

Isolation and Identification of Cellulolytic and Lignolytic Bacteria from the Gut *Oryctes rhinoceros* L. Larvae Decomposition of Oil Palm Empty Fruit Bunches

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Abstract. One of the organisms that helps in decomposition of oil palm empty fruit bunches is *Oryctes rhinoceros* L. larvae. This is because of in the gut of the larvae there are many cellulolytic bacteria and lignolytic. The process of accelerating the decomposition of oil palm empty fruit bunches into compost can be done by optimizing the work of the bacteria. The aim of this research is to obtain cellulolytic and lignolytic bacteria from larvae *O. rhinoceros* L.. The research succeeded to isolate 24 isolates of cellulolytic bacteria and lignolytic bacterial isolates from the gut of *O. rhinoceros* larvae. Based on qualitative test, 9 isolate bacteria produced cellulolytic index above 2. The highest cellulolytic index was generated by ORL19 isolate, while the lignolytic index obtained ORL6 isolate. Based on macroscopic and microscopic identification of bacteria, biochemical and physiological tests, it was found ORL 6 belongs to the genus *Bacillus* sp. while the ORL 19 belongs to the genus *Citrobacter* sp..

Keywords: cellulolytic index, lignolytic index, oil palm empty fruit bunches, *O. rhinoceros* larvae.

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1. Introduction

Oil palm empty bunches is one of the waste generated from the processing of oil palm. Every processing of a ton of fresh fruit bunches (FFB) produces 23-25% oil palm empty fruit bunches, 6.5% shell, 13-15% fiber 5.5-6% seeds and 16-20% crude palm oil (CPO) [1]. Data from the Riau Province in 2014, the total CPO production in Riau Province is 1.4 million tons. It is expected to increase by 7% every year in line with increased palm oil production [2].

Utilization of waste that has not maximized causing much accumulation of oil palm empty fruit bunches in factories and oil palm plantations. Composting it naturally takes about 3 months, this is because the composition of it is difficult to decompose, which consists of 33.25% cellulose,

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56.49% holocellulose, 23.24% hemicellulose and 25.83% lignin [3]. So far oil palm empty fruit bunches used as raw material for compost fertilizer, but constrained in the length of the composting process.

Efforts that can be done to accelerate the process of decomposition of organic materials one of them is by the addition of microorganisms. Microorganisms that can accelerate the decomposition process of oil palm empty fruit bunches are suspected to be in the digestion of larvae *O. rhinoceros* L., this is because it is a food source for the larvae. The results of study, successfully isolated 28 bacterial strains from the larval digestive tract *O. rhinoceros* L. and 3 of them showed the highest cellulolytic activity, *Bacillus pumilus*, *B. cereus* and *B. Subtilis* [4]. The ability of cellulolytic bacteria in degrading oil palm empty fruit bunches is influenced by the enzyme cellulase produced by the bacteria that can break down cellulose into its sugar derivative compound [5]. Sugar formed during the decomposition process can be a source of energy for the growth of these cellulolytic bacteria so as to accelerate the decomposition process of oil palm empty fruit bunches [6].

The constituents of oil palm empty fruit bunches that are difficult to decompose other than cellulose are hemicellulose and lignin. Hemicellulolytic bacteria are also present in the digestive tract of larvae *O. rhinoceros* L. According to the results of the total 11 isolates isolated from the digestive tract of *O. rhinoceros* L [7]. larvae were all capable of hydrolyzing mannan and xylan which were the components of the hemicellulosic composer, where the highest mannanolytic activity by *Bacillus aryabhatai* and the highest xylanolytic activity by *Bacillus pumilus*.

Larvae *O. rhinoceros* L. is one of the sources of cellulose and hemicellulose degrading bacteria [7], but research on the bacteria's ability to degrade lignin has never been done. The objective of this research was to obtain bacteria from *O. rhinoceros* L. larvae which have potential as oil palm empty fruit bunches composition.

