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Optimization of Cocoa Bean Husk Fiber Delignification Using Marasmius sp. via Solid-State Fermentation

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Abstract: Cocoa bean shell fiber is an abundant lignocellulosic by-product from the cocoa agroindustry with high cellulose potential but limited utilization due to its lignin content. This study aimed to optimize the delignification of cocoa bean shell fiber using Marasmius sp through solid state fermentation (SSF) as an environmentally friendly approach to obtain cellulose-rich fibers as precursors for agro-industrial applications. Delignification was evaluated under various fermentation parameters, including pH (4–6), glucose concentration (2–5 g/L), temperature (27–37°C), and incubation time (7–14 days). Lignin content was determined using the Van Soest method, while fiber morphology was observed using Scanning Electron Microscopy (SEM). The optimum SSF conditions were achieved at pH 5, glucose concentration of 5 g/L, temperature of 32°C, and incubation time of 14 days, resulting in lignin reduction of 70.29% ± SD without significant damage to the cellulose structure. These results indicate that SSF using Marasmius sp is an effective and sustainable delignification strategy to produce cellulose-rich fibers suitable as nanocellulose precursors for agricultural and agro-industrial materials.

Keywords: Agro-industrial material; Cocoa bean shell fiber; Delignification; *Marasmius* sp; Solid state fermentation.

Introduction

Indonesia is one of the world's largest cocoa producers, with production volumes continuing to increase. However, this increase in production is directly proportional to the accumulation of biomass waste. This large amount of biomass waste remains largely unutilized (Guo et al., 2024; Tripathi et al., 2019). One such waste is cocoa bean hulls, which originate from post-harvest and cocoa processing. This waste is classified as lignocellulosic biomass with a high cellulose content, thus offering potential use as a value-added raw material in the agricultural and derivative industries (Ojo, 2023; Okolie et al., 2020; Periyasamy et al., 2022).

Cocoa bean hulls (CBC) are a major byproduct that are often discarded or used as low-quality animal feed (Yuan et al., 2018). Cocoa bean hulls are waste containing fibers classified as lignocellulosic biomass (Diaz et al., 2022). This waste is generally not optimally utilized and has the potential to cause environmental problems (Rasyid et al., 2020). Cocoa bean hull fiber contains high levels of cellulose, but the presence of complex and resistant lignin is a major obstacle to its utilization (Makaveckas & Šimon, 2025). Structurally, CBC contains significant lignocellulosic components, namely cellulose, hemicellulose, and lignin (Panahi-Sarmad, 2024; Ali et al., 2023). Lignin acts as a physical and chemical barrier (recalcitrance) that protects cellulose from degradation, therefore cellulose isolation requires effective pretreatment (Brienza et al., 2024).

Delignification is a crucial step in breaking down the lignocellulose structure, allowing further utilization. Chemical delignification methods using strong acids or bases have been widely used, but they pose environmental challenges and increase waste treatment costs (Siefkes et al., 2020). Therefore, alternative, more

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environmentally friendly and sustainable (green) methods are needed, or a biological approach using microorganisms that produce ligninolytic enzymes is a more environmentally friendly alternative.

White-rot fungi such as *Marasmius* sp. are known to produce ligninolytic enzymes, including laccase, lignin peroxidase, and manganese peroxidase, which effectively degrade lignin. Solid-state fermentation (SSF) provides suitable conditions for fungal growth and enzyme production and is considered more efficient for solid biomass processing. Biological delignification using white-rot fungi (WROF) offers a selective approach. Fungi from the genus *Marasmius* sp. are known to possess a robust ligninolytic enzyme system, particularly laccase (Lac) and manganese peroxidase (MnP), capable of degrading lignin polymers without damaging the crystalline structure of cellulose (Leonowicz et al., 2001; Nahas et al., 2021; Torres-Farradá et al., 2024). The use of the Solid-State Fermentation (SSF) method in this process offers the advantages of minimal water use, higher enzyme concentrations, and an environment that mimics the fungus's natural habitat, resulting in a more efficient delignification process (Mannina et al., 2020).

