The effect of malt extract and incubation time on ethanol production from lignocellulose degradation of oil palm empty fruit bunches (OPEFB) using *Phlebia sp*. MG-60

I Nurika, T Indirahayu and S Suhartini

Department of Agro-industrial Technology, Faculty of Agricultural Technology, Universitas Brawijaya, Malang, Indonesia

E-mail: irnia@ub.ac.id

**Abstract.** This study aimed to evaluate the effects of the addition of malt extract and incubation time on ethanol production from lignocellulose degradation of OPEFB using *Phlebia sp*. MG-60. The first experiment was conducted to figure out the best treatment of the addition of malt extract (0; 0.1; 0.2; 0.3 g/L) for the degradation of lignocellulose using *Phlebia sp*. MG-60 for 28 days, then followed with the ethanol production which incubated into different time courses (0, 3, 5, and 7 days). Total reducing sugar (TRS), total soluble phenol (TSP), pH, lignin concentration, weight loss and ethanol concentration were analyzed to identify the changes on the breakdown of lignocellulose. The best treatment was obtained from the addition of malt extract 0.2 g/L with the highest amount on TRS (26.489 mg/g) incubated for 15 days. While weight loss (19.01%) and the lowest lignin residue (12.61%) were obtained from culture incubated for 28 days. Meanwhile the highest ethanol concentration was 0.28% obtained from 5 days culture.

1. Introduction

The production of Indonesian palm oil industry showed an increase where in 2010 palm oil production in Indonesia reached 21.95 tons per year and increased to 33.50 million tons per year in 2016 [1]. The main products produced in the palm oil industry are crude palm oil (CPO), whereas the palm oil industry also produce waste consisting of liquid waste originating from steaming and hydrocyclone waste, solid waste in the form of oil palm empty fruit bunches (OPEFB), shells and sludge and gas waste from the burning shells [2]. OPEFB fibers ranged from 25 to 26 percent of each palm fruit bunches (FFB) supplied to processors [3]. In 2013, Indonesia produced 37 million tons of OPEFB and was estimated to increase by 7% every year. OPEFB contain high amount of lignocellulosic compounds. The lignocellulose component in OPEFB consist of 24–65% cellulose, 21–34% hemicellulose and 14–31% lignin [4]. The cellulose and hemicellulose content are high on OPEFB, and therefore OPEFB has a potential source of high sugar which can be fermented into biofuels such as ethanol using microorganism as biological pretreatment [5]. Lignocellulose can be degraded by extracellular enzymes of white rot fungus, such as manganese peroxidase (MnP), lignin peroxidase (LiP), versatile peroxisade and laccase [6]. In white rot fungus *Phlebia sp*. MG-60, the MnP enzyme dominates the lignocellulose degradation process [7]. *Phlebia sp*. MG 60 is one of the white rot fungus which has the ability to depolimerised lignocellulose and use the breakdown of cellulose and...
hemicellulose compounds to be converted to produce ethanol in a single semi aerobic fermentation condition [8].

The addition of several nutrients, such as nitrogen, manganese and copper and as well as the growth conditions will influence the fungal activities [9]. Malt extract consists of 78% maltose as an energy source, 17% dextrin as a polysaccharide, 14% glycerol as a source of carbon and 0.05% peptone as a source of nitrogen [10]. According to Izmirlioglu and Dermici [11], using malt extract as substrate could promote the fungal metabolism of fungal growth which then increase the enzyme production for producing high amount of ethanol. Meanwhile, the incubation time will greatly affect the levels of ethanol produced [12]. The optimum of incubation time will increase amount of fungal biomass and this will affect on the yield of ethanol production. However, the fermentation process will stop at certain time when the ethanol level achieved cannot be tolerated anymore by the microorganism, because the high ethanol concentration will inhibit the growth of the inoculum. Only a few inoculums are tolerant to the high concentration of ethanol [13]. It is therefore the aim of this study was to identify the effect of addition malt extract and the length of incubation to the ethanol production from lignocellulose degradation of OPEFB using the white fungus Phlebia sp. MG-60.

2. Materials and Method

2.1. Microorganism and culture conditions
White rot fungi Phlebia sp. MG-60 was obtained from Department of Forest and Environmental Sciences, University of Miyazaki Japan, which was then maintained on potato dextrose agar (PDA) medium in Laboratory of Bioindustry at Agro-industrial Technology Department, Universitas Brawijaya, Indonesia. The OPEFB was obtained from PT. Sawit Arum Mardani, Indonesia.

2.2. Research methods
Experiments were conducted into two steps, first the effect of addition malt extract (M) (M0=0.0; M1=0.1; M2=0.2; 0.3 g/L) through the ability of Phlebia sp. MG-60 incubated for 28 days to breakdown lignocellulose, then followed with the effect of incubation time (I) (I0=0; I1=3, I2=5, and I3=7 days) toward the alcohol production.

