The effect of MnSO$_4$ and unrefined sea salt on bioethanol production by lignocellulose degradation of oil palm empty fruit bunches (OPEFB) using *Phlebia* sp. MG-60

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**Abstract.** This study aimed to determine the effects of MnSO$_4$ and sea salt against the degradation lignocellulose and ethanol production generated from Oil Palm Empty Fruit Bunches (OPEFB) using *Phlebia* sp. MG-60. The factorial experiment in a randomized block design was used, consists of two factors: MnSO$_4$ concentration (0, 300, and 600 mg/L), sea salt concentration (0%, 3%, and 5% (w/v). The results revealed that the addition of MnSO$_4$ and sea salt to substrate, and its interaction were significantly different (p value < 0.05) on the change of total soluble phenols (mg/g), total reducing sugar (mg/g), pH, lignin concentration (%), weight loss (%) and ethanol concentration (%). The best treatment from this study was obtained from the addition of MnSO$_4$ 300mg/L, sea salt 3% incubated for 20 days. This treatment has the highest value of total reducing sugars (22.471 mg/g), total soluble phenols (0.334 mg/g), weight loss (16.23%), ethanol (0.71%), lignin (19.17%) and pH (6.1).

1. Introduction

Most oil palm supplies are currently produced from Malaysia and Indonesia which approximately produced 85% of the world oil palm production [1]. Each processing of 1 ton of fresh fruit bunches (FFB) of palm oil could produce 22-23% oil palm empty fruit bunches (OPEFB) [2]. The OPEFB contains high amount of lignocellulose (55-60%) which consist of cellulose (38.76%), hemicellulose (26.69%) and lignin (22.23%) [3]. The OPEFB can be chemically or biologically hydrolysed to release amount of sugars and a range of value added chemicals which can be depolymerised from lignocellulose [4]. The conversion of biomass using biologically pre-treatment have shown to offer most environmentally friendly for the production of bioethanol or other derivative product of lignocellulose [5].

Using the consolidated biological processing (CBP) for ethanol production is become challenging as single microorganism in a bioreactor produces all the enzymes required for both lignocellulose degradation and ethanol production. CBP can potentially reduce the cost of ethanol production by eliminating operating cost and capital investment associated with the needs of number of enzymes required for ethanol production [6]. The decomposition of lignocellulose using enzymatically pre-treatment is mostly carried out by the brown and white rot fungi from Basidiomycetes [7].

White rot fungi generally degrade lignin by secreting enzymes, such as manganese peroxidase (MnP), lignin peroxidase (LiP), versatile peroxidase (VP), and laccase [8]. Furthermore the white rot fungus *Phlebia* sp. MG-60 shows properties which are capable to produce ethanol with high yield of
The production of MnP lignin degrading enzymes can be optimized by adding Mn^{2+} to the substrates. According to Kamei et al. [10] addition of 3% (w/v) sea salt and MnSO_{4} to the substrate of Phlebia sp. MG-60 can increase MnP activity 4 times compared to medium without adding sea salt. The addition of MnSO_{4} with a concentration of 300 mg/L can increase MnP activity. However, the addition of MnSO_{4} in certain concentration can also inhibit the growth of fungus. Therefore, the aim of this study was to obtain the best amount of the addition MnSO_{4}, sea salt and incubation time of Phlebia sp. MG 60 for the production of ethanol using OPEFB.

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. Fungal pre-treatment and OPEFB solid-state fermentation
10 g of OPEFB was chopped into small pieces and homogenised using a disk mill 40-100 mesh. 30 mL of distilled water were added to 200 ml honey jar and autoclaved. A mycelial disk of was taken from two weeks old fungal colony and placed into OPEFB powder in honey jar. Certain amount of MnSO_{4} (M) (M0=0 mg/L, M1=300 mg/L, and M2=600 mg/L) and sea salt (S) (S0=0%, S2=3% and S3=5% w/v) were added. The inoculated of OPEFB was incubated at 28 °C for 0, 10, 20 and 30 days. Each treatment consisted of 3 replications.

2.3. Extraction of OPEFB
100 mL of distilled water was heated at 80 °C and added into each jar. These samples were shaked using water bath at 40°C, 150 rpm for 1 hour and filtered using Whatman paper. The filtrate was then used for analysis.