Several studies have described the successful delignification of lignocellulosic biomass using SSF however, studies on optimizing the fermentation conditions of Marasmius sp on cocoa bean husk fiber as a raw material for nanocellulose precursors are still limited. Therefore, this study aims to optimize the delignification conditions of cocoa bean husk fiber using *Marasmius* sp through SSF and evaluate the characteristics of the delignified fiber as a nanocellulose precursor that has the potential to be applied in the food sector. The novelty of this research lies in the specific use of *Marasmius* sp. on cocoa bean hull substrates with SSF parameter optimization as the initial step (precursor) for nanocellulose production. Nanocellulose has great potential in the agricultural industry (García et al., 2016).

Method

Materials

Cocoa bean husk (CBH) used in this study was obtained from cocoa plantation waste in Gunung Kidul, Yogyakarta, Indonesia. The husks were cleaned to remove adhering impurities, dried in a hot-air oven at 60°C until a constant weight was achieved, ground using a laboratory mill, and sieved to obtain particle sizes ranging from 40 to 60 mesh. The chemicals used included glucose, sodium chloride (NaCl), distilled water, and reagents required for lignin determination following the Van Soest method. A pure culture of *Marasmius* sp. was used as the delignifying microorganism. The fungal culture was rejuvenated on Potato Dextrose Agar (PDA) medium and incubated at room temperature for 7 days prior to inoculum preparation.

Determination of Optimum Inoculum Age

The optimum inoculum age was determined by monitoring fungal growth in Potato Dextrose Broth (PDB) medium for 7 days. Fungal biomass development was observed daily to construct a growth profile. The inoculum age corresponding to the middle of the exponential (logarithmic) growth phase was selected for subsequent fermentation experiments to ensure maximum metabolic activity during the delignification process.

Delignification of Cocoa Bean Husk by Solid-State Fermentation

Delignification was carried out using the solid-state fermentation (SSF) method. A predetermined amount of cocoa bean husk substrate was adjusted to the desired moisture content and supplemented with glucose as an additional carbon source. The substrate was inoculated with *Marasmius* sp. and incubated under various fermentation conditions. The experimental variables consisted of pH (4, 5, and 6), glucose concentration (2, 3.5, and 5 g/L), incubation temperature (27, 32, and 37°C), and incubation period (7, 10.5, and 14 days). Fermentation was conducted in triplicate for each treatment combination. At the end of the incubation period, the fermented substrate was dried and subjected to lignin analysis.

Lignin Analysis

Lignin content before and after fermentation was determined using the Van Soest fiber analysis method. The percentage of lignin reduction was calculated using Equation (1):

$$\text{Lignin reduction (\%)} = [(L_0 - L_t) / L_0] \times 100 \quad (1)$$

where L_0 represents the initial lignin content (%) of untreated cocoa bean husk and L_t represents the lignin content (%) after fermentation treatment.

Morphological Characterization

Morphological changes in cocoa bean husk fibers before and after delignification were examined using Scanning Electron Microscopy (SEM). Samples were dried, mounted on aluminum stubs, coated with a thin conductive layer, and observed at appropriate magnifications to evaluate surface structure alterations resulting from fungal degradation.

Experimental Design and Statistical Analysis

The experiment was conducted using a completely randomized design (CRD) with variations in pH,

glucose concentration, incubation temperature, and incubation time as treatment factors. Each treatment was performed in triplicate. Experimental data were expressed as mean ± standard deviation. Statistical analysis was performed using analysis of variance (ANOVA) at a 95% confidence level to evaluate the significance of treatment effects. When significant differences were detected ($p < 0.05$), further comparison among means was conducted using Duncan's Multiple Range Test (DMRT). The optimum fermentation conditions were determined based on the highest percentage of lignin reduction.

Result and Discussion

Inoculum Determination

The dry weight of Marasmius sp was observed for 156 hours (7 days). In each batch of Marasmius sp growth curves whose dry weight was to be measured, it was found that the growth of Marasmius sp in PDB (Potato Dextrose Broth) produced round and hairy pellets. The dry weight of Marasmius sp is depicted and can be observed in Figure 1.