2.3. Culture incubation
Phlebia sp. MG-60 was grown on 10 g of OPEFB, which consist of 75% moisture content and sterilised using autoclave at 121°C for 15 minutes 1 atm. The sample was then incubated at 28°C for 28 days for the delignification process.

2.4. The extraction of OPEFB
After the samples were incubated at certain times (0-28 days) and extracted, then the samples were measured for total soluble phenols (TSP) [14], total reducing sugar (TRS) [15], pH, lignin concentration [16] and weight loss [17].

2.5. Total reducing sugar
Total reducing sugar and soluble phenols assays were performed on the aqueous extract samples. Reducing sugars were determined colourimetrically by the dinitrosalicylic acid (DNS) method using glucose as the standard [15] and the absorbance was read at 540 nm using a UV-Vis spectrophotometer. In order to minimize sugar variation, each samples contained 3 replications.

2.6. Total soluble phenols
Phenols were measured colourimetrically using the Folin-Ciocalteau method with gallic acid as the standard and the absorbance was read at 760 nm using a UV-Vis spectrophotometer. This colorimetric method is based on chemical reduction of the reagent, a mixture of tungsten and molybdenum oxides.
which results in a blue colour at measurement at 765nm [14]. The intensity of light absorption at that wavelength is proportional to the concentration of phenols. The concentration of phenols and reducing sugar was expressed per gram of substrate (dry weight).

2.7. pH
pH testing was carried out on the incubated extract using a pH meter.

2.8. Weight loss (dry weight)
Samples were taken at different times (0, 10, 20, and 30 days), and three samples were collected at each sampling time. Three samples were removed from the cultivation jars and were oven-dried at 100 °C till a constant weight was reached. Weight loss was estimated as the difference between the weight of the whole culture in the medium at the beginning and at the end of the pretreatment.

2.9. Amount of lignin
1 gr sample (a) was refluxed with the addition of 150mL distilled water 100 °C for 2 hours using waterbath and filtered. The residue was washed using hot water and dried. The residue was dissolved in 0.5 M H$_2$SO$_4$ (150 mL) and refluxed for 2 hours at 100 °C. 10mL of 72% H2SO4 was added to dry residue and soaked at room temperature for 4 hours. 150 mL of 0.5M H$_2$SO$_4$ was added and refluxed at 100 °C for 2 hours. The residue was filtered and washed using distilled water then heated on the oven at 105 °C and weighed (d), the ash content was measured (e).

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\text{Lignin concentration} = \frac{(d-e)}{a} \times 100\% \tag{1}
\]

2.10. Ethanol assay [18]
The samples were obtained from the extraction process was placed into the evaporator. Evaporation was carried out at 55 °C with 65 rpm 100 bar for 5 minutes. The ethanol concentration was then measured using alcoholmeter.

3. Results and Discussion
3.1. Water content, ash content, volatile solid (VS) and total solid (TS)
The average water content of OPEFB was 3.5%, the TS (96.5%) shows the total amount of solids in material both organic and inorganic (Table 1). According to Kamei et al. [19], the optimum level of water content needs to optimize the growth of Phlebia sp. MG-60 is 75%. Mehmian et al. [20] showed that the water content below the standard white rot fungus requirement will hinder its growth, but if it is too high it will cause inter-particle space to decrease, inhibit air diffusivity through lignocellulose biomass and inhibit fungal growth, so that lignin degradation becomes low. This can affect fungal growth and the production of enzymes that will significantly affect lignin degradation [21]. OPEFB ash content used in this study was 4.9% and VS 95.1%. Determination of VS can be used as an organic parameter in a biomass waste, which will then be used in a biological process such as biodegradation substrate [22].

| Parameter                | Initial content (%) |
|--------------------------|---------------------|
| Water content            | 3.5                 |
| Ash content              | 4.9                 |
| Volatile solid (VS)      | 95.1                |
| Total solid (TS)         | 96.5                |

Table 1. Physical characteristics of oil palm empty fruit bunch
3.2. Total reducing sugars (mg/g)
Total reducing sugars is one indicator for pretreatment process, which is used to see how the white rot fungus *Phlebia sp.* MG-60 is able to breakdown cellulose and hemicellulose. The highest TRS (26,489 mg/g) was obtained from substrate contains 0.2 g/L malt extract and incubated for 14 days. The highest TRS from all samples was mostly released at 14 days and decrease afterwards, indicated that the optimum incubation time for *Phlebia sp.* MG-60 to release high amount of sugars is at day 14. The presence of malt extract can increase the metabolism of *Phlebia sp.* MG-60 that then affect on the increasing of TRS due to the delignification process. In fermentation process, the addition of malt extract which consist of carbon and nitrogen, can increase fungal metabolism [10]. Kamei et al. [19] showed that the decrease in TRS could be caused by the use of simple carbohydrates released during delignification process as nutrient source by the fungus *Phlebia sp.* MG-60. Therefore, by adding some nutrients such as carbon and nitrogen, will help the decreasing of TRS [23].

