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Selection of Lignin Degrading Bacteria from Soil, Kitchen Waste, Leaf Litter, and Cow Dung Based on Lignin Peroxidase and Manganese Peroxidase Activities

Taruna Dwi Satwika*, Dwiana Muflihah Yulianti, Galang Anahatta Hidayat, Afifah Mariana

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ABSTRACT

Lignin is a complex chemical heterogeneous polymer that forms a physical barrier to lignocellulose's biological and chemical hydrolysis, making lignocellulosic biomass challenging to degrade. Ligninolytic microorganisms play an essential role in lignin degradation by producing extracellular enzymes. Lignin peroxidase and manganese peroxidase are enzymes that play a role in lignin degradation. Forty-one bacterial isolates have been isolated from soil, kitchen waste, leaf litter, and cow dung. However, the ligninolytic activity of these isolates has yet to be discovered. This research aimed to determine the ligninolytic ability of bacteria isolated from soil, leaf litter, kitchen waste, and cow dung based on lignin peroxidase and manganese peroxidase activity. The study was conducted stages: isolate recultured, qualitative and quantitative testing of lignin peroxidase activity based on degradation of methylene blue dye, and qualitative and quantitative testing of manganese peroxidase activity based on degradation of phenol red dye. A total of four bacterial isolates from soil (Tn9, Tn14, Tn16, and Tn17) and two bacterial isolates from cow dung (KS2 and KS5) showed qualitative and quantitative lignin peroxidase activity. Manganese peroxidase activity was also shown by four isolates from soil (Tn2, Tn6, Tn14, and Tn16), one isolate from kitchen waste (SD1), and one isolate from cow dung (KS5) both qualitatively and quantitatively. The nine bacterial isolates that showed lignin peroxidase and manganese peroxidase activity have potential as lignin-degrading biological agents.

Keywords: bacteria, ligninolytic, peroxidase

INTRODUCTION

Organic waste, such as agricultural and household waste, is a source of lignocellulose waste that can have economic value if further processed. Waste can be processed into other products such as compost, organic fertilizers, bioethanol, and animal feed (Akhtar *et al.* 2015; Sutini *et al.* 2020). However, the presence of lignin can be a limiting factor in the utilization of plant fibers (Permata *et al.* 2022). Lignin is intertwined with two other significant polymers, cellulose and hemicellulose, in lignocellulosic biomass that provides structural strength, durability, and water transport in the cell wall, as well as protection from degradation by microorganisms and enzymes (Brink *et al.* 2019). Lignin is a complex, chemically heterogeneous polymer that forms a physical barrier to cellulose's biological and chemical hydrolysis, making plant biomass challenging to degrade (Himmel *et al.* 2007).

Lignin can be eliminated using delignification, which breaks down the structure of lignin and makes cellulose and hemicellulose easier to use. Physical and chemical pretreatments for delignification include mechanical comminution, pyrolysis, physicochemical pretreatment (steam explosion, ammonia fiber explosion, carbon dioxide explosion), chemical pretreatment (ozonolysis, acid hydrolysis, alkaline hydrolysis, oxidative delignification, organosolv process), and pulsed electric field pretreatment (Gilca *et al*. 2014; Kumar *et al*. 2009; Yang *et al*. 2015). Most of these approaches involve expensive equipment, more energy, or may produce harmful substances (Yang *et al*. 2017). Biological delignification with ligninolytic enzyme-producing microorganisms is a popular alternative (Permata *et al*. 2022). Biological alternatives, as opposed to physical and chemical approaches, are less expensive, more environmentally friendly, and reduce pollution levels (Kumar and Chandra 2020).

The degradation of lignin by microbes in nature has been extensively studied. Lignin-degrading microbes are

Faculty of Biology, General Soedirman University, Purwokerto 53123, Indonesia
* Coressponding Author:
Email: tarunadwisatwika@unsoed.ac.id

generally from the group of bacteria and fungi. White and brown decaying fungi have been extensively studied to degrade lignin (Karim *et al.* 2016; Schmidt *et al.* 2016). However, commercial biocatalytic processes using mushrooms have not been available to date. Compared to fungi, bacteria have an advantage due to lower ethical issues associated with using bacteria, better environmental adaptation, faster growth, and easier handling of genetic manipulation (Suman *et al.* 2016; Tian *et al.* 2016). Most lignin-degrading bacteria belong to the phylum Actinobacteria and Proteobacteria (Tian *et al.* 2014). Various enzymes have been reported to be involved in the degradation of lignin by bacteria, including laccase, glutathione *S*-transferase, dioxygenase (Allocati *et al.* 2009; Masai *et al.* 2003), monooxygenase, phenol oxidase (Ferreira-Leitão *et al.* 2007; Perestelo *et al.* 1989), Lignin peroxidase (Bandounas *et al.* 2011; Tian *et al.* 2016), and manganese peroxidase (Kumar and Chandra 2018). The use of ligninolytic enzyme-producing bacteria has an excellent opportunity to be utilized in the biological decomposition process of lignin.

The bacteria used in this study were isolated from soil, kitchen waste, and cow dung (Satwika *et al.* 2021). However, the isolates have yet to be discovered for its ligninolytic ability. The origin of bacterial isolates containing lignin can obtain ligninolytic isolates. Screening of the potential of these isolates needs to be carried out to get potential lignin-degrading bacterial isolates

The purpose of this study is to investigate the ligninolytic ability of soil, leaf litter, kitchen trash, and cow dung bacterial isolates using lignin peroxidase and manganese peroxidase enzyme activity as indicators. This investigation is expected to offer information on the ability of those isolates to degrade lignin, as well as the activity of ligninolytic enzymes.

METHODS

Isolates Rejuvenation

A total of one ose isolate was inoculated into 10 mL of Nutrient Broth (NB) medium. Incubation was carried out for 24-48 hours at room temperature. The active isolates were then stocked on the Nutrient Agar (NA) medium by scratching one on the NA medium at an angle and incubated for 24-48 hours at room temperature. The isolates tested in this study and the origin of the isolates are listed in Table 1.

Qualitative Lignin Peroxidase Activity Test

Qualitative screening of lignin peroxidase activity was carried out using the dye decolorization assay (Bandounas *et al.* 2011). The medium was Luria Bertani Agar, which contains 150 mg/L of methylene blue (LB-MB). The bacterial isolate was inoculated on LB-MB medium and then incubated for 3 days. Formation of clear zones was observed daily. A clear zone around the bacterial colony indicates lignin peroxidase activity.

Quantitative Lignin Peroxidase Activity Test

Lignin peroxidase activity was quantified using the dye decolorization technique (Bandounas *et al*. 2011). The medium was Luria Bertani Broth, which contains 100 mg/L of methylene blue. The test bacterial isolate was inoculated into 15 mL of medium and incubated for 108 h under aerobic conditions at 30°C and 120 rpm. Cultures without inoculum were employed as a control to assess dye degradation in the medium. Dyes-free media inoculated with bacterial isolate serves as a control for measuring changes in media absorption caused by bacterial growth. All control and treatment tests were conducted three times. The cultures were centrifuged at 10,000 g for 10 min. The resultant supernatant was tested for absorbance at a wavelength of 665 nm. The percentage of decolorization was determined by the formula (Tian *et al.* 2016):

$$\%\ \text{decolorization} = \frac{\text{A kontrol} - (\text{A observasi} - \text{A media})}{\text{A awal}} \text{x} 100\%$$

where

$A_{control}$: Absorbance of media without inoculum
$A_{observed}$: Absorbance of bacterial isolate treatment
A_{media} : Absorbance of dye-free media
$A_{initial}$: Media absorption before incubation

Qualitative Manganese Peroxidase Activity Test

The dye decolorization assay was used to conduct a qualitative screening of lignin peroxidase activity. The medium was mineral salt medium agar with 0.01% phenol red (w/v). MSM composition (in g/L): Na_2HPO_4 2.4; KH_2PO_4 2.0; NH_4NO_3 0.1; $MgSO_4$ 0.01; $CaCl_2$ 0.01;

