Characterization of lignocellulolytic fungi isolated from cocoa rhizosphere under different cropping patterns

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Abstract. Cocoa plantations may provide a considerable amount of fungal biodiversity with high potential as soil organic matter biodecomposer agents. Therefore, the isolation and characterization of potential fungi is the first step to obtain the wide variety of lignocellulolytic enzymes. This study aimed at identifying potential lignocellulolytic fungi isolated from different type cocoa cropping pattern. The lignocellulose-degrading enzymes was determined by growing the isolated fungi on Guaiacol containing PDA media, and CMC media with additional of Red Congo solution. Their potential index (IP) was scored by measuring the diameter of the clear zone around the fungal colonies. Our identifications suggest the presence of both ligninolytic, and cellulolytic enzymes through a reddish, and clear zones produced around the colonies. Additionally, we identified two types of potential fungi with higher potential index both ligninolytic and cellulolytic, including strain fungal CSF#5 and CSF#15 which belongs to the group of fungi Ascomycetes.

1. Introduction

The diversity of shade vegetation types and specific microclimate characteristics in cocoa plantations provides a large amount of biodiversity, especially in the soil. Various types of fungi have diverse roles and functions and synergize with each other, one of which is as an agent of organic waste in cocoa plantations. One of the main obstacles in the processing of cocoa plant waste is the long decomposition time due to the structure of cocoa organic matter which is rich in lignocellulose. Lignocellulosic wastes represent about 60% of plant biomass and their disposal usually poses difficulties due to their volume and characteristic hard structure [1]. Biological biodegradation using microbes is considered the safest and most environmentally friendly [2].

In general, fungi have a better ability than bacteria in breaking down plant residues (hemicellulose, cellulose, and lignin) [3]. The growth of hyphae from fungi class Basidiomycetes and Ascomycetes (hyphal diameter 5–20 m) more easily penetrates the walls of tubular cells which are the main constituents of wood tissue accompanied by the release of enzymes that dissolve the cell walls of wood tissue (polymer complexes of lignin, cellulose and hemicellulose) [4]. Several enzymes involved in the breakdown of organic matter include -glucosidase, lignin peroxidase (LiP), manganese peroxidase (MnP), laccase and versatile peroxidase. These enzymes are produced by rot fungi such as Pleurotus eryngii, P. ostreatus, and Bjekandera adusta [5] and Phanerochaete chrysosporium [6] and Trichoderma sp [7].
The use of microbes, especially fungi as bioactivators in lignocellulosic degradation, is a technique that can be applied to increase the effectiveness of lignocellulosic waste degradation. Wood rot fungi and *Trichoderma* sp. can reduce the lignin and cellulose content in OPEFB by 20.83% and 33.77% [8]. Based on the description above, it can be seen that fungi play an important role in the process of biodegradation of lignocellulosic waste. Therefore, in this study, qualitative exploration and selection of potential lignocellulolytic fungi will be carried out on solid media. The results of the study can be used as a recommendation for the use of these fungal inoculants for decomposer agents in the treatment of lignocellulosic waste from cocoa plantations and other organic wastes.

2. Material and Methods

2.1. Isolation of fungi from cocoa plantation

Soil samples from 5 cocoa growing locations with different shade patterns were isolated using PDA media with the dilution method. A total of 10 g of soil samples were suspended in 90 ml of physiological solution, i.e. NaCl 0.85%, with 5 series of dilutions, namely 10⁻¹ to 10⁻⁵. The last three dilution series, 10⁻³, 10⁻⁴ and 10⁻⁵, were taken 100 µl and poured into sterile petri dishes containing PDA media and then incubated at room temperature for 4-8 days [9][10]. After that, the total colonies that grew were calculated using the Total Plate Count method, then purification was carried out with the isolates were coded based on the type of fungus. Each selected fungal strain from the purification results was then selected for its potential in degrading lignin and cellulose.

2.2. Screening test of lignocellulolytic activity

The primary selection was performed using a qualitative method by measuring the ligninolytic and cellulolytic potency indices on petri dishes. Selection of ligninolytic potential was carried out using PDA media supplemented with Guaiacol reagent. The selection of cellulolytic potential using CMC + Congo red media.

2.3 Ligninolytic activity test

Fungal cultures aged ± 7 days were inoculated into PDA media, each of which was added with 0.05% Guaiacol [11][12], then incubated at room temperature for 3-7 days. A positive reaction on Guaiacol media is indicated by the presence of a reddish brown zone around the colony which is an indication of the production of the extracellular enzyme phenol oxidase (laccase) by oxidizing guaiacol [13]. The ligninolytic potency index can be obtained by the following formula:

\[
\text{Lignolytic Potency Index} = \frac{\text{Colored zone area}}{\text{Colony zone area}}
\]

2.4 Cellulolytic activity test

Fungal cultures were inoculated onto 1% Carboxymethyl Cellulose (CMC) media and incubated for 3-7 days at room temperature. Then the petri dish was soaked with 15 ml of 0.1% Congo red solution and let stand for 30 minutes, then rinsed twice using 15 ml of 1 N NaCl and let stand for 15 minutes [14]. A positive reaction was indicated by the presence of a clear zone around the colony which was an indication of cellulase enzyme activity.

\[
\text{Cellulolytic Potency Index} = \frac{\text{Clear Zone Diameter (mm)} - \text{Colony Diameter (mm)}}{\text{Colony Diameter (mm)}}
\]

From the two selections above, fungal isolates that showed positive results from each selection method were morphologically identified to determine the characteristics of fungi in more detail.

