Potential of basidiomycetes *Marasmiellus* sp. and *Ganoderma lucidum* in xylanase enzyme production and its activity using agroindustry waste

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Abstract. Nowadays, agro-industrial waste such as kemiri sunan (*Reutealis trisperma* (Blanco) Airy Shaw) shell is abundant along with the development of biodiesel production made from kemiri sunan. The production of xylanase from kemiri sunan waste was carried out by solid state fermentation method using Basidiomycetes *Marasmiellus* sp and *Ganoderma lucidum*. Enzyme activity of xylanase was tested by measuring the amount of reducing sugar liberated from the medium by reactants using DNS method. The highest of enzyme activity resulted by fermentation using 4% of substrate concentration either by *Marasmiellus* sp or *G. lucidum*. Fermentation by *Marasmiellus* sp produced the highest xylanase enzyme activity amounted for 190.5 U ml⁻¹ within 48 hours. Meanwhile, the highest enzyme activity of xylanase fermentation by *G. lucidum* was at the value of 121.9 U ml⁻¹ within 72 hours and it still showed a tendency to increase.

1. Introduction
Currently biodiesel has been known as a diesel fuel substitute so it can also be called biosolar. Biodiesel was previously produced from palm oil, considering that palm oil is also used in the production of edible oil, of course its use as a biodiesel feedstock can disrupt the stability of prices and the availability of feedstock oil. One of natural sources that can be used as raw material for biodiesel production is kemiri sunan (*Reutealis trisperma* (Blanco) Airy Shaw). Kemiri sunan is known as toxin hazelnut that cannot be consumed so that its use as a raw material for biodiesel will not affect the availability of food. Kemiri sunan consists of shells (35 - 45%) and fruit flesh (55-65%). The portion that is used in biodiesel production is fruit flesh, while in the processing process the shell becomes waste. With the increasing use of kemiri sunan in biodiesel production, kemiri sunan shell waste will certainly increase.

Kemiri sunan shell is a lignocellulose material with consisting components of cellulose, hemicellulose and lignin which can be reused in producing biochemical materials. Xylanase enzyme is one of the economically valuable biochemical materials that can be produced from hemicellulose
components. Xylanase enzyme can be used in the food and chemical industries as a reaction catalyst in the production of xylose sugar, paper bleach, improving animal feed nutrition, and purifying in fruit juice and wine production. Nowadays, Indonesia still imported xylanase with a value of $1.4 billion per year. In the other hand, Indonesia has the potential to produce xylanase independently. The high hemicellulose content in kemiri sunan shell waste makes it possible to produce xylanase by fermenting microorganisms with xylan substrates. In this experiment, microbial xylanase production was carried out by solid state fermentation (SSF). SSF is a method of substrate fermentation in high humidity conditions with growth media that has sufficient water availability [1]. The advantages of SSF are cheaper fermentation media, equipment and simple operating arrangements but the high number of products produced [13], low energy requirements, easier scaling up process, high product stability and easier containment control due to low moisture content during fermentation [9]. Microorganisms used in xylanase production are groups of fungi including *G. lucidum* and *Marasmiellus* sp. Both of these fungi can be used to produce enzymes using lignocellulose substrate [12].

### 2. Materials and methods

#### 2.1. Raw materials preparation

Kemiri sunan shell waste was collected from Post-harvest Laboratory Department of Agriculture Industrial Technology, Universitas Padjadjaran located in Jatinangor, Bandung-Indonesia. Shell was the part unused material in preparation of kemiri sunan in order to produce biodiesel. Shell was washed using tap water then oven dried at 105°C overnight. Dried shell waste was grinded and sieve using tyler 80 mesh.

#### 2.2. Inoculum fungal preparation

The fungal used in this research were *G. lucidum* and *Marasmiellus* sp, both of fungal were preserved in potato dextrose agar (PDA) slants followed by incubated at 30°C within 72 h. Inoculum was prepared by suspending the fungal spores in 50 ml of sterile 5% sodium chloride.