Table 1 List of tested bacterial isolates and the respective sources

Source of isolate	Isolate code
Soil	Tn1, Tn2, Tn3, Tn4, Tn5, Tn6, Tn7, Tn8, Tn9, Tn10, Tn11, Tn12, Tn13, Tn24,
Leaf litter	Sr14, Sr15, Sr16, Sr17, Sr18, Sr19, Sr20, Sr21, Sr22, Sr23
Kitchen waste	SD1, SD2, SD3, SD4, SD5. SD6, SD7, SD8, SD9, SD10
Cow dung	KS1, KS2, KS3, KS4, KS5, KS6

D-glucose 10.0; peptone 5.0 (Chandra *et al*. 2007). A total of one bacterial isolate was inoculated in a continuous streak on MSM+phenol red medium and incubated for two days. The formation of clear zones was observed daily. A clear zone surrounding the bacterial colony shows manganese peroxidase activity.

Quantitative Manganese Peroxidase Activity Test

Manganese peroxidase activity was quantified using the dye decolorization technique (Zainith *et al*. 2019). The medium used was Mineral Salt Medium Broth (pH 7.6) with 0.01% phenol red (w/v). A total of 99 mL of medium was inoculated with 1 mL of the test isolate and incubated for 72 h under aerobic conditions (32°C, 120 rpm agitation). Cultures without inoculum were employed as a control to assess dye degradation in the medium. Dyes-free media inoculated with isolate serves as a control for measuring changes in media absorption caused by bacterial growth. All control and treatment tests were conducted three times. The culture was centrifuged at 10,000 g for 10 min. The resultant supernatant was tested for absorbance at a wavelength of 600 nm. The percentage of decolorization is determined by the formula (Tian *et al.* 2016):

$$\%\ \text{decolorization} = \frac{\text{A kontrol} - (\text{A observasi} - \text{A media})}{\text{A awal}} \text{x} 100\%$$

where

$A_{control}$: Absorbance of media without inoculum
$A_{observed}$: Absorbance of bacterial isolate treatment
A_{media} : Absorbance of dye-free media
$A_{initial}$: Media absorption before incubation

RESULTS AND DISCUSSION

Bacterial isolates of soil origin, leaf litter, kitchen waste, and cow dung, totaling 41 isolates, have been tested for ligninolytic activity qualitatively. Four isolates of bacteria of soil origin (Tn9, Tn14, Tn16, and Tn17) and two of cow dung origin (KS2 and KS5) showed lignin peroxidase activity (Figure 1). The index of lignin peroxidase ranges from 0.209 to 0.296. Lignin peroxidase activity is indicated by a clear zone around the colony grown on the medium of Luria Farming Methylene Blue (LBMB) (Figure 2ke). These results are in line with previous studies where it has been reported that lignin peroxidase activity was found in bacterial isolates of soil origin and cow dung (Bandounas *et al.* 2011; Dube *et al.* 2023).

The six isolates that demonstrated lignin peroxidase activity were subsequently examined quantitatively. The methylene blue decolorization measures lignin peroxidase activity quantitatively. The six bacterial isolates had varying degrees of decolorization, ranging from 56.31 to 79.44% (Figure 3). Tn17 isolate has the highest percentage of decolorization.

Methylene blue decolorization was utilized in this work to determine lignin peroxidase activity. According to Ferreira-Leitão *et al*. (2003), the lignin peroxidase can oxidize methylene blue. Methylene blue is a thiazine dye that cannot be broken down by oxidase enzymes with low redox potential, like manganese peroxidase and lactase. Decolorization requires high redox potential oxidase enzymes, particularly lignin peroxidase (Archibald 1992). This suggests that methylene blue can be used to selectively detect lignin peroxidase activity.