2.5 Identification of lignocellulolytic fungi

Identification was carried out morphologically, both macroscopically and microscopically. Macroscopically with the top and bottom surfaces of agar petri dishes by observing the shape, color,
Table 1. Distribution of lignocellulolytic fungal species diversity in 5 different cacao cropping patterns

| Isolate Code | Isolate Source | Zone |
|--------------|----------------|------|
|              | Soil-Lamtoro Shade | Soil-Lamtoro + Coconut Shade | Soil-Lamtoro + Palm Oil Shade | Soil-Lamtoro + Betel Nut Shade | Soil-Paranet Shade | Lignolytic | Cellulolytic |
| CSF#1        | -               | +                | -                | -                | -                | -        | +         |
| CSF#2        | -               | +                | -                | -                | -                | -        | +         |
| CSF#3        | -               | +                | -                | -                | -                | -        | +         |
| CSF#4        | +               | +                | +                | +                | -                | -        | +         |
| CSF#5        | -               | +                | -                | -                | -                | +        | +         |
| CSF#6        | +               | +                | -                | -                | -                | -        | +         |
| CSF#7        | +               | -                | -                | -                | -                | -        | +         |
| CSF#8        | +               | -                | -                | -                | +                | -        | +         |
| CSF#9        | +               | -                | -                | -                | +                | -        | -         |
| CSF#10       | -               | -                | +                | -                | -                | +        | +         |
| CSF#11       | -               | -                | +                | -                | -                | -        | -         |
| CSF#12       | +               | +                | -                | -                | -                | -        | +         |
| CSF#13       | +               | -                | +                | -                | -                | -        | +         |
| CSF#14       | -               | +                | -                | -                | -                | -        | -         |
| CSF#15       | +               | +                | +                | -                | -                | +        | +         |
| CSF#16       | +               | -                | -                | -                | -                | -        | +         |
| CSF#17       | +               | -                | -                | -                | -                | -        | -         |
| CSF#18       | +               | +                | +                | -                | -                | -        | -         |
| CSF#19       | +               | -                | -                | -                | +                | -        | -         |
| CSF#20       | +               | -                | -                | -                | +                | -        | -         |
| CSF#21       | +               | +                | +                | -                | +                | +        | +         |
| CSF#22       | +               | +                | -                | -                | -                | -        | -         |
| CSF#23       | +               | +                | -                | -                | +                | -        | +         |
| Total Types of Fungi | 15 | 10 | 9 | 2 | 7 | 4 | 17 |

CSF#1: Cocoa Soil Fungal No. 1…etc ; +: present; -: absent

The data on the diversity of 23 types of fungi from cocoa plantations in the table above describes the distribution pattern of locations and their ability to degrade lignin and cellulose. The cacao cropping pattern with lamtoro shading had the most diversity, there are 15 species compared to other
shading patterns, while the lamtoro + betel nut shading patterns had the smallest diversity of fungi, namely 2 types of fungi. This is in accordance with the research of Tondok et al. [15] that in cocoa (Theobroma cacao), the presence of shade and leaf age affected the diversity of endophytic fungi isolated from the site, but was not affected by the geographical location of the plant and the presence or absence of pathogen infection. As for the ability of fungi to degrade lignin and cellulase, there were 4 types of fungi that had ligninolytic abilities, namely CSF#5, CSF#10, CSF#15 and CSF#21. While fungi that have cellulolytic ability there are 16 types of fungi. Fungi that have ligninolytic abilities also have cellulolytic abilities, so that 4 types of fungi are potential that can degrade lignin and cellulose or lignocellulosic fungi.

3.2. Screening result of lignocellulolytic activity in solid medium

3.2.1. Screening result and enzyme potency index of ligninolytic activity

Pure cultures (single strain) aged ± 7 days from the isolated samples were selected using PDA media supplemented with 0.05% Guaiacol. The results showed that among the 23 isolates tested, there were 4 isolates that showed a color change reaction to reddish brown after being incubated for 3-7 days at room temperature.

![Figure 1](image-url)  
**Figure 1.** Selection results of lignolytic fungal on PDA media supplemented with Guaiacol 0.05% after 7 days. (a) CSF#5; (b) CSF#10; (c) CSF#15; (d) CSF#21

As shown in Figure 1, there were differences between fungal colony growth, area and thickness of the reddish-brown zone produced after incubation for 7 days. The CSF#15 isolate produced a wider colored zone which occupied the entire petri dish, while the CSF#5 isolate produced a narrower colored zone but the color was more intense. The growth of CSF#5 and CSF#15 fungi colonies was positively correlated with the production of colored zones where the wider the colony growth, the more colored zones were produced. While the isolates of fungi CSF#10 and CSF#21, the growth of fungal colonies and the production of colored zones were negatively correlated, because the growth of colonies was faster than the production of colored zones. CSF#10 isolate had faster fungal colony growth and a wider and more concentrated color zone than CSF#21 isolate. The reddish-brown color was produced because the polymer oxidation was enhanced by guaiacol which started to appear in the media along with mycelium growth fungi on solid media as incubated [16].