3.3. Total soluble phenols (mg/g)
The measurement of total soluble phenols was used to indicate the breakdown or depolymerised of lignin compounds by biological pretreatment using the white rot fungus *Phlebia sp.* MG-60. The highest amount of phenols released (0.627 mg/g) was obtained from substrate consist of 0.1 g/L malt extract and incubated for 21 days (Figure 1b). The increasing of TSP showed the existence of some ligninolytic enzymes such as MnP or VP produced by the white rot fungus *Phlebia sp.* MG-60 to breakdown the structure of lignin. However, the amount of TSP decreased after 21 days incubation, which possibly caused by the decreasing of enzyme activity due to lack of nutrients.

3.4. pH
The pH cultures gradually decrease from an initial pH 7 to pH 5.8 after 28 days incubation (Figure 1c). pH value indicates the presence of organic acids released by the fungus during the fungal metabolism. The lowest pH value was obtained from substrate consist of 0.2 g/L malt extract which incubated at 28 days, while the highest pH value (7.0) was found at initial growth. The longer the incubation the more organic acids produced by the fungus, causing pH to decline (Figure 1c). The breakdown of lignocellulose and other organic compounds from OPEFB substrate caused the production of organic acids. The optimum pH needs for the MnP enzyme activity ranged from 4.5 to 5.5 [24].

3.5. Weight loss
The weight reduction in delignification process is due to depolymerised of lignin, which affected to the mass of the substrate. Percentage of heavy losses associated with degradation of cellulose and hemicellulose components, resulting from the release of lignin bonds by *Phlebia sp.* MG-60. Figure 1d showed all the weight loss percentage gradually increase from initial growth to 28 days incubation. The highest percentage of weight loss obtained from sample treated with 0.2 g/L malt extract, incubated for 28 days (19.01%). According to Zhang et al. [25], together with the production of lignocellulose degradation products will be followed by a reduction in the weight of the material. This shows that the increase in lignocellulose degradation will have an impact on reducing the weight of the material and therefore it shows an increase in the weight loss of the material.

3.6. Lignin concentration
The length of the incubation influences the amount of lignin released on the breakdown of lignocellulose. It is known that the longer the incubation, the more lignin could be degraded. In this study, the smallest percentage of lignin (12.61%) was achieved at 28 days from sample treated with 0.2g/L malt extract, while the largest percentage of lignin 17.64% was obtained from sample treated with addition malt extract 0.3 g/L. The decreasing percentage of lignin in pretreatment process is due to the break down of lignin bonds [26]. Providing carbon and nitrogen as nutrients on the substrate can
improve the delignification process [6], because the content of nitrogen can affect ligninolytic enzyme ability [27].

![Figure 1](image_url)

**Figure 1.** The effect of addition malt extract (M) to the release of: (a) total reducing sugars (mg/g), (b) total soluble phenols (mg/g), (c) pH, (d) weight loss (%), (e) lignin concentration (%) on the lignocellulose breakdown of OPEFB using *Phlebia sp.* MG-60
3.7. Ethanol concentration (%)
The highest ethanol yield (0.28%) was obtained from substrate consist of 0.2 g/L malt extract and incubated for 5 days. The lowest yield of ethanol production on the 5th day is sample 0 g/L (M0) of 0.15%. The production of ethanol during fermentation by Phlebia sp. MG-60 is due to the presence of genes associated with glycolysis, pyruvate production, and synthesis of ethanol [28].

![Figure 2](image_url)

Figure 2. The effect of different incubation time (days) on the production of ethanol (%)

According to Khuong et al. [6], ethanol production by providing nutrients can increase the ethanol concentration compared to without additional nutrient. Malt extract is a medium that commonly used as a carbon and nitrogen sources [11]. Study by Kamei et al. [19] showed that the highest ethanol production (0.35%) released from bagasse pretreated using Phlebia sp. MG-60 in semi-aerobic conditions at 10 days incubation. In this study the optimum amount of ethanol produced from substrate treated with 0.2 g/L malt extract revealed that the amount of nitrogen and carbon needed were suitable for ethanol production by Phlebia sp. MG-60 [6]. The content of malt extract containing nitrogen sources influenced the degradation process of lignin. Nitrogen metabolism plays a role in regulating the degradation of lignin as part of secondary metabolism in white rot fungi. Low nitrogen concentration stimulates enzyme production, whereas high nitrogen concentrations will suppress enzyme production [23].

4. Conclusions
The addition of malt extract using different concentration have an effect on the breakdown of lignocellulose by Phlebia sp. MG-60, indicated by the change of total reducing sugars, total soluble phenols, pH, weight loss and lignin concentration during cultured. While the fermentation time has shown to influence the ethanol content produced by Phlebia sp. MG-60.

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