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The Existence And Characterization Of Potential Cellulolytic And Lignolytic Bacteria From Spent Mushroom Substrate Of Pleurotus Ostreatus L.

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Abstract – Spent mushroom substrate (SMS) of P. ostreatus is the residual compost waste generated by the mushroom production industry. The main component of P. ostreatus cultivation is sawdust that high of lignocellulosic material. The presence of cellulose and lignin can be the carbon source of indigenous cellulolytic and lignolytic bacteria. The study was conducted to know the existence of indigenous cellulolytic and lignolytic bacteria and determine their activity or potential character. This research was conducted using the purposive sampling method and analyzed descriptively. The result showed the existance of cellulolytic bacteria (41–51×10^5 cfu/g) and lignolytic bacteria (60-80 × 10^5) in SMS. PD04S has a higher cellulolytic index and PYK2L has a higher lignolytic index.

Keywords – Cellulolytic index; lignolytic index; Pseudomonas; Paracoccus

I. INTRODUCTION

Pleurotus ostreatus L. *or oyster mushroom* is a consumable mushroom that is in great demand by the public. This is because oyster mushrooms contain a lot of nutrition and can be a source of protein for the body. Astuti and Kuswytasari [1] reported that 100 grams of Oyster Mushrooms dry-weight contain 128 calories, 16 grams of protein, 0.9 grams of fat, 64.6 mg of carbohydrates, 51 mg of calcium, 6.7 mg of iron, and 0. 6 mg of carbohydrates. and 1 mg of B vitamins

High demand from consumers causes the production of Oyster mushrooms is increasing every year. Based on BPS-Statistics Indonesia in 2018, the production of oyster mushrooms in Indonesia reached 3.701,956 tons in 2017 and increased to 31,051.571 tons in 2018. In several harvest cycles, oyster mushroom cultivation can produce 6 tons of SMS [2]. So it has negative impacts such as increasing waste due to unused SMS. Growth medium of oyster mushroom consists of sawdust, wheat bran, gypsum (CaSO4), corn flour, and calcium carbonate (CaCO3). SMS is formed due to substrates in growth medium that are not used up when producing oyster mushrooms, thus leaving ineffective residues for the growth of oyster mushrooms. Sawdust is the main component of oyster mushroom cultivation with a high lignocellulosic material.

The lignocellulosic material in SMS consisted of cellulose 46%, lignin 31%, and hemicellulose 16% [3]. Cellulose and lignin are components that are difficult to degrade because of their complex structures and interlocking bonds [4]. The availability of these polysaccharides can be utilized by microorganisms such as cellulolytic and lignolytic bacteria as a source of carbon and nutrients for their growth.

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Cellulolytic bacteria produce cellulase as extracellular enzymes that hydrolyze cellulose into simpler products [5]. The cellulase enzyme consists of three main types of enzymes, there are endo-β-1,4-glucanase complex (CMCase, Cx cellulase endocellulase, or carboxymethyl cellulase), the exo-β-1,4-glucanase complex (aviselase, selobiohydrolase , C1 cellulase), and β-1,4- glucosidase or cellobiase [6]. Bacteria also have the ability to degrade natural lignin through their lignolytic activity. There are three lignolytic enzymes from microbes, there are Lignin Peroxidase (LiP), Manganese Peroxidase (MnP), and Laccase [7]. The utilization of extracted enzymes from bacteria is extensive. In addition to food and industry, using cellulase and ligninase enzymes from bacteria can solve pollution problems, especially in reducing the amount of lignocellulosic waste. Research on the potential of cellulose- and lignin-degrading bacteria from SMS of oyster mushrooms has never been carried out, so research is needed to screen and identify the activity of cellulolytic and lignolytic bacteria which have a high potency index.