In addition to lignin peroxidase activity, manganese peroxidase activity was assessed in this study. Four isolates of soil bacteria (Tn2, Tn6, Tn14, and Tn16), one isolate of kitchen waste bacteria (SD1), and one isolate of cow dung bacteria (KS5) all exhibited manganese peroxidase activity (Figure 4). The manganese peroxidase index ranges from 0.054 to 0.233. A clear zone around the colony grown on a Mineral Salt Medium (MSM) medium with phenol red indicates manganese

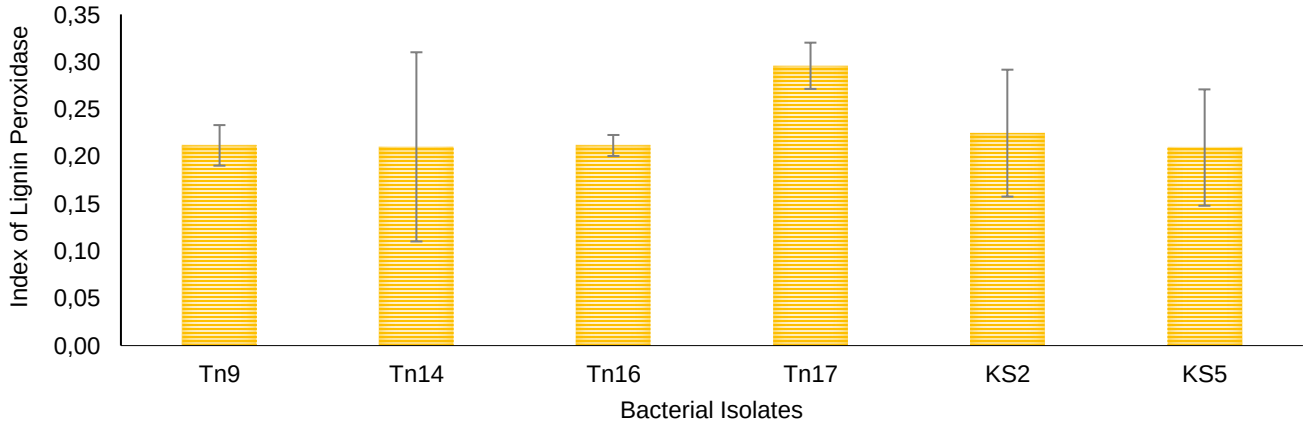

Figure 1 Lignin peroxidase index qualitatively.

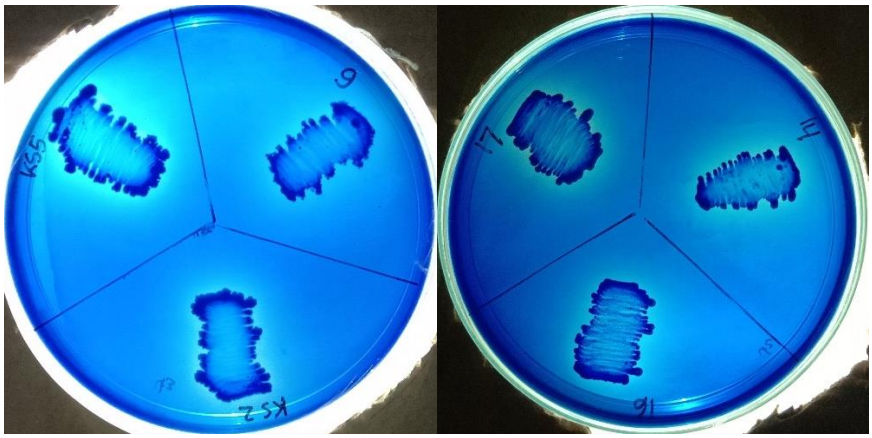

Figure 2 Qualitative lignin peroxidase activity in LBMB media.

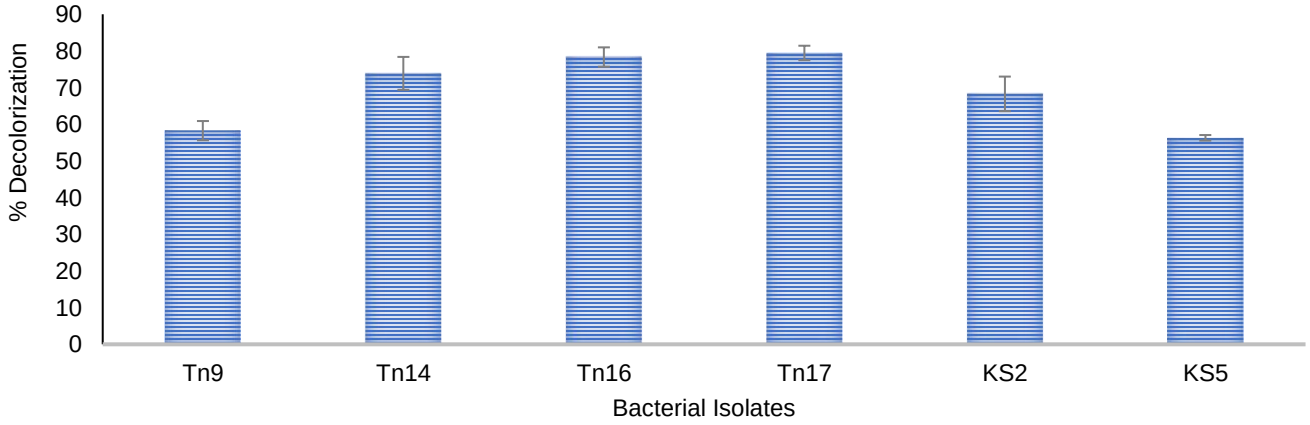

Figure 3 Quantitative lignin peroxidase activity

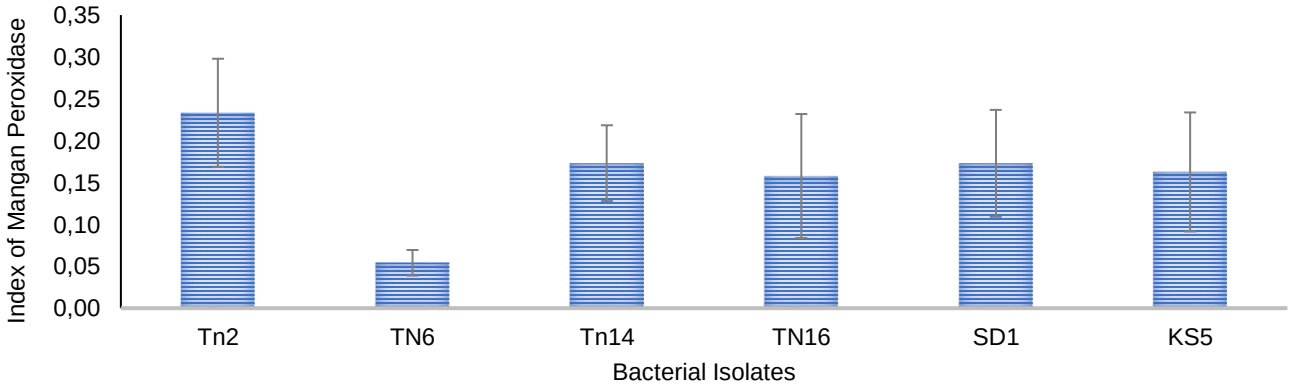

Figure 4 Manganese peroxidase index qualitatively.

peroxidase activity (Figure 5). Manganese peroxidase activity has already been observed in soil-derived microorganisms (Nayanashree and Thippeswamy 2015). Meanwhile, there have been no reports of manganese peroxidase activity in bacteria found in kitchen waste or cow dung.

The six isolates that demonstrated manganese peroxidase activity were subsequently examined quantitatively. The decolorization percentage of phenol

red reveals manganese peroxidase's quantitative activity. The six bacterial isolates had varying decolorization percentages, ranging from 12.3% to 89.52% (Figure 6). The Tn16 isolate shows the highest percentage of decolorization. Phenol red has been commonly utilized to measure manganese peroxidase activity (Ishak *et al*. 2018; Kumar and Chandra 2018; Zainith *et al*. 2019). Phenol red is a lignin-like dye that specifically targets manganese peroxidase activity.

In this investigation, three bacterial isolates from soil and cow dung, Tn14, Tn16, and KS5, demonstrated two lignin-degrading enzyme activities. Some bacteria containing lignin peroxidase and manganese peroxidase activity have been successfully isolated from other materials. *Klebsiella* sp., *Serratia* sp., *Pseudomonas* sp., and *Enterobacter* sp. from the gastrointestinal tract of the wood-eating beetle *Rynchophorus ferrugineus* have been found to contain lignin peroxidase and manganese peroxidase activities (Ishak *et al*. 2018). Several bacterial isolates from weathered wood have also been reported to contain lignin peroxidase and manganese peroxidase activity (Yang *et al*. 2017).

The study also found that soil bacteria had higher levels of lignin peroxidase and manganese peroxidase activity than bacteria from cow dung and kitchen waste. Soil is the most studied sample for the genesis of ligninolytic bacterial isolates (Brink *et al*. 2019) and the most anticipated ecosystem for ligninolytic microbial ecological niches (Cragg *et al*. 2015). This is due to the presence of lignocellulose at varying stages of decomposition in the soil. The input of lignocellulosic sources in the soil is more than that of kitchen waste and cow dung, thus ligninolytic microorganisms in the soil have been driven to proliferate, and the population is high.

CONCLUSION

A total of nine isolates showed ligninolytic activity. Four bacterial isolates from soil and two from cow dung

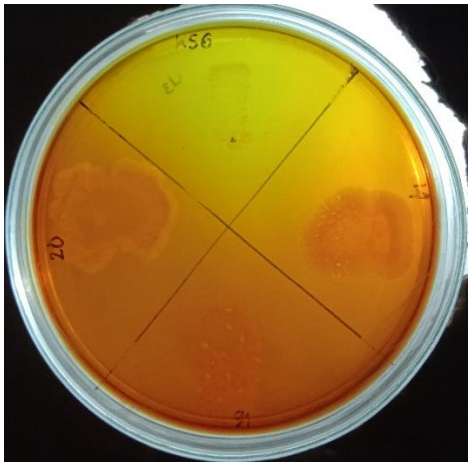

Figure 5 Qualitative activity of manganese peroxidase in MSM+phenol red media.

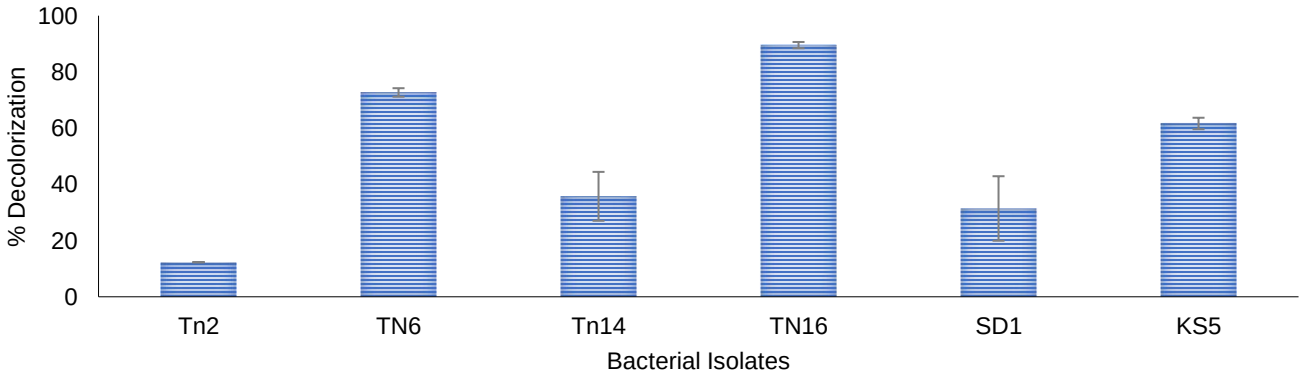

Figure 6 Manganese peroxidase activity quantitatively.

showed lignin peroxidase activity, with the percentage of methylene blue decolorization ranging from 56.31 to 79.44%. Meanwhile, manganese peroxidase activity was demonstrated by four isolates from the soil, one isolate from kitchen waste, and one from cow dung, with a percentage of phenol red decolorization ranging from 12.3 to 89.52%.

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