added. Manganese and nitrogen as nutrition had effects on *Pleurotus floridanus* LIPIMC996 growth. On day 35, it could be observed that 200 and 400 $\mu\text{g Mn}^{2+}/\text{g OPEFB}$ concentrations had nearly the same amount of fungal biomass. This trend was also found for the 600 and 800 $\mu\text{g Mn}^{2+}/\text{g OPEFB}$ concentrations, which resulted in higher fungal biomass. With the addition of 20 mM and 40 mM nitrogen, the fungal biomass yields were much higher than that in which nitrogen was added at lower concentration.

The highest fungal biomass, of 31 g/g OPEFB, was obtained from the sample harvested on day 21, to which 800 $\mu\text{g Mn}^{2+}/\text{g OPEFB}$ was added. Nitrogen supplementation of 20 mM resulted in 29 g/g of OPEFB fungal biomass, which was harvested on day 28. Based on this result, the optimum time to grow *Pleurotus floridanus* LIPIMC996 in OPEFB in solid state fermentation with the addition of manganese was 21 days of incubation and 28 days for the addition of nitrogen.

The results of the fungal growth examined in this work, which was assessed by measuring the glucosamine content, showed that the higher the

quantity of nutrition added to the substrate, the higher the extent of growth achieved. Ammonium sulfate is a nitrogen source, which stimulates fungal growth during solid state fermentation. Boyle³¹ suggested that supplementation with nitrogen source ammonium sulfate can stimulate and increase fungal growth. Pretreated OPEFB with a higher manganese addition (600 and 800 $\mu\text{g Mn}/\text{g OPEFB}$) and a higher nitrogen addition (20 mM and 40 mM) showed high glucosamine content.

CONCLUSION

Ligninolytic enzyme activities were detected on the solid state fermentation of OPEFB using *Pleurotus floridanus* LIPIMC996. The addition of manganese was not needed in order to enhance the activity of laccase and manganese peroxidase, however, manganese and nitrogen are necessary for the fungal growth. The addition of manganese and nitrogen did not significantly affect lignin degradation by the fungus.

Therefore, it is possible that there is no direct correlation between fungal growth, lignin degradation and enzyme activities. The fungal growth increased along with the incubation time and resulted in approximately 25-27% of lignin removal. From this result, it could be concluded that oil palm empty fruit bunch could be pretreated using *Pleurotus floridanus* LIPIMC996 without

any nutritional supplementation in order to obtain lignin biodegradation.

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Abbreviations

OPEFB: oil palm empty fruit bunch
MnP: manganese peroxidase
LiP: lignin peroxidase

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