Several genera of bacteria that have cellulolytic abilities are *Achromobacter, Angiococcus, Bacillus, Cellulomonas, Cytophaga, Clostridium, Cellivibrio, Flavobacterium, Pseudomonas, Poliangium, Sorangium, Sporocytophaga, Vibrio, Cellfalcicula, Citrobacter, Serratia, Klebsiella, Enterobacter* and *Aeromonas* [8][9]. Meanwhile, some researchs found several genera of bacteria that have lignolytic abilities, there are *Staphylococcus, Pseudomonas, Bacillus, Ochrobacterium,* and *Leucobacter* [10, 11]. Research on the potential of cellulose- and lignin-degrading bacteria in SMS of *P. ostreatus* has never been done, so it is necessary to carry out research to screen and identify cellulolytic and lignolytic bacteria that have a high potency index.

II. RESEARCH METHODS

2.1. Sample Collection

Samples of *P. ostreatus* spent mushroom substrate (SMS) were obtained from three locations in West Sumatra, there are Padang, Payakumbuh and Padang Panjang. Sampling was carried out by purposive sampling, which are unproductive SMS in producing fruit bodies of aged up to 4 months after harvest and not contaminated by other mushrooms. The process was performed at the Research Laboratory of Microbiology Andalas University, Padang.

2.2. Existence of indigenous microflora from SMS of P. ostreatus

The existence of indigenous microflora was observed by colony bacteria that grew on Glucose Peptone Agar (GPA) medium and the existence of cellulolytic was observed in Carboxymethyl cellulose agar (CMCA) medium and lignolytic bacteria was observed in Luria bertani + Methylene blue agar (LBMBA) medium [12]. The existence of bacteria signed with the formation of a clear zone around the colony after 24 hours of incubation. The smallest with the largest clear zone from each different colony grew on the CMCA and LBMBA medium and were selected to be cultured using streak method in GPA. The isolates obtained were coded with the location's initial (PD, PYK and PP), ability initial (S and L) and the number of isolates.

2.3. Evaluation of cellulolytic index from selected isolates

Selected isolates were grown in 10 ml of GPB media with the same cell density of 10^7 cfu/g for 24 hours. 1 ml culture isolates were grown on CMCA with pour plate method and incubated at 37°C for 48 hours. Observations of the halo zone were carried out after 48 hours. cellulolytic and lignolytic index of indigenous bacteria was calculated by comparing the diameter of the clear zone formed by the colony of bacteria and the diameter of the colony of bacteria [13]. Choi *et al.,* [14] reported that the determination of qualitative criteria of degradation index was based on assumptions if less than ≤1 given low criteria, equal to 1-2 given the criterion of medium and above ≥2 given high criteria.

2.4. Evaluation of lignolytic index from selected isolates

Selected isolates were grown in 10 ml of GPB media with the same cell density of 10^7 cfu/g for 24 hours. 1 ml culture isolates were grown on LBMBA with pour plate method and incubated at 37°C for 48 hours. Observations of the halo zone were carried out after 48 hours. Lignolytic index of indigenous bacteria was calculated by comparing the diameter of the clear zone formed by the colony of bacteria and the diameter of the colony of bacteria [13]. Nahrowi *et al*. [10] reported that the determination of qualitative criteria of degradation index was based on assumptions if less than 1.5 given low criteria, equal to 1.5 given the criterion of medium and above 1.5 given high criteria.

2.5. Characterization of potential cellulolytic and lignolytic bacteria isolates

The isolates were characterized morphologically by observing the colony shape, elevation, color, margin, and surface of each isolates. Characterization was also carried out by observing cell morphology with Gram staining and biochemical tests (Catalase test with H_2O_2 3%, KOH 3%, TSIA, and Motility test in Sulfid Indol Motility Agar medium).

2.6. Data Analysis

The data in this study were the existence of bacteria, morphological character (Microscopic and Macroscopic), cellulolytic and lignolytic Index. The data was analyzed with descriptive and qualitative method.

III. RESULTS AND DISCUSSION

3.1. The existence of indigenous microflora from SMS of *P. ostreatus*

Mapping the existence of bacteria in SMS of *P. ostreatus* was taken from 3 different locations. The results of the enumeration of cellulolytic, lignolytic and total bacteria in each sample can be seen in Table I.