#### 2.3. Medium cultivation preparation

Media for fungal cultivation was prepared by mixing dried kemiri sunan shell waste in liquid moistening solution with optimization according to Mardawati [11]. The moistening solution were contained (NH₄)₂SO₄, KH₂PO₄, Urea, CaCl₂ and MgSO₄.7H₂O. After the medium was homogenously mixed, the process was continued with sterilisation using autoclave at 121°C for 15 minutes.

#### 2.4. Production of xylanase enzyme

Xylanase enzyme produced by solid state fermentation and optimized various substrate concentration (30%, 40%, 50% and 60%) and cultivation time (24 h, 48 h and 72 h). Medium cultivation during fermentation was incubated at 30°C within interval times. Xylanase enzyme harvested by mixing the fermented solid solution using 40 ml of distilled water then rotate it with orbital shaker at 1000 rpm for 1 h continued by filtered solution and filtrate which defined as crude xylanase enzyme extract.

#### 2.5. Analysis of xylanase activity

The xylanase activity was determined with DNS methode described by Mardawati [10]. Substrate used in this method was larch wood xylan (Sigma Co., USA).

DNS test starting by create standard xylose curve, making 400 ppm xylose stock solution (10 mg xylose in 100 ml distilled water) continuous by pour it into 0 ppm, 20 ppm, 40 ppm, 60 ppm, 80 ppm, 100 ppm, 120 ppm, and 160 ppm. This procedure was carried out by taking 0 ml, 0.2 ml, 0.4 ml, 0.6 ml, 0.8 ml, 1.1 ml, 1.2 ml and 1.4 ml from the 400 ppm solution previously prepared. Each of stock solution was placed in a test tube and sterile distilled water was added to a total volume of 4 ml of solution. Taken 2 ml of the mixed solution from each of the test tubes into a new test tube, then reacted with 2 ml of DNS solution by heating the mixture in a waterbath for 5 minutes at 100°C.
After the solution cooled following by measurement of absorbancy at \( \lambda = 550 \text{ nm} \). The absorbance value of each concentration was plotted into graphical form.

The DNS test was conducted by preparing of xylose standard through mixing 0.5 ml of distilled water with 0.5 ml of 1% xylan and adding 0.5 ml of acetate buffer pH 5 and incubated with a water bath at 40°C for 15 minutes. The reaction was stopped by adding 1.5 ml of DNS and then boiling it on the water bath for 5 minutes, the measured absorbance solution with a spectrophotometer at \( \lambda = 550 \text{ nm} \). The xylose concentration on sample was calculated using Equation 1.

\[
y = ax + b
\]  

The absorbance value obtained from is converted to xylose concentration (mg/ml) with the xylose standard curve. Xylose analysis in the sample was carried out by dissolving 0.5 ml of crude xylanase enzyme extract in 0.5 ml sterile distilled water and mixing it with 0.5 ml of 1% xylan. The mixture was added with 0.5 ml acetate buffer pH 5 and incubated with a water bath at 40°C for 15 minutes. The reaction was stopped by adding 1.5 ml of DNS then boiling on a water bath for 5 minutes, the measured absorbance solution with spectrophotometer at \( \lambda = 550 \text{nm} \). Then the absorbance value is converted to xylose concentration (mg/ml) with a standard xylose curve. The equation tested for xylanase enzyme activity shown in equation 2:

\[
U = (K_{sp} - K_{ks}) \frac{1000 F_p}{B M_{xylose} t . V}
\]  

Noted:
- \( U \): enzyme activity (U/ml or \( \mu \text{mol}/(\text{minute, ml}) \))
- \( K_{ks} \): xylose on enzyme extract (mg/ml)
- \( K_{sp} \): xylose on standard (mg/ml)
- 1000: conversion factor (\( \mu \text{mol} \))
- \( F_p \): xylanase enzyme dilution factor
- \( B M_{xylose} \): molecular weight of xylose (150.13 g/mol)
- \( T \): incubation time (minute)
- \( V \): enzyme volume used in the analysis (ml)

The research method used was an experimental method which was analyzed descriptively.

3. Results and discussion

Xylanase enzyme activity was measured based on the concentration of reducing sugar (xylose) formed from pure xylan as a substrate. The higher of xylose content measured, the higher of xylanase enzyme activity. The measurement method used DNS (3,5-dinitrosalicylic acid), an aromatic compound that will react with reducing sugars and other reducing components to form 3-amino-5-nitrosalicylic acid, a compound that is able to absorb electromagnetic wave radiation strongly at 540 nm [10]. The average of obtained enzyme activity produce in each substrate concentration is describe in Table 1.

| Cultivation Time (hour) | Xylanase Enzyme Activity in Each Substrate Concentration (U/ml) |
|------------------------|---------------------------------------------------------------|
|                        | 3%                      | 4%                      | 5%                      | 6%                      |
| GL                     | ML                      | GL                      | ML                      | GL                      | ML                      |
| 24                     | 3,178.4                 | 2,616.0                 | 13,539.8                | 13,919.7                | 3,030.4                 | 2,771.4                 | 3,341.3                 | 3,237.6                 |
| 48                     | 2,949.0                 | 6,090.7                 | 14,462.5                | 12,707.2                | 3,000.8                 | 3,015.6                 | 2,542.4                 | 2,916.5                 |
| 72                     | 3,400.5                 | 2,764.0                 | 15,737.9                | 9,754.2                 | 2,654.6                 | 1,943.7                 | 3,044.8                 | 2,547.7                 |
Substrate concentration that can produce crude xylanase extract with higher activity among some substrate concentrations tested is at 4% substrate concentration. Substrate concentration is related to the water content of the cultivation medium. Water content is the most important factor determining the success of solid phase fermentation processes [16]. Higher substrate concentration of water content of cultivation medium will be lower. If the water content of the SSF process is too high, the substrate porosity will decrease as a result the particle size and texture of the substrate change and oxygen transfer becomes low. Conversely, if the water content is too low it will reduce the nutrient solubility of the substrate so that microbial growth is disrupted and enzyme production is inhibited. Moisture content affects the physical properties of solid substrates used which also influence microbial growth and product biosynthesis [20].

![Figure 1](image.png)

**Figure 1.** Xylanase enzyme activity *G. lucidum* (a) and *Marasmiellus sp* (b) at some of cultivation time.

Changes in xylanase enzyme activity over time can be seen in Figure 1. Enzyme production by using *G. lucidum* still increased until the end of the cultivation time at 72 hours of fermentation. It showed that *G. lucidum* has higher viability than *Marasmiellus sp*. *G. lucidum* is known as a medical mushroom that has antitoxic properties [19]. Antitoxic properties can make *G. lucidum* reacted more optimally than that *Marasmiellus sp* because this fungus as naturally has a mechanism of resistance against other microorganisms that can interfere with other product biosynthesis and growth. Laccase enzymes are widely distributed in organisms among the fungus, bacteria, plants, and insects [4]. Basidiomycota, including *Ganoderma* dan *Marasmiellus* are commonly suggested for potent degradation of lignin and laccase production [2,5]. Various biomasses have been employed for delignification through microbial systems such as *Ganoderma lucidum, Schizophyllum commune, Marasmiellus palmivorus, Peniophora sp* [6, 7, 14, 17]. To produce high volume of synthesis of enzymes, the agricultural waste residues utilization has been an attractive and cost-effective approach. Some of the agroindustry waste residues potentially used include banana peel, apple pomace, brewery waste, wheat straw, wheat bran, rice straw, rice bran, sugarcane bagasse, etc. [3, 8, 15, 18]. Kemiri sunan shell as biodiesel production waste has also potentially used as a substrate in xylanase enzyme production. Thus, the xylanase production using kemiri sunan shell waste can be more effective, eco-friendly and low cost.
4. Conclusion
The optimum condition of fermentation using kemiri sunan shell waste pre-treated with autoclave was at 4% substrate concentration (4 g substrate /10 ml moistening solution) and cultivation time of 72 hours by *G. lucidum* and 24 hours by *Marasmiellus* sp.

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