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Effectivity of locally wood rot fungal isolates in decomposition of leaf and cocoa pod husk waste

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A B S T R A C T

Cocoa pod husk is a major waste of cocoa plants that can be used either as an organic fertilizer or as animal feed. For 972.400 hectares of cocoa plantation, produce as much as 572.900 tons of cocoa beans, while the waste generated reached 1.8766 million tons/year. However, only 94.515 tons of cocoa waste has been utilized. Given the composition of twigs, leaves and cocoa pods that contain lots of lignin and cellulose, further research is needed to find microbes that effective in decomposing of cocoa waste in field conditions, kinds of media that can enhance the growth of rot fungi and production of cellulolytic enzymes. Totally 25 various isolates of white rot fungi and brown rot have been isolated and collected. To select its growth rate, these 25 isolates were grown in Potato Dextrose Agar medium. Thirteen best isolates were then cultivated on Malt Peptone Agar media and measure their colony diameter in two days interval. Eight isolates achieve maximum growth on PDA medium 3 days after inoculation while other isolates were 5 and 6 days after inoculation. The analysis of the cellulose, hemicellulose and lignin content in leaf and cocoa pod after treatment with fungal isolates *in vitro* were conducted according to van Soest (1976). The result showed that all of isolates were capable in cocoa waste degradation. Treatment with E isolate caused highest percentage reduction of hemicellulose components on cocoa leaf (70.5%) whereas KSH isolate was only 11%. Highest percentage reduction of cellulose component was observed in the treatment with E isolate (31.11%) and the lowest at KSB isolate (4.25%). Lignin component generally has not experienced a significant reduction in all fungal treatments. On cocoa pod, decrease of lignin components 30 days after inoculation was still very low in all treatments. Most decreasing component was hemicellulose, which reached up to 61.7% followed by cellulose component (31%).

Introduction

White- and brown rot fungi play an important role in plant litter decomposition in agriculture ecosystems through nutrient recycling and humus formation in soil (Swift, 1979) because they attack the lignocellulose matrix in litter that other organisms are unable to assimilate (Kjøller and Struwe 1982, Cooke and Rayner, 1984).

Some of the most important and well-studied white-rot fungi are *Pleurotus ostreatus* (Sannia *et al.*, 1986), *Phlebia*

radiate (Niku-Paavola *et al.*, 1988), *Coriolus* (Trametes, Polyporus) *versicolor* (Rogalski *et al.*, 1991), and *Pycnoporus cinnabarinus* (Eggert *et al.*, 1996). Based on the substrate utilization patterns of fungi in decomposing organic matter Miyamoto *et al.* (2000) and Osono and Takeda (2002) divided the fungi into three functional groups : lignocellulose decomposers that attack both lignin and cellulose in various proportions, cellulose decomposers that preferentially attack carbohydrates and sugar fungi that rely on soluble sugar for growth.

In the process of degradation of lignin, white rot fungi produce a unique extracellular oxidative enzyme. The secretion of the enzyme systems of microorganisms as biodegradation agents has ability to breaking down lignocellulose material into simpler molecules. This enzyme is also excellent to degrade pesticides and toxic waste compounds (Srebotnik *et al.*, 1998).

Ligninolitic fungi use not only lignin as a sole carbon and energy source for growth, but also some polysaccharide of the existing lignocellulolytic substrate. The main function of ligninolysis is to open polysaccharide into cellulose and hemicellulose that can be solved by fungi (Hammel, 1997).

White rot fungus depolymerize lignin with oxidative way by secreting several enzymes, such as lignin peroxidase, manganese peroxidase and laccase (Acunzo *et al.*, 2002). Lignin peroxidase and manganese peroxidase oxidizes the major components of the lignin polymer in non-phenolic aromatic form compounds with high oxidation reduction potential. While laccases oxidize phenolic lignin structures which constitute a minor content of lignin polymer (Srebotnik *et al.*, 1998).

In nature, lignocellulose is a constituent for the major part of biomass and, consequently, its degradation is essential for the operation of the global carbon cycle. Lignocellulose, such as wood, is mainly composed of a mixture of cellulose (ca. 40%), hemicellulose (ca. 20± 30%), and lignin (ca. 20±30%) (Sjostrom, 1995). Lignin is an integral cell wall constituent, which provides plant strength and resistance to microbial degradation (Argyropoulos and Menachem, 1997).