2.4. Total reducing sugar
Total reducing sugar and soluble phenols analysis were performed on the aqueous extract samples. Reducing sugars were determined colourimetrically by the dinitrosalicylic acid (DNS) method using glucose as the standard [13] and the absorbance was read at 540 nm using a UV-Vis spectrophotometer. In order to minimize sugar variation, each samples contained 3 replications.

2.5. Total soluble phenols
Phenols were measured colourimetrically using the Folin-Ciocalteu 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 765 nm [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).

2.6. pH
pH testing was carried out on the incubated extract using a pH meter.
2.7. Amount of lignin
1 g sample (a) was refluxed with the addition of 150 mL distilled water 100 °C for 2 hours using water bath 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. 10 mL of 72% H$_2$SO$_4$ 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), and the ash content was measured (e).

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

(1)

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 until 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 pre-treatment.

2.9. Ethanol concentration
CBP was performed after delignification with the addition of 200 mL of fermented culture (10 g/L yeast extract, 10 g/L KH$_2$PO$_4$, 2 g/L (NH$_4$)$_2$SO$_4$, and 0.5 g/L MgSO$_4$·7H$_2$O, pH 6.0) and incubated at 28 °C for 5 days. After the CBP stage finish, the extracts of OPEFB were filtered and tested for ethanol concentration using alcoholmeter.

2.10. Statistical analysis
The data obtained was analysed using multivariate variance (MANOVA) and followed with Tukey test.

3. Results and Discussion
3.1 Total reducing sugars (TRS), total soluble phenols (TSP), pH, lignin concentration and weight loss
The amount of TRS released from the breakdown of OPEFB lignocellulose using Phlebia sp. MG-60 could be used as indicator the effectiveness of fungus breakdown cellulose and hemicellulose from OPEFB. The effect of addition MnSO$_4$ and sea salt during the fungal growth showed significantly different on the increasing of the amount of sugars released (range from 17.287-22.477 mg/g) (M1S1) (Figure 1a). Using the addition of 300 mg/L MnSO$_4$ and 3% of sea salt, the results showed the highest amount of sugar produced by Phlebia sp. MG 60 (22.477 mg/g). However, the lowest amount of sugars was obtained from the substrate which was incubated for 30 days and added with 600 mg/L MnSO$_4$; 5% sea salt (M2S2) (Figure 1a). This indicated that the addition certain amount of MnSO$_4$ and sea salts on substrate of OPEFB could potentially increase the production of enzymes (manganese peroxidase), which were found to play a role in the breakdown of cellulose and hemicellulose.

Similar to the pattern of TRS, the highest amount of total soluble phenols was produced by the addition of 300 mg/l MnSO$_4$ and 3% sea salt incubated for 20 days. The amount of total soluble phenols released representing to the ability of the fungus depolymerizes lignin. In contrary, when the MnSO$_4$ was added at higher concentration (600 mg and 900 mg) to the OPEFB substrate, followed by the addition of sea salt 3-5%, then the results of amount soluble phenolics compounds released are lower compared to the control (M0S0) (Figure 1b).
**Figure 1.** The effect of addition MnSO$_4$ (M) and sea salt (S) to the pattern of lignocellulose degradation from OPEFB using *Phlebia* sp. MG-60, which showed to the production of a). Total reducing sugars (mg/g); b). Total soluble phenols (mg/g); c). pH; d). lignin concentration (%); and e). weight loss (%).
The value of pH (Figure 1c) representing to the amount of organic acid releases during lignocellulose degradation using fungus. Some organic acids are extracellularly produced during fungal metabolism. The present organic acid could potentially help on generating the hydroxyl radicals through Fenton reaction. The decreasing of pH during 30 days of cultures could be generated by the accumulation of organic acid produced during the period of cultures (30 days). The range of the pH from the lignocellulose started from pH 7.0 in the beginning of incubation periods and reached the lowest pH 6.2 at 30 days incubation. The decrease in pH was suspected because of the fungus has the ability to form secondary metabolites such as organic acids so that it can reduce pH [15]. According to Tuomela et al. [16], pH decreases due to accumulation of organic acids during degradation, including oxalic and malic acids.

The measurement of lignin concentration representing the amount of lignin remains after 30 days of incubation. The percentage of lignin in all treatments decreased due to degradation of lignocellulose by the Phlebia sp. MG-60. The addition MnSO₄ and sea salts at certain incubation time had significant differences (significance value <0.05). The changes of lignin percentage during the fungus growth in OPEFB biomass can be seen in Figure 1d. The lowest percentage of lignin (18.033%) occurred at 30 days incubation (in all treatments).