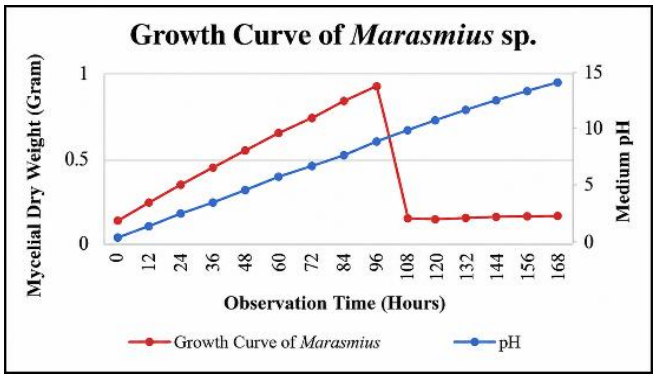


Figure 1. Growth Curve of Marasmius Sp

The growth curve of *Marasmius* sp at 0-24 hours did not show a significant increase. *Marasmius* sp at 0-24 hours was a lag phase. The 24th hour to the 72nd hour showed a significant increase, and appeared to be a log phase, while at the 72nd to the 96th hour there was no increase in the growth graph, but at the end of the 96th hour there was a significant spike in the graph until the 144th hour. The research data showed the occurrence of a diauxic affect event.

The PDB medium used in this study is a PDB medium made manually from potato extract. Potatoes contain a wide variety of nutrients, including carbohydrates (glucose, dextrose, and starch), fat, minerals, and vitamins (Beals, 2018; Zaheer & Akhtar, 2016). Potato extract contains a greater variety of these nutrients, albeit at very low concentrations, resulting in fungal growth exhibiting a diauxic phenomenon due to the increased diversity of carbon (C) sources in the medium during the observation process. The increase in pH occurs due to the activity of Marasmius sp which can break down glucose and is able to produce polysaccharides that are basic in nature (Yuliana et al., 2024). By determining the optimum inoculum age of ½ log phase, the maximum inoculum age of *Marasmius* sp was 84 hours.

Delignification Process with Solid State Fermentation

Based on the results of measuring lignin content from 7 variations of optimization of the cocoa bean skin fiber delignification process treatment. The SSF method by *Marasmius* sp. was incubated at the same time for 14 days. The results of lignin content measurements in each variation of fermentation condition optimization showed different decreases in lignin content. It appears that the greatest decrease in lignin content occurred when the SSF delignification method by Marasmius sp. was under regulated substrate conditions, namely at pH 5, the addition of a glucose concentration of 5 g/L and the fermentation process took place at a temperature of 32°C. Under these conditions, the fermentation process was able to produce a decrease in lignin content of 70.29% as shown in Figure 2.

The laccase enzyme is the enzyme with the highest production produced by *Marasmius* sp. compared to manganese peroxidase (MnP) and lignin peroxidase (LiP) (Ali et al., 2023; Shi et al., 2020). All three are extracellular enzymes produced when the fungus enters the secondary (idiophasic) metabolic phase driven by a lack of nitrogen, carbon, and sulfur nutrients. Generally starting when glucose levels are low, this condition triggers *Marasmius* sp. to produce extracellular enzymes (Daly et al., 2019). The presence of these three extracellular enzymes plays a role in degrading lignin in the lignocellulose and hemicellulose structures so that cellulose and its contents can be used as a carbon source again for the growth of *Marasmius* sp. (Ramadiyanti et al., 2020)