The only one microorganism that capable for degrading lignin is wood-rot fungi which are classified in the class Basidiomycetes. Wood-rot fungi distinguished by white rot fungi, brown-rot and soft-rot. White rot fungi attack lignin and polysaccharides. Degraded wood becomes white and soft. Brown-rot fungi degrade wood polysaccharides and degrade lignin slightly so that the wood becomes brown and brittle. While the soft-rot fungus prefer cellulose and hemilcellulose as its substrate (Fengel and Wegener, 1995).

With regard to the reform process of wood by fungi, Brauns (1952) suggested that there are two types of fungi that play an active role, ie brown rot and white rot fungi. Brown rot fungus prefers to attack the cellulose, leaving residual lignin and transform decomposes to brown. In white mushrooms, lignin and cellulose are overhauled, so that the attacked wood color becomes pale white (Martawidjaja, 1988).

Cocoa plant produces biomass of leaves and twigs that reach 6.85 tons/ha/yr for cocoa without shade and 11.88 tons/ha/yr with shade. In addition, harvest 1kg of cocoa beans will be left 10 kg of cocoa pods, pulp and placenta. Mineral nutrient content of cocoa pods is quite high, especially potassium and nitrogen. Abundantly cocoa

waste however, if not managed properly will lead to seriously problems such as environmental pollution sources (methane, CO_2 and NO_2) and become as breeding places of plant pest organisms ie; *Phytopthora palmivora*, *Diplodia* sp and several kind of insect pests.

Base on these facts, the purpose of this study is to assess by a pure-culture test the best growth ability in culture media, as well as their ability in decomposing of cocoa leaf and cocoa pod husk in controlled condition.

Materials and Methods

Source of fungi

Twenty-five isolates were collected from decayed wood and living trees of *Theobroma cacao*, at Soppeng, Gowa regency and Makassar, South Sulawesi, Indonesia. The fruit bodies were collected along with supporting wood. Fruit bodies were wrapped in paper bags and brought to the laboratory. The fruiting bodies were separated, sterilized with 1% mercuric chloride solution, repeatedly washed with sterile distilled water and inoculated on 2 % of malt agar medium. Pure culture were subcultured and maintained in malt agar slants until used.

Growth ability tests on malt peptone agar media

Collected isolates were grown first on Potato Dextrose Agar (PDA) to select their growth ability. Selected isolates were then cultivated on Malt Peptone Agar (MPA). Pieces of each culture isolates (φ 7 mm) were grown aseptically on media and then incubated at room temperature. Observations were made every day by measuring the diameter of the colony to fulfill a Petri dish grown mycelia.

Decomposition of Cocoa Pod husk and cocoa leaves *in vitro*

Lignocellulolytic character of best isolates was further tested on a sterile organic media (cocoa pod and leaves) in a plastic baglog. Isolates were first cultivated in medium contain of 23 % barley, 3 % bran, 0.4 % lime and 73.6 % of sawdust and water as needed, for producing inoculum. Each cocoa pod husk and cocoa leaf were mixed first with rice bran and lime in ratio of 5:1:0.1, filled with 400 g in a plastic baglog then autoclaved twice for 20 minutes at 1.5 atm. Each baglog inoculated with 5 g fungal inoculum then incubated in a chamber, until 4 weeks at room temperature. This test was done with 4 replications. To determine the levels of lignin, cellulose and hemicellulose after 4 weeks inoculation of fungal isolates, organic media were first removed from the spawn baglog then dried for 2-3 days. To determine the levels of lignin, cellulose and hemicellulose, ADF (Acid Detergent Fiber) and NDF (Neutral Detergent Fiber) were first determined using the method of van Soest(1976).

Results and Discussion

There were totally 25 fruiting bodies of rot fungi were successfully collected from field. Based on the color of infected wood and their morphological characters, the fungi were separated into 2 groups, white rot and brown rot fungi. Samples were then taken to the laboratory to be isolated and purified on PDA medium.

Initial screening was done by growing of isolates on Potato Dextrose Agar medium. There were 13 best isolates from 25 collected isolates. These isolates were further cultured on Malt Peptone Agar (MPA). The results showed that each isolate has a different growth rate in culture media.

Best growth was marked by the achievement of maximum growth after third day incubation. Generally all isolates showed very rapid growth on MPA medium, except one isolate (KSH) exhibited a restricted growth. The colony growth was achieved only 2,63 cm after 7 days incubation Table 1).

Reduction of Lignocellulose content in all treatment is shown on Table 2. Each isolate had different ability to decompose pod husks and cocoa leaf. The highest percentage reduction in the hemicellulose content was observed by JM isolate that reaches up to 61.69 %, while the lowest percentage (6.61 %) was observed on B isolate. Highest reduction of cellulose component can be seen on MKS isolates with 31.07 % and the lowest was E isolates (1.96%). Reduction of lignin content after inoculation for 30 days, generally restricted in all treatment with fungal rot isolates. Highest reduction was only 10.73 % in B isolate followed by JM isolate with 10.31% cellulose reduction.

On cocoa leaves, the highest reduction of hemicellulose components was observed in treatment with E isolates and C isolates, while the lowest was observed in the treatment with KSH isolate. Highest percentage reduction of hemicellulose reached 70.5 %, (Isolate E) while the lowest (KSH isolate) only reaches 11 %. The highest reduction of cellulose was observed in the treatment with E isolates (31.11 %) and the lowest was KSB isolate (4.25 %). Lignin generally had not significantly reduced in all fungal treatments. However, treatment with B isolates causing decreased lignin content (8.21 %), while the other isolates ranged from 0.13 % to 2.31 % (Fig. 1). Thirty days after treatments with all fungal isolates, decreasing of lignin component was still not so evident, but the highest reduction was observed by JM and MKS isolates. Most reduced components was hemicellulose, which reached 61.7 % in JM isolate, while the highest reduction of cellulose component was observed in isolates MKS (31.0 %) (Fig. 1).

Growth is one of the important characteristics of living cells. Growth of microorganisms can be defined as events increase the volume of an organism with increased biomass. On fungi, growth is characterized by elongation of the hyphae. Growth of white rot fungi as well as other microorganisms to follow a certain pattern and the specific growth rate is one of the important parameters to evaluate the performance of a microorganism in a culture. Another important parameter is the colony radial growth rate (Kr) (Reeslev and Kjøller, 1995).

Biodegradation of lignin is the unique ability and possessed by rot fungi that are not shared by other microorganisms. Lignin is broken down by enzymes with oxidative processes, whereas cellulose and hemicellulose will split through hydrolytic process. Oxidative separation between carbon-carbon and ether bonds between the ether linkages include units phenylpropane performed by peroxidase enzymes. Extracellular H_2O_2 resource is necessary for the survival of enzymatic reactions (Zabel and Morel, 1992).

According to Howard *et al.* (2003), the cellulose degradation by fungi is the result of a group of cellulolytic enzymes that work synergistically. Cellulose is a polymer of glucose with β-1,4-glucoside bonds in the linear chain, waking up in the form of a cellulose base that is a dimer of glucose cellobiose. Long chains of cellulose are connected together through hydrogen bonds and van der Waals forces. B-1,4-glucoside bond in the cellulose fibers can be broken down into glucose monomers by means of acid or enzymatic hydrolysis.

Cellulase is an enzyme that can degrade cellulose. This enzyme is capable of hydrolyzing cellulose into simple sugars or glucose. According to Schlegel (1994), the breakdown of cellulose by cellulases consist of at least three enzymes: (1) endo-β-1, 4 glucanase influence simultaneously β-1, 4 bonding in macromolecules and produce large pieces shaped chain with free ends, (2) enzyme exo-β-1, 4 glucanase cut ends of the chain disaccharide and cellobiose, (3) enzyme β-glucosidase hydrolize cellobiose to form glucose.

Degradation of hemicellulose into monomeric sugars and acetic acid is done by the enzyme hemicellulase. Hemicellulase like most other enzymes that can hydrolyze plant cell wall is a multi-domain protein. Xylan is the major carbohydrate constituent of hemicellulose and xylanase is a major hemicellulase hydrolyze Lignocellulolytic enzyme consists of a set of enzymes that are divided into two categories: hydrolytic and oxidative. Hydrolytic enzymes to degrade cellulose and hemicellulose and each enzyme acts on a specific substrate. Oxidative enzymes are non-specific enzyme, and work through a mediator is not a protein that plays a role in the degradation of lignin.

Table.1 Growth Rate of 13 fungal isolates on MPA (Media Malt Pepton Agar) 2-6 dpi

Isolates	Days after inoculation- (cm)				
	2	3	4	5	6
A	7.93^{fg}	9.00^{d}	9.00^{e}	9.00^{bc}	9.00^{bc}
B	5.87^{de}	9.00^{d}	9.00^{e}	9.00^{bc}	9.00^{bc}
C	5.97^{e}	9.00^{d}	9.00^{e}	$9.00b^{c}$	9.00^{bc}
D	5.47^{cd}	9.00^{d}	9.00^{e}	9.00^{bc}	9.00^{bc}
E	4.73^{c}	9.00^{d}	9.00^{e}	9.00^{bc}	9.00^{bc}
F	7.73^{g}	9.00^{d}	9.00^{e}	9.00^{bc}	9.00^{bc}
G	6.30^{ef}	9.00^{d}	9.00^{e}	9.00^{bc}	9.00^{bc}
KSA	0.10^{a}	0.23^{a}	0.63^{a}	1.43^{a}	2.63^{a}
KSB	1.03^{ab}	2.70^{b}	4.57^{b}	6.67^{b}	9.00^{bc}
KSD	3.50^{b}	9.00^{cd}	9.00^{e}	9.00^{bc}	9.00^{bc}
KSH	4.57^{bc}	7.77^{c}	8.10^{de}	8.20^{bc}	8.20^{b}
MKS	0.93^{a}	2.60^{b}	5.30^{bc}	6.93^{bc}	9.00^{bc}
JM	0.80^{a}	3.65^{bc}	6.70^{cd}	9.00^{bc}	9.00^{bc}

a,b,c,d values followed by the same letter in the same column means are not significantly different at the ($p \leq 0,05$) LSD test

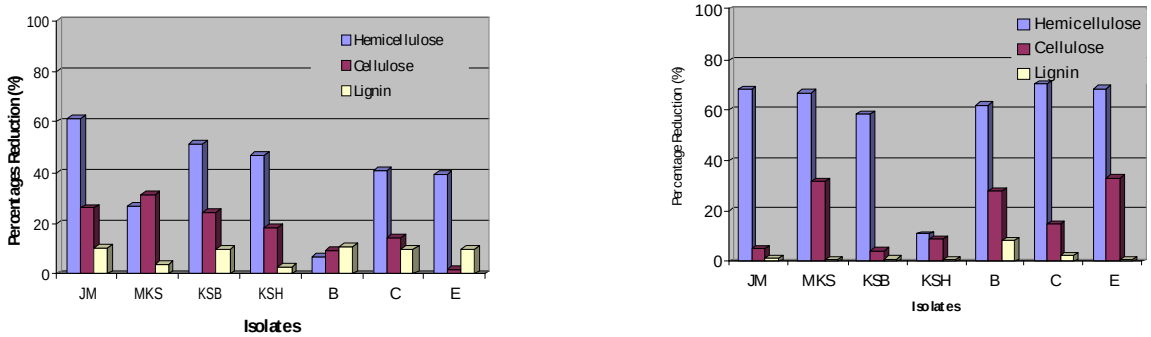

Fig. 1 Percentage reduction of hemicellulose, cellulose and lignin on leaf (left) and cocoa pod (right), 30 dpi with 7 fungal rot isolates.

Enzymes that degrade lignin is generally composed of two main groups, namely laccase (Lac) and composed of lignin peroxidase peroxidase (LiP) and manganese peroxidase (MnP). All three of these enzymes are responsible for the initial breakdown of the lignin polymer and produce products with low molecular weight in white-rot fungi, white rot fungus example the fungus *Phanerochaete chrysosporium* (Perez *et al.*, 2002) β-1,4 bond of xylan chains into oligosaccharides. Hemicellulose is a heterogeneous group of polysaccharides with low molecular weight. The amount of hemicellulose is usually between 15 and 30 percent of the dry weight of lignocellulosic materials. Hemicellulose is relatively easier to be hydrolyzed by acid monomers containing glucose, mannose, galactose, xylose and arabinose (Perez *et al.*, 2002).

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