The highest percentage of lignin breakdown by the fungus was achieved from the treatment M2S0 which means the higher amount of ligninolytic enzymes produced in white rot Phlebia sp. MG-60 is when the substrate contains 300 mg/L MnSO₄ with no addition of sea salt. While when MnSO₄ was not present on the substrate or when the substrate contains high amount of MnSO₄ (600 mg/L) with addition 5% of sea salt, the results showed that the lignin concentration are still high compare to other treatment. This indicates that in order to breakdown or depolymerized lignin effectively the substrate needs certain amounts of MnSO₄ and sea salt to guarantee the fungus growth optimally. The presence of lignin degrading enzymes in the form of laccase, LiP and MnP showed that the enzymes are able to degrade lignin. LiP oxidizes lignin non-phenolic units through the release of one electron and forms a cation radical, which then decomposes chemically. LiP can break the Cα-Cβ bonds of lignin molecules and be able to open lignin rings [17, 18]. While MnP oxidizes Mn²⁺ to Mn³⁺ [19]. The high reactive properties of Mn³⁺ then oxidize lignin phenolic rings into unstable free radicals and are followed by spontaneous lignin decomposition [18, 19].

The significant weight loss on the lignocellulose degradation by Phlebia sp. MG.60 was obtained after 10 days of incubation. Weight loss is one of parameter showed the level of lignocellulosic sample degrade by the fungus. The higher the degradation of lignocellulose, the greater the weight loss achieved [20]. The highest weight loss of the OPEFB substrate (16.23%) was obtained from sample consists 600 mg/L of MnSO₄ without the presence of sea salt.

3.2. Ethanol production (%)
The results of the MANOVA analysis of bioethanol levels were found that the addition MnSO₄, sea salt, at certain time incubation, have significantly affected to the ethanol content of fermented OPEFB extract. The highest amount of ethanol was obtained from M1S1 (0.713%), followed by treatment M1S0 (0.630%), M2S0 (0.533%), and M0S0 or control (0.52%). The increase in ethanol levels occurs with the increase of time incubation, which then significantly enhance the ethanol production in medium [21]. The ethanol content of alkaline pre-treatment bagasse degradation and CBP fermentation using Phlebia sp. MG-60 in semi-aerobic conditions obtained the highest value of 0.35% at 10 days incubation period [22]. The highest ethanol concentration was obtained from OPEFB substrate added with of 300 mg / L of MnSO₄ and 3% sea salt (Figure 2).
The ethanol production from fermentation of OPEFB is thought to be correlated to the amount of TRS released from substrate. It was expected that the addition of MnSO₄ and sea salt can increase the ability of *Phlebia* sp. MG. 60 to degrade lignocellulose. The present of certain amount of MnSO₄ added on the substrate in this study have stimulated the manganese peroxidase enzyme activity, it is therefore the ability of the fungus to breakdown lignocellulose is increased. The structure of cellulose and hemicellulose were breakdown by the fungus to produce glucose or xylose (simple sugars), which then were converted to ethanol through the fermentation stage using *Phlebia* sp. MG-60. According to Wang et al. [9] white rot fungus *Phlebia* sp. MG-60 produces ethanol via the glycolytic pathway which is the same as the mechanism in *Saccharomyces cerevisiae*, and produces ethanol efficiently by involving all genes involved in glycolysis. Some enzymes involved on the glycolysis of *Phlebia* sp. MG-60 are hexokinase, glucose-6-phosphate isomerase, 6-phosphofructokinase, fructose-bisphosphate aldolase, triosephosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, 2,3-bisphosphoglycerate-independent phosphoglycerate mutase, enolase, and pyruvate kinase.

4. Conclusions
The addition of MnSO₄ and sea salt on substrate revealed to increase the ability of *Phlebia* sp. MG-60 to breakdown lignocellulose and increase ethanol concentration. Based on MANOVA analysis all assays proved that the effect of the addition MnSO₄, sea salt, and its interactions showed significantly different (p <0.05). The highest percentage ethanol (0.713%) was obtained from OPEFB substrate added with 300 mg / L MnSO₄ and 3% sea salt.

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