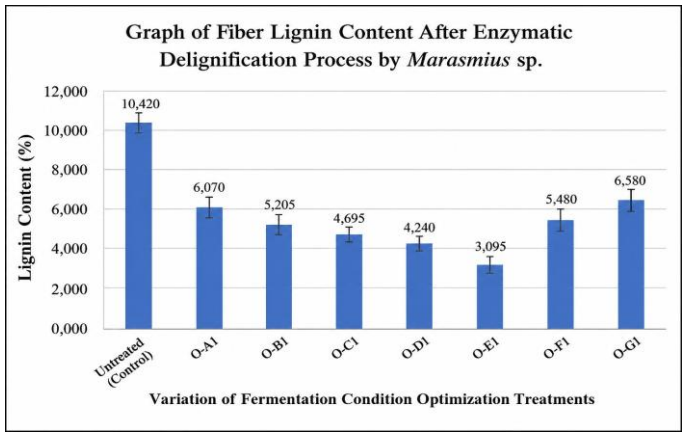


Figure 2. Variation of Fermentation Condition Optimization Treatment

Therefore, the lower the glucose concentration added to the medium, the higher the induction of laccase, MnP, and LiP enzyme production. However, the study found that the combination of adding a glucose concentration of 5 g/L actually resulted in the highest decrease in lignin levels compared to the lowest glucose concentration of 2 g/L. This is because the appearance of each enzyme is largely determined by the combination of C and N source limitations in the medium (Mita, 2020). Beginning with the growth of *Marasmius* sp., the C source contained in the medium will be utilized for the growth and increase of fungal biomass. When the C source concentration begins to be limited, the growth of *Marasmius* sp. will begin to slow and stop temporarily. The transition of the *Marasmius* sp. metabolic system is initiated to seek other C sources by degrading or damaging the lignin structure in lignocellulosic waste used as a growth substrate, thus facilitating access to cellulose and hemicellulose in lignocellulosic waste which will be used as a C source instead of simple sugars such as cellulose and hemicellulose. The first extracellular enzyme that is actively working and produced in degrading lignin is the laccase enzyme, the highest production of which is produced by *Marasmius* sp (Yuliana et al., 2024).

This study reduced lignin levels in variations of 5 g/L glucose concentration additions by the laccase enzyme after 7 days of incubation. The growth substrate of Marasmius sp containing glucose supports rapid hyphal growth, the formation of extracellular glucans, and the de novo synthesis of secondary metabolites, such as veratryl alcohol. The presence of veratryl alcohol can catalyze ligninase in vitro (Ramadiyanti et al., 2020). The best pH and temperature parameters that show the best reduction in lignin levels are medium pH 5 at an incubation temperature of 32°C. According to Tri et al (2020), a good medium pH for the growth of Marasmius sp is at pH 4, especially for the production of extracellular enzymes and the induction of enzyme activity. The experimental results are less in accordance with the literature, which states that in the experiment the best growth of Marasmius sp was found to be seen at a medium pH of 5. The growth and induction of lignin degrading enzyme performance continued to work well in the fermentation process because, before inoculating fungal growth on the substrate, the substrate containing cocoa bean husk fiber and a pH-adjusted alkaline medium was sterilized first using moist heat at a temperature of 121°C. This condition makes it so that in the early stages of the delignification process, lignin levels have been degraded and lignin is released which makes it easier for Marasmius sp to re-degrade the lignin structure, so that the medium pH setting range of 4-5 does not affect growth Figure 2.

Marasmius sp in the delignification process as in Figure 2, the data is strengthened based on the measurement of lignin optimization content for the long fermentation time of the purpose of delignification fermentation day found that the 0th fiber, without inoculum has shown a decrease in lignin of 3% of the levels shown in Figure 2. While incubation on the 14th day showed the highest decrease in lignin levels in accordance with Shao research (2019) which obtained laccase enzyme production after 7 days and on the 10th day was the highest activity of the MnP and LiP enzymes that work when induced by the addition of a mediator in the form of ABTS.

The incubation temperature of the fermentation process shows a temperature of 32°C as the best incubation temperature because at an incubation temperature of 27°C there was no visible growth of *Marasmius* sp and at a temperature of 37°C the growth of *Marasmius* sp was very slow, in accordance with the literature, that *Marasmius* sp is a mesophilic fungus that can grow at a maximum temperature of 35°C and optimum growth at a temperature of 30°C which correlates with the optimum conditions for Marasmius sp to produce MnP and LiP enzymes (Mita, 2020). Based on the research results, it was found that the decrease in lignin levels in cocoa bean skin fibers occurred during the 14-day incubation period under various group conditions, with a medium pH of 5, the addition of a glucose concentration of 5 gr/L and incubation at a temperature of 32°C of 70.29%.