2. Materials and Methods

The organisms used as the isolates source were instar 2 and 3 instars of *O. rhinoceros* extracted from PTPN V Sei Plantation Sei Galuh. The isolation medium used is Nutrient Agar (NA). Media screening of bacteria using cellulolytic media 0,02% $MgSO_4 \cdot 7H_2O$; 0,03% NH_4NO_3 ; 0,05% K_2HPO_4 ; 0,1% KH_2PO_4 ; 0,002% $FeSO_4 \cdot 7H_2O$; 0,004% $CaCl_2 \cdot 2H_2O$; 0,2% yeast extract, 1% CMC, and 1.5% for bacto. Lignolytic bacterial screening using 1% trypton, 1% NaCl, and 0.5% yeast extract and 1.5% agar bacto [8].

2.1. Isolation of Bacteria from Larva *O. rhinoceros*

A total of 1 g of the gastrointestinal tract of larvae was homogenized using a physiological solution (NaCl 0.85%) by a series dilution method. The diluted solution of 10^{-4} - 10^{-7} was each taken as 100 μ l using a micro pipette then dripped and leveled over the surface of the NA medium. Isolates were incubated for one to three days at room temperature [9]. Subsequently isolates were grown purified onto a new NA medium to obtain pure isolates.

2.2. Qualitative Test of Cellulolytic Capability

A qualitative bacterial test was performed by bottling one ose of pure isolate on a CMC medium and incubated for 5 days at 37 °C. Furthermore, the isolate growing on the media was flooded with 15 ml of congo red 0.1% for 30-60 minutes. After that was rinsed 2 times with 15 ml of 1% NaCl and silenced again for 15 minutes [10]. Cellulolytic index or Cellulolytic Activity Index (IAS) was obtained by using [11] in [10].

2.3. Qualitative Test of Lignolytic Ability

The lignolytic ability test was performed by bottling 1 ose isolate which was 48 hours old 1 ose then inoculated on LB medium and methylene blue for 24 hours at 30°C. The resulting clear zone indicates that the microb is producing an enzyme that can degrade lignin [12]. Observation of ligninase activity was done by measuring the diameter of the colony and the diameter of the clear zone formed using the sliding term. The ligninase activity index is calculated as the ratio between the diameter of the clear zone and the diameter of the colony.

2.4. Identification of Potential Bacteria

Identification of potential bacteria cellulolytic and lignolytic conducted several stages that was by looking at the microscopic character through gram staining and physiological and biochemical tests. Gram staining was done by the method of [13]. Purple bacteria are classified into Gram positive bacteria and red bacteria are classified into Gram-negative bacteria [14]. Biochemical tests consist of carbohydrate fermentation test (mannitol, maltose, sucrose, and glucose test), MR test and test VP. Physiological test was oxygen requirement test.

3. Results and Discussion

3.1. Test of Cellulolytic and Lignolytic Potential

The isolation of bacteria from the digestion of *O. rhinoceros* L. larvae was obtained by 24 pure isolates with the code of ORL 1, ORL 2, ORL 3 to ORL 24. Of these 24 isolates, 9 were able to grow on cellulolytic and lignolytic test media, ie ORL 1, ORL2, ORL4, ORL6, ORL 7, ORL8, ORL17, ORL 19 and ORL24. The result of potential bacteria isolate test can be seen in Figure 1.