The quantification test was carried out by measuring the colony growth area and the colored zone which was measured twice on the 3rd and 7th incubation days to measure the ligninolytic potential index of each fungal isolate. The lignolytic potency index indicates the lignolytic enzyme as shown in Figure 2 below.
As shown in figure 2, on the 3rd day of observation, isolate CSF#15 produced a higher potency index than the other three isolates, but isolate CSF#21 did not have a ligninolytic index because the colored zone had not been produced. On the 7th day of observation, the lignolytic potential index decreased in isolates CSF#10 and CSF#15, while isolates CSF#5 experienced a significant increase, and isolates CSF#21 began to form colored zones with the lowest ligninolytic potential index, than other isolates. Based on the results of the ligninolytic potency index measurement, isolate CSF#5 produced the largest potency index on the 3rd and 7th day of observation, followed by isolates CSF#15, CSF#10 and CSF#21.

On the colony growth parameters, isolate CSF#5 had the slowest colony growth and isolate CSF#15 had the fastest colony growth compared to other isolates on the 3rd and 7th day of observation. This is because the production of the reddish-brown zone produced by isolate CSF#5 expanded until the 7th day compared to colony growth which tended to be slow, resulting in the highest ratio of ligninolytic potential index compared to other isolates. A higher potency index value indicates a higher ligninolytic enzyme activity and vice versa.

### 3.2.2. Screening and enzyme potency index of cellulolytic activity

Pure culture (single strain) aged ± 7 days from the isolated sample was selected using 1% CMC media then incubated for 7 days and soaked in 0.1% Congo red for 30 minutes to determine the cellulolytic ability. The results showed that among the 23 isolates tested, there were 17 isolates that produced a clear zone around the colony after being incubated for 7 days at room temperature.
Figure 3. Relationship of fungal colony growth diameter, clear zone and cellulolytic potential index of 17 isolates of fungi on the 7th day of observation

As shown in table 3 and figure 3, the diameter of fungal colony growth, clear zone production and cellulolytic potency index were related to each other. The fastest colony growth diameter after 7 days of observation was found in isolates CSF#10, CSF#15 and CSF#21 and CSF#7, with values of 80 to 85 mm. This was positively correlated with the production of a wider clear zone than colony growth along with the length of incubation time, but resulted in a low ratio below 1.50 mm after 7 days of incubation. However, there is a unique difference from isolate CSF#5 which has the highest cellulolytic potential index ratio of 2.60 with colony growth which is classified as low/slow, namely 10 mm on the 7th day of observation. CSF#5 isolate produced a clear zone that was relatively fast compared to colony growth, resulting in a higher ratio than other isolates. The low potency index is because fungi require a longer adaptation time to produce enzymes for oxidation. The difference in the cellulolytic activity index is thought to be due to the cellulase enzyme excreted by each fungal isolate with different potential to decompose substrates in growth media [20]. The greater the cellulase index in the isolate, the greater the cellulolytic activity produced [21].

Figure 4. Selection results of cellulolytic fungal on 1% CMC media after soaking in 0.1% Congo red on the 7th day of observation. (a) CSF#5; (b) CSF#10; (c) CSF#15; (c) CSF#21
Based on the results of the lignolytic and cellulolytic selection, it shows that there are 4 types of fungi that have two abilities, both lignolytic and cellulolytic or called lignocellulolytic. The largest lignolytic and cellulolytic potency indices were found in CSF#5 isolate after the 7th day of incubation, but this isolate had a lower/slower colony growth than the other 3 lignocellulolytic isolates. While the fastest growth of fungal colonies was found in isolate CSF#15 both on the 3rd and 7th day of observation. This isolate had the highest lignolytic potential index on the 3rd day of observation, but decreased on the 7th day along with colony growth. The isolates CSF#10 and CSF#21 had faster colony growth than isolates CSF#5, but had low lignocellulolytic abilities. Based on the qualitative test of the lignolytic and cellulolytic abilities of each fungus accompanied by the speed of colony growth, it can be concluded that the most potent isolates were CSF#5 and CSF#15.

3.3. Identification of strain
Based on the morphology of isolates CSF#5 and CSF#15, it was known that the two isolates belonged to the Ascomycetes group strain due to the presence of insulated conidium and hyphae.

4. Conclusion
The results showed that 23 species of fungi were isolated from the soil at 5 cocoa planting locations with different types of shade. The results of the lignolytic selection showed that there were 4 types of fungal strains that had the ability to produce lignolytic enzymes, and there were 17 types of fungal strains that had the ability to produce cellulolytic enzymes. From the results of the two qualitative selections on solid media, it can be concluded that there are two potential isolates that have the highest lignolytic and cellulolytic abilities, namely strains CSF#5 and CSF#15 which are thought to come from the Ascomycetes fungi group.

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