TABLE I. THE EXISTENCE AND PERCENTAGE OF SEVERAL BACTERIAL GROUPS FROM SMS OF *P. OSTREATUS* IN SOME DIFFERENT LOCATIONS

Bacteria	Total colony (…×10^5cfu/g)		
	Padang	Payakumbuh	Padang Panjang
Cellulolytic (CMCA)	41	51	48
Lignolytic (LBMBA)	80	60	66
Total bacteria (GPA)	196	184	164

The total existence of bacteria in *P. ostreatus* SMS ranged from 164–196×10^5 cfu/g. Substrate composition and nutrients in SMS affect the existence of bacteria. Badu *et al*. [3] reported that SMS mainly consists of 46% cellulose, 31% lignin, 16% hemicellulose, and 17% protein. Several related studies showed that the total bacteria in SMS had different values. Ntougias *et al.* [15] obtained the total bacterial population on SMS substrates which is 180×10^7 cfu/g, and ***Kamelia*** *et al*. [16] reported an abundance of bacteria in *P. ostreatus* SMS reached 94.6×10^5 cfu/g.

In the SMS of *P. ostreatus*, there were cellulolytic bacteria that detected in CMCA media. The results indicated the existence of cellulolytic bacteria in a range of 41–51×10^5 cfu/g. The remnants of cellulose in SMS affect The existence of cellulolytic bacteria. A group of cellulolytic bacteria makes cellulose compounds a carbon source. Based on Kamelia *et al*. [16], the SMS of Oyster Mushrooms left remnants of sawdust substrate rich in cellulose.

The existence of lignolytic bacteria was also found in *P. ostreatus* SMS, which was detected using LBMBA media. The results indicated that lignolytic bacteria were in the range of 60-80 × 10^5 cfu/g. lignolytic bacteria have a high amount of abundance. It is because the growth media of mushrooms mostly consists of sawdust, a source of lignocellulose. Morales *et al*. [17] reported that the lignocellulosic compound in sawdust consist of 52.68% cellulose, 10.68% hemicellulose and 25.98% lignin.

3.2. Evaluation of Cellulolytic Index

Twelve isolates of cellulolytic bacteria are characterized cellulolytic index (Table II).

TABLE II. THE CELLULOLYTIC INDEX OF POTENTIAL ISOLATES FROM *P. OSTREATUS* SMS

Isolates	Cellulolytic Index
PD01S	4,20
PD02S	2,40
PD03S	2,12
PD04S	**4,50**
PYK01S	1,66

PYK02S	1,11
PYK03S	1,26
PYK04S	**3,54**
PP01S	1,92
PP02S	3,25
PP03S	2,00
PP04S	**3,40**

Specific media of cellulolytic bacteria contain CMC (Carboxy Methyl Cellulase) substrates which can be degraded by cellulase enzymes (CMCase). Munifah [18] explained that cellulase enzymes hydrolyze cellulose by breaking β-1,4 bonds in cellulose, cellodectrin, cellobiose, and other cellulose derivatives into simple sugars or glucose. The cellulolytic index of the bacterial isolates was calculated through the clear zone formed around the colonies after dripping with congo red solution. Anand *et al.* [9] explained that congo red would bind to β-1,4 glycosidic bonds in CMCA media so that the media turns red.

Each bacterial isolate produces a different cellulase enzyme (Table 2). index values are low to high categories, ranging from 1 to 4.5. The difference in index value from each isolate was due to the diversity of bacterial isolates. Thus the number of cellulase enzymes produced varied. Sudiana *et al.* [19] explained that the genus or species of bacteria affect the cellulolytic index. Each group of bacteria has a different ability to produce cellulase enzymes that hydrolyze CMC substrates. Three isolates with the highest cellulolytic index values from each location (PD04S, PYK04S, and PP04S) belong to the high cellulolytic index category (Figure 1).

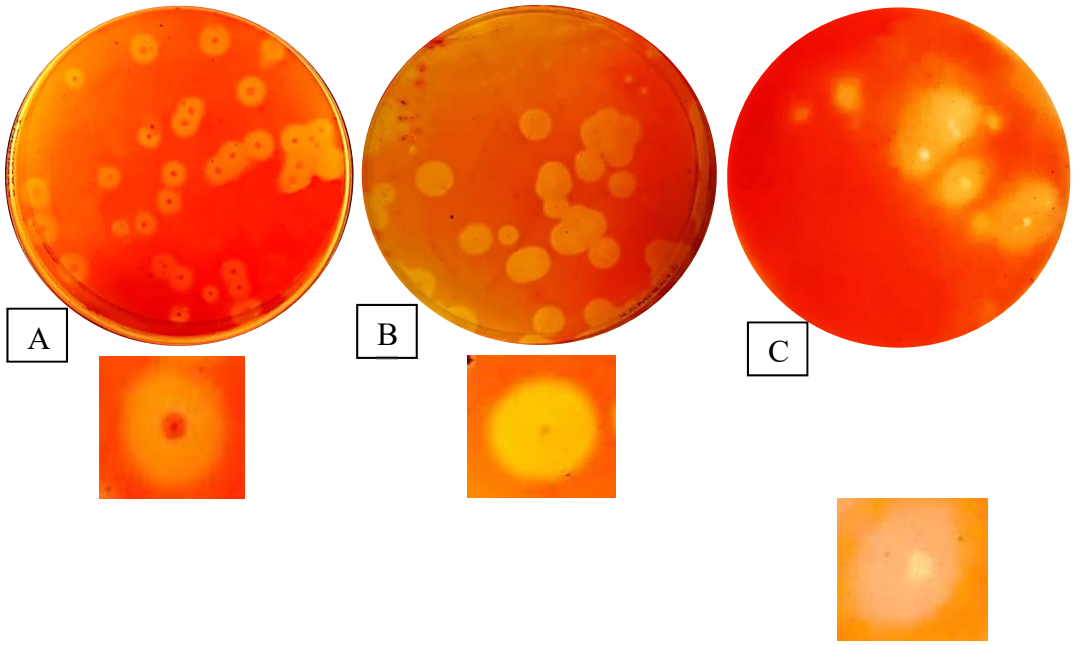

Figure 1. The clear zone areas produced by each potential cellulolytic bacteria isolates on CMCA media

A. PP04S, B. PYK04S and C. PD04S.

3.3. Evaluation of Lignolytic Index

six isolates of cellulolytic bacteria are characterized lignolytic index (Table III).

TABLE III. THE LIGNOLYTIC INDEX OF POTENTIAL ISOLATES FROM P. OSTREATUS SMS

Isolates	Lignolytic index
PD1L	1,63
PD2L	**2,56**
PYK1L	1,48
PYK2L	**2,66**
PP1L	1,55
PP2L	**2,00**

Colonies that showed lignolytic activity were determined by clear zones forming on LBMBA media. The clear zone was formed due to the degradation and decolorization of methylene blue as a lignin-like compound. Bandounas *et al*. [12] explained that lignin peroxidase (LiP) enzyme of bacteria could degrade Methylene Blue (MB), which is known as a dye derived from lignin derivatives. Oxidation of MB by lignin peroxidase (LiP) occurs through gradual N-demethylation, followed by cleavage of the aromatic ring [20]. The three isolates with the highest lignolytic index values from each location (PD2L, PYK2L, and PP2L) belong to the high lignolytic index category (Figure 2).

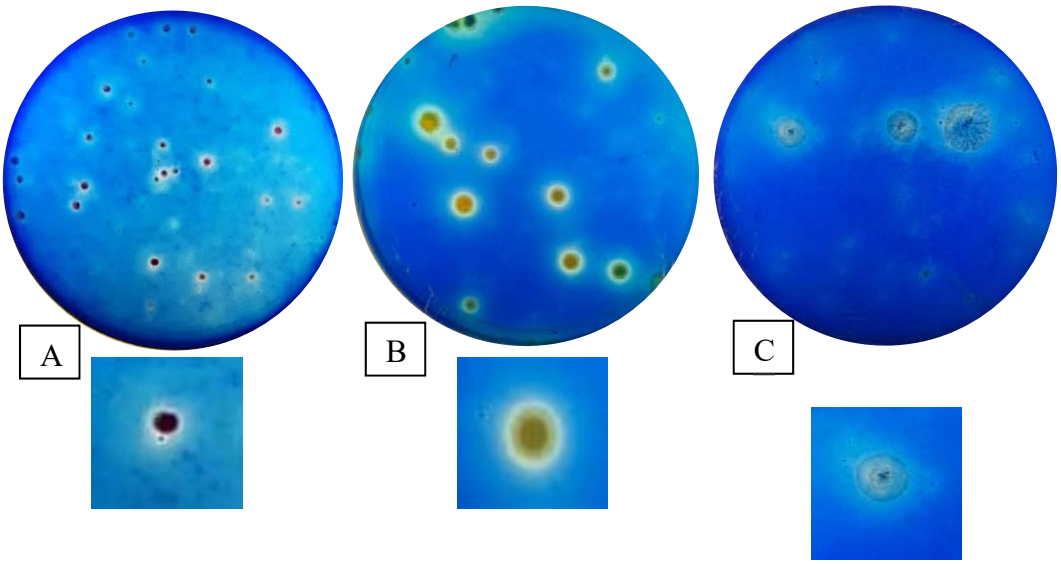

Figure 2. The clear zone area produced by each potential lignolytic bacteria isolates on LBMBA media

A. PD2L, B. PYK2L and C. PP2L

3.4. Characterization of potential cellulolytic and lignolytic bacteria

Selected cellulolytic and lignolytic bacteria isolates continued to be characterized by partial morphology (macroscopic, microscopic, and biochemical) (TABLE IV).

TABLE IV. PARTIAL CHARACTERS OF POTENTIAL CELLULOLYTIC AND LIGNOLYTIC BACTERIA FROM P. OSTREATUS SMS

Characteristics	Isolate					
	PD04S	**PYK04S**	**PP04S**	**PD2L**	**PYK2L**	**PP2L**
Macroscopy						
a. Colony shape	Circular	Circular	Circular	Circular	Circular	Circular
b. Colony margin	Entire	Entire	Entire	Entire	Entire	Entire
c. Colony elevation	Raised	Flat	Flat	Flat	Flat	Flat
d. Colony colors	White	Yellowish white	White	Red	Yellow	White
Microscopy						
a. Cell shape	Coccus	Bacil	Coccus	Coccus	Bacil	Bacil
b. Gram	-	-	+	+	-	-
c. Motility	-	-	-	-	-	-
Biochemical						
a. Catalase	+	+	+	+	+	+
b. KOH 3%	+	+	-	-	+	+
c. TSIA (B/S)	A/K	K/K	A/A	A/A	K/K	A/K
d. Glucose	+	-	+	+	-	+
e. Sucrose	-	-	+	+	-	-
f. Lactose	-	-	+	+	-	-
g. H_2S	-	+	-	-	+	-
h. Gas	-	-	-	-	-	-
Cellulolytic index	4,50	4,00	3,40	-	-	-
Lignolytic index	-	-	-	2,00	2,66	2,56
Genus	*Paracoccus*	*Pseudomonas*	*Staphylococcus*	*Micrococcus*	*Pseudomonas*	*Alcaligenes*

(+) : positive, (-) : negative, B: *butt*, S: *slant*, A/A:*acid/acid*, A/K: *Acid/Alkaline*, K/K: *Alkaline/Alkaline*

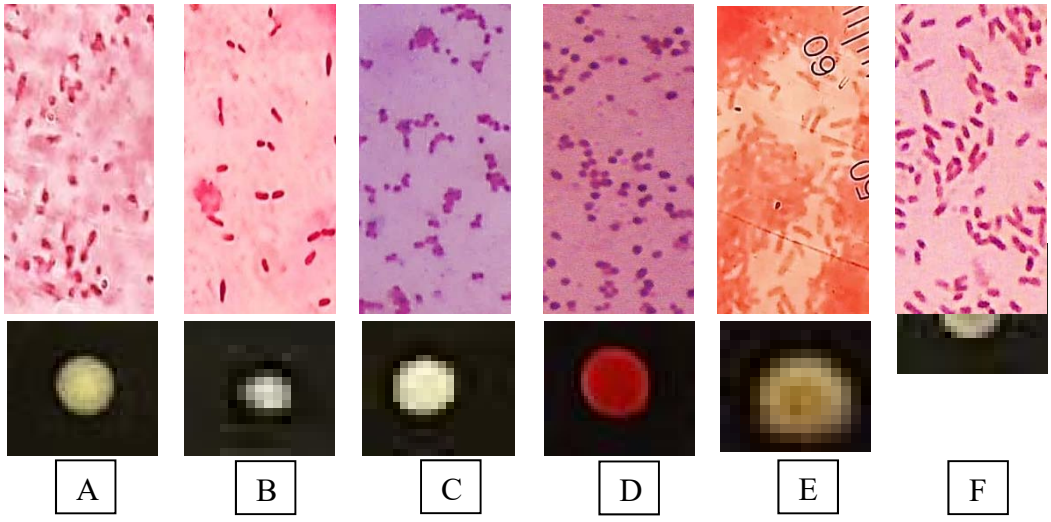

Figure 5. Morphological characters of six bacterial isolates from *P. ostreatus* SMS

A. PD04S, B. PYK04S, C. PP04S, D. PD2L, E. PYK2L, and F. PP2L

Based on morphological, physiological, and biochemical characterizations adapted to the determination key of bacterial identification "Bergey's Manual of Systematic Bacteriology, Second Edition" [21] PYK04S and PYK2L isolates has similarities to *Pseudomonas*. From morphological characters and biochemical tests of bacteria, it has rod-shaped cells and belongs to Gram-negative. In contrast, the biochemical test results were positive catalase, negative oxidase, positive motility, inability to ferment sugar, and ability to produce H2S. Previous research reported that *Pseudomonas* was also found in *P. ostreatus* SMS [22, 23].

PP04S isolate belongs to the genus of *Staphylococcus*. *Staphylococcus* is a gram-positive group that has a coccus cell shape with 0.5-1.5 mm diameter. It appears singly, in pairs, tetrads, or short chains (3-4 cells), and typically forms clusters like an irregular grape. *Staphylococcus* is nonmotile, catalase-positive, and oxidase-negative [21]. In previous studies, *Staphylococcus* was found as a cellulolytic bacteria that isolated from microbial fuel cell reactors [24] and peat soil [25].

Furthermore, PD04S belongs to *Paracoccus* genus. *Paracoccus* is gram-negative group, round or short rods shape, nonmotile, catalase positive, oxidase positive and reduce nitrate [26]. Moreover, *Paracoccus* only ferments glucose [27]. *Paracoccus* bacteria from PD04S isolate belong to cellulolytic bacteria because it has a high cellulolytic index. Ferbiyanto *et al*. [28] found *Paracoccus* bacteria as cellulolytic bacteria that isolated from the intestine of Macrotermes gilvus.

PP2L isolate is known as *Alcaligenes* group of bacteria. *Alcaligenes* has bacilli-shaped cells and belongs to gram-negative group [29]. *Alcaligenes* bacteria have a circular colony morphology and produce positive catalase and positive oxidase [30]. Ahlawat *et al.* [31] found 50 types of bacterial isolates on mushroom substrates, one of which is *Alcaligenes*. In addition, related research conducted by Rizk *et al.* [32] reported that *Alcaligenes* bacteria could degrade lignin compounds.

The characterization showed that PD2L isolate belongs to the Micrococcus genus. Micrococcus bacteria has cocci or tetrad shaped cells, gram-positive bacteria, positive catalase, negative oxidase, motile and able to produce acid through glucose and sucrose fermentation [33]. Gbolagade [23] found Micrococcus genus in growing media for the *Pleurotus tuber-regium* mushroom.

IV. CONCLUSIONS

Spent mushroom compost of *P. ostreatus* has indigenous cellulolytic and lignolytic bacteria. PD04S isolate, which refers to *Paracoccus* genus was chosen as the potential isolate of the indigenous bacteria with the greatest cellulolytic index. PYK2L isolate, which refers to *Pseudomonas* genus was chosen as the potential isolate of the indigenous bacteria with the greatest lignolytic index.

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