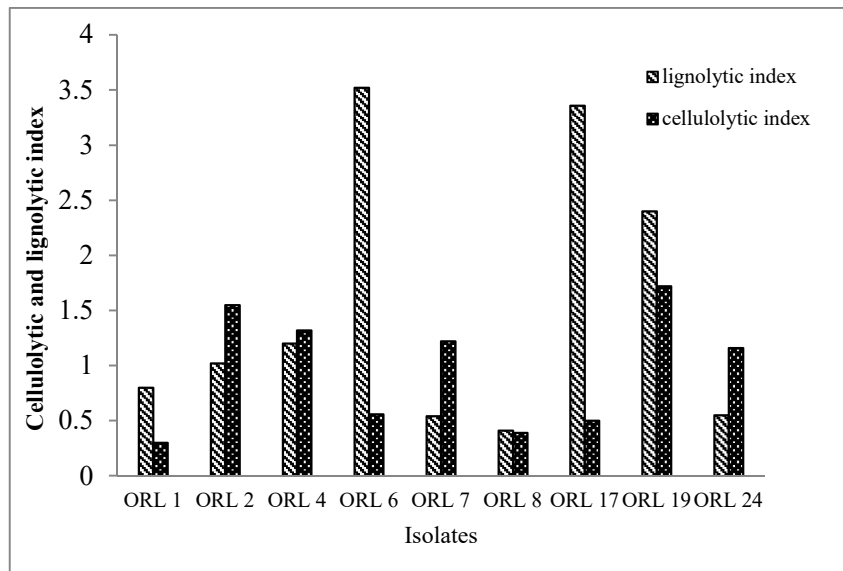


Figure 1. Cellulolytic and Lignolytic Index of Bacterial Isolates

Figure 1 showed that 9 bacterial isolates have the potential to produce various cellulases and lignases in each isolate. The value of cellulolytic and lignolytic activity is strongly influenced by the ability of bacteria to produce its enzymes. The bacteria isolate produced the highest index of cellulolytic activity ie isolate with the code of ORL 19 of 2, whereas the bacteria isolate yielding the highest lignolytic activity index was ORL 6 isolate of 3.52.

The ability of bacteria in degrading cellulose is characterized by the formation of clear zones on CMC media. According to [15], the use of CMC as an activity test medium because CMC is a derivative of water-soluble cellulose that triggers bacteria to excrete cellulase enzymes that break down cellulose into glucose. [16] added that due to the hydrolysis of cellulose into glucose in CMC solid medium, around the colony there appears a brighter region called a cleared zone. This bright zone is the indicator that inoculated bacterial isolates are capable of decomposing cellulose.

Clear zone formation is enhanced by the addition of Congo Red reagents. Congo Red is able to bind ((1-4) - β -D-glucan causing red colour [17]. The presence of hydrolysis (1-4) - β -D-glycosidic causes a decrease in the intensity of the resulting colour. Clearly coloured areas around the colonies indicate the presence of cellulose hydrolysis by cellulase enzymes [18]. The clear zone formed due to enzymes secreted by bacterial cells either cellulase, pectinase or lignase does not occur binding by Congo Red [19].

The diameter of the cellulolytic bacteria clear zone is generally larger than the diameter of the bacterial colony. This is because cellulase enzymes are secreted into the environment around bacteria [20]. Cellulolytic activity of bacteria was measured by calculating index value of cellulolytic activity based on [11] method. The cellulolytic activity index is the ratio between the diameter of the clear zone and the diameter of the bacterial colony.

According to [21], the large or small cellulolytic index produced by bacteria signifies the strong ability of bacteria in degrading cellulose. [22] adds that the greater the clear zone formed the higher the cellulolytic activity.

The magnitude of the ability of 9 bacteria in producing cellulase is also seen in lignolytic, namely the ability of bacteria to decompose lignin. Lignin is a complex compound comprising subimmethoxylated, monomethoxylated and non-methoxyphenyl phenylpropane units [23]. Lignin is found mostly in secondary plant cell walls. The compound fills the gap between cellulose, hemicellulose and pectin, making the cell wall more rigid, hydrophobic and difficult to degrade by microorganisms [24].

Testing of lignolytic ability qualitatively by measuring the clear zone produced on Luria Bertani (LB) medium which is given lignin derivative dye that is methylene blue [8]. The formation of clear zones indicates that the bacterial colonies produce enzymes that degrade the surrounding lignin [12]. [25] reported that methylene blue may also be used for lignolytic performance testing quantitatively by looking at the degree of lignin peroxidase demethylation to methylene blue.

The results showed that bacteria isolated from the digestive tract of *O. rhinoceros* L. larvae had different abilities in degrading lignin. This is evident from the size of the clear zone formed on the test medium and the lignolytic index value index. Isolate with code ORL 6 has the highest lignolytic index value index that is 3.52, followed by ORL 17 with value 3,36 and ORL 19 with value 2,4. The index value of the lowest lignolytic activity is ORL 8 with a value of 0.41. According to [12] clear zone formed indicates the presence of methylene blue degradation by bacterial isolates.

3.2. Identification of Potential Bacterial Isolates

Identification of bacteria from the digestive larvae of *O. rhinoceros* L. was performed to determine the nine types of potential bacteria in producing cellulase and lignase. Identification is done to include macroscopic and microscopic morphology. Macroscopic colony morphological observations were seen in a single colony of pure cultures. [26] states that observations of bacterial morphology in macroscopic terms include shapes, edges, colours and bacterial elevation. Characteristics of bacterial microscopic morphology were observed by Gram staining. Gram staining is one of the most widely used procedures for classifying various bacteria [27]. According to [28], Gram staining is used to determine the morphology of bacterial cells as well as to distinguish Gram positive and Gram negative bacteria. The results of morphological observations of bacterial colonies both macroscopically and microscopically can be seen in Table 1.