Optimization of incubation time

The results of this study indicate that Marasmius sp. in a Solid State Fermentation (SSF) system can reduce lignin levels by up to 70.29% under optimum conditions (pH 5, glucose 5 g/L, temperature 32°C, and 14 days of incubation). This finding aligns with recent research showing that white-rot fungi have a high capacity to degrade lignin through the production of ligninolytic enzymes such as laccase and peroxidase under acidic conditions and mesophilic temperatures (±30–35°C).

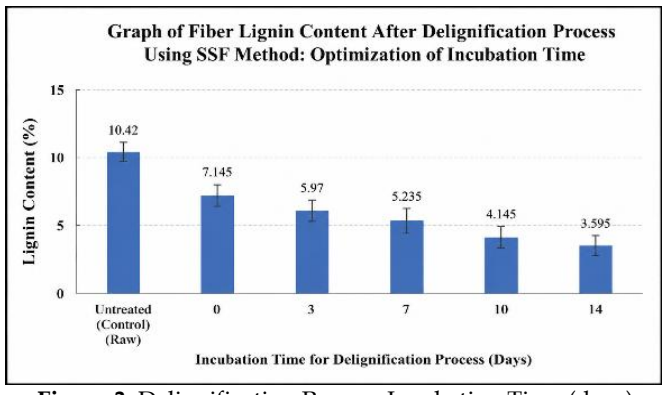


Figure 3. Delignification Process Incubation Time (days)

Table 1. Optimization of delignification conditions of cocoa bean husk fiber using the SSF method by *Marasmius* sp.

pH	Glucose (g/L)	Temperature (°C)	Time (days)	Lignin Reduction (%)
4	2	27	14	42.15
4	5	32	14	55.30
5	2	32	14	61.80
5	5	32	14	70.29
6	5	32	14	48.60
5	5	27	14	39.75
5	5	37	14	45.10

Research by Hidayatullah et al. (2021) on the delignification of oil palm empty fruit bunches using Marasmius sp. also reported that this fungus was able to achieve significant lignin degradation (approximately 26.67% at 20 days of incubation), with further increases with longer incubation times of up to 30 days under optimized SSF conditions. This supports the findings of this study that incubation time and nutritional conditions play a significant role in increasing delignification efficiency.

Furthermore, a study by (Z. Wang et al., 2023) showed that inoculation of Marasmius tricolor onto a lignocellulosic substrate significantly increased the lignin degradation rate and altered the microbial community structure during the decomposition process, indicating the adaptive capacity of the Marasmius genus to degrade complex lignocellulosic materials. This confirms the effectiveness of this genus in the SSF bioprocess for biomass delignification.

Optimization of SSF Delignification Conditions

The results showed that optimal SSF fermentation conditions at pH 5, glucose concentration 5 g/L, temperature 32°C, and incubation time 14 days resulted in a lignin reduction of 70.29% ± SD. This finding aligns with various recent studies confirming that white rot fungi, including the genus Marasmius, exhibit optimal ligninolytic activity at a slightly acidic pH and mesophilic temperature (30–35°C), as these conditions support the activity of enzymes such as laccase, lignin peroxidase, and manganese peroxidase in the delignification process.

Research by (Laokor & Juntachai, 2005) showed that Marasmius sp. was able to degrade up to 26.67% of lignin in oil palm empty fruit bunches biomass through the SSF process, and degradation efficiency increased with optimization of the incubation time to 30 days. This reinforces the findings of this study that incubation time is a critical factor in enhancing lignin degradation, although this study demonstrated higher efficiency, likely influenced by additional optimizations such as pH and glucose supplementation.

Furthermore, a study by (Wang et al., 2023) on Marasmius tricolor showed that inoculation of this fungus onto a lignocellulosic substrate increased the rate of lignin degradation and altered the microbial community structure during the decomposition process, indicating that the Marasmius genus has a high adaptive capacity for biodelignification of complex lignocellulosic materials. Other studies have also shown that the addition of simple carbon sources such as glucose in low amounts can increase the production of ligninolytic enzymes, but excessive concentrations can cause catabolite repression, which actually reduces lignin degradation activity. Therefore, the glucose concentration of 5 g/L in this study is within the optimum range that supports enzyme activity without inhibiting the delignification process.

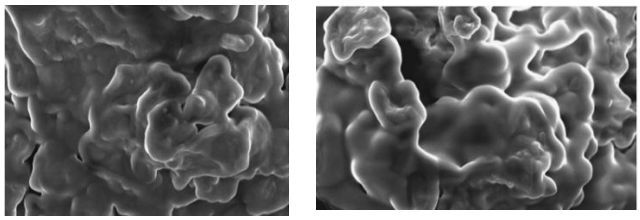
Fiber Morphology Analysis by Scanning Electron Microscopy (SEM)

The morphology of cocoa bean husk fibers resulting from chemical and enzymatic delignification treatment processes is shown in Figure 4 - Figure 5. Observation of the morphology of cocoa bean husk fibers resulting from treatment was carried out using Scanning Electron Microscopy (SEM), showing the morphological structure of cocoa bean husk fibers before the delignification process at a magnification of 3000 times, seen in the morphological structure of cocoa bean husk fibers still in an intact state. On the other hand, Figure 5 shows the morphological structure of cocoa bean husk fibers after the delignification process, the SSF method by Marasmius sp at a magnification of 3000 times, seen in the morphological structure of cocoa bean husk fibers which are larger but still similar to untreated cocoa bean husk fibers.

These results align with research by Okolie et al., (2020), which reported that biological delignification using white rot fungi tends to cause gradual changes in the lignocellulose structure, where lignin is degraded first without directly damaging the primary cellulose structure. This results in fibers that retain their basic shape but become more porous due to the reduction of lignin as a structural "binder." Furthermore, research by (Rohrbach & Luterbacher, 2021) also showed that enzymatic and SSF treatments on lignocellulosic biomass do not always result in extreme structural damage, but rather increased porosity and weakening of the lignin-hemicellulose matrix. These conditions favor further enzyme diffusion into the fiber network.

Another study by Qi et al., (2023) on lignocellulosic biomass showed that white-rot fungi such as Marasmius and Trametes tend to undergo selective delignification, decomposing lignin before cellulose, so that the basic

fiber structure remains visible despite internal changes. However, these results differ from several studies that have shown much more significant morphological changes. For example, research by (Meenakshisundaram et al., 2021) reported that SSF treatment of certain lignocellulosic biomass using white-rot fungi can cause very obvious damage to the fiber structure, such as cell wall rupture, loss of fiber integrity, and a drastic increase in porosity under SEM magnification. Furthermore, (Wang et al., 2020) found that under more aggressive fermentation conditions (extreme pH and longer incubation times), delignification can cause the degradation of not only lignin but also some cellulose, resulting in the fiber structure becoming very brittle and fragmented.



Before After

Figure 4. SEM images of lignocellulosic fiber surface before and after biological delignification

SEM analysis showed that the delignified fibers experienced damage to the lignin matrix and increased openness of the fiber structure compared to the untreated sample. The cellulose fiber structure remained intact, indicating that the SSF process is selective for lignin. These results indicate that biological delignification is able to reduce lignin significantly without damaging cellulose, so that the resulting fibers are suitable for use as nanocellulose precursors.

Conclusion

This study demonstrates that solid-state fermentation using *Marasmius* sp. under optimum conditions (pH 5, glucose concentration 5 g/L, temperature 32°C, and incubation time of 14 days) can achieve a lignin reduction of up to 70.29% in cocoa bean husk fiber. This level of delignification highlights the potential of *Marasmius* sp. as an effective biological agent for lignin removal and contributes to the growing body of research on the valorization of agro-industrial residues through fungal bioprocessing. The findings provide scientific evidence that cocoa bean husk, an underutilized agricultural by-product, can be transformed into a cellulose-enriched material suitable for further development as a nanocellulose precursor for food-related applications. Beyond the observed lignin reduction, this study contributes to the understanding of the interaction between fermentation parameters and fungal delignification performance in lignocellulosic biomass. The results support the feasibility of biological pretreatment as an alternative to conventional chemical methods, which are often associated with higher chemical consumption and environmental burdens. Future research is recommended to evaluate the scalability of the process, characterize the physicochemical properties of the resulting cellulose and nanocellulose, and assess their functional performance and safety in specific food applications.

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Author Contributions

All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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