Table 1. Morphology of Bacterial Colonies Both Macroscopically and Microscopically

Isolate code	Macroscopic morphology of bacteria				Microscopic bacteria	
	Colonies	Colony Edge	Colour	Elevation	Gram	Cell Forms
ORL 1	Spherical with a tangled surface	Wavy	Milky white	Arises	+	Bacil
ORL 2	Irregular spread	Lobal	Milky white	Convex	-	Bacil
ORL 4	Filiform	Wavy	Clear white	Convex	-	Bacil
ORL 6	Round	Smooth	Milky white	Convex	+	Bacil
ORL 7	Irregular spread	Branch	Clear white	Arises	-	Bacil
ORL 8	Rhizoid	Irregular	Milky white	Convex	-	Bacil
ORL 17	Bulat	Smooth	Clear white	Arises	-	Bacil
ORL 19	Round with edges emerge	Irregular	Clear white	Flat	-	Bacil
ORL 24	Filiform	Wavy	Milky white	Arises	-	Bacil

Based on observations of colonies of selected bacterial isolates, it is dominated by rounded colonies with different shapes. Based on [13], the edge of the colony seen in the petri dish can be a slippery, irregular, shimmering, jagged and choppy edge. According to [29], the difference in the shape of growth of each bacterial isolate in the cup medium to indicate that all isolates come from different types of bacteria, where the colony form can be used as the basis for bacterial identification. [26] added that based on the morphological characteristics of bacterial colonies and pure cultures can be done the process of identification of the types of microorganisms. Table 1 also shows that there are seven bacterial isolates from the digestive tract of *O. rhinoceros* L. larvae which are Gram negative (ORL 2, ORL 4, ORL 7, ORL 8, ORL 17, ORL 19, ORL 24) and two other isolates are Gram positive (ORL 1 and ORL 6) with the cell form entirely of bacil.

3.3. Biochemical and Physiological Test of Bacteria

Biochemical and physiological tests are the tests used to determine the properties of bacteria isolated from the digestive tract of *O. rhinoceros* L larvae. Each bacterial isolate is tested on a series of media to view its biochemical and physiological activity. Biochemical tests performed include carbohydrate fermentation test (glucose, sucrose, maltose and mannitol), MR (Methyl Red) test, and VP (Voges Proskauer) test, while physiological test is oxygen demand test. The result of biochemical test can be seen in Table 2.

Table 2. Biochemical Test Results of Bacteria

Isolate Code	Carbohydrate Fermentation Test				MR Test	VP Test
	Glucose	Sucrose	Maltose	Mannitol		
Control	-	-	-	-	-	-
ORL 1	+	+	+	+	-	+
ORL 2	+	+	+	+	+	-
ORL 4	+	+	+	+	-	+
ORL 6	+	+	+	+	-	-
ORL 7	+	+	-	+	+	-
ORL 8	+	+	+	+	-	-
ORL 17	+	+	+	+	-	-
ORL 19	+	+	+	+	-	-
ORL 24	+	+	+	+	+	-

Information:

ORL : *O. rhinoceros* larvae

+ : Change colour

- : It does not change colour

The nature of bacterial metabolism in biochemical tests is usually seen from the interaction of metabolites produced against chemical reagents. The nature of bacterial metabolism can also be seen from the ability of bacteria in using certain compounds as a source of carbon and energy sources [30]. The result of biochemical test in Table 3 shows that each isolate has different biochemical activity. According to [30], the biochemical activity of each type of bacteria is different because each bacterium has different enzymatic activity, therefore the biochemical test stage is very influential in distinguishing the genus found.

The results of bacterial carbohydrate fermentation test showed positive results, except isolate ORL 7 which showed negative result on maltose test. Similarly, MR test showed 2 isolates which showed positive result that ORL 2 and ORL 24 and 7 other isolates showed negative result while in VP test there were 2 isolates showing positive result that ORL 1 and ORL 4 and 7 other isolates showed negative result.

3.4. Oxygen Needs Test

Oxygen demand test is performed to see the ability of bacteria in utilizing oxygen using Oxidative-Facultative (OF) media. According to [26], bacteria can be sorted according to the need for oxygen, including obligate aerobes, obligate anaerobes, facultative anaerobes and microaerophils. The oxygen demand test results can be seen in Table 3.

Table 3. Results of bacterial oxygen demand test from larvae *O. rhinoceros* L.

Isolate Code	Colour Change		Information
	Anaerob Test	Aerob Test	
Kontrol	-	-	-
ORL 1	+	+	Facultative Anaerob
ORL 2	+	-	Anaerob
ORL 4	+	-	Anaerob
ORL 6	+	+	Facultative Anaerob
ORL 7	+	-	Anaerob
ORL 8	+	-	Anaerob
ORL 17	+	+	Facultative Anaerob
ORL 19	+	+	Facultative Anaerob
ORL 24	+	-	Anaerob

Information:

ORL : *O. rhinoceros* larvae

+ : Changed colour

- : It does not change colour

Based on the morphological, biochemical, and physiological identification of nine bacterial isolates, ORL 1, ORL 2, ORL 4, ORL 6, ORL 7, ORL 8, ORL 17, ORL 19 and ORL 24 refer to the book Bergey's Manual of Determinative Bacteriology 7th edition, the ORL 1 and ORL 6 belong to the genus *Bacillus* sp., ORL 2 belong to the genus *Proteus* sp., ORL 4 belong to the genus *Ochrobactrum* sp., ORL 7 belong to the genus *Erwinia* sp., ORL 8 included in the genus *Aeromonas* sp., ORL 17 and ORL 19 belong to the genus *Citrobacter* sp. and ORL 24 belong to the genus *Pseudomonas* sp..

The results of [4] study found 28 bacterial strains isolated from the digestive tract of the *O. rhinoceros* L. larvae, three of which yielded the highest cellulolytic activity then based on morphological and biochemical characteristics indicating these bacteria were *Bacillus pumilus*, *Bacillus cereus* and *Bacillus subtilis*. The results of research [7] added that 11 bacteria isolated from the digestive tract of larval *O. rhinoceros* L. were identified, 10 of which belong to the genus *Bacillus* sp. and one isolate belongs to the genus *Citrobacter* sp.

Cellulolytic and hemicellulolytic bacteria can be found almost in all insect digestive tracts, such as in the gut of *Oryctes rhinoceros* larvae [7], in *Bombix mori* larvae [31], *Holotrichia parallela* larvae [32], on insects like *Reticulitermes flavipes* [33], *Anoplophora glabripennis* [34], and *Attacus atlas* L. [35], capable of degrading plant biomass with the aid of microorganisms found in the digestive tract.

Herbivorous animals are able to utilize cellulose as a source of energy. This ability is supported by the presence of microorganisms in the digestive tract that mampu produce cellulase enzyme. Based on the results of the study of [7], from a total of 11 isolates isolated from the digestive tract of *O. rhinoceros* L. larvae, 63.6% were cellulolytic bacteria, 72.7% xylanolytic and 100% mannanolytic. [7] added that the *O. rhinoceros* L. larvae are the best source for studying

cellulolytic, xylanolytic and mannanolytic microorganisms and their enzymes that are particularly useful for degrading lignocellulose.

4. Conclusion

The results of bacterial screening from the digestive larvae of *O. rhinoceros* L. were obtained as many as 24 bacterial isolates, nine of which were potential as oil palm empty fruit bunches compositions which were demonstrated by the cellulolytic and bacterial lignolytic potential test with ORL 1, ORL 2, ORL4, ORL 7, ORL 8, ORL 17, ORL 19 and ORL 24. Isolates yielding the highest cellulolytic index on ORL 6, while lignolytic in ORL19.

Based on the results of macroscopic and microscopic identification of bacteria and bacterial biochemical and physiological tests, it was suspected that the isolates of ORL 1 and ORL 6 belong to the genus *Bacillus* sp., ORL 2 belonging to the genus *Proteus* sp., ORL 4 belongs to the genus *Ochrobactrum* sp., ORL 7 belongs to the genus *Erwinia* sp., ORL 8 belongs to the genus *Aeromonas* sp., ORL 17 and ORL 19 belongs to the genus *Citrobacter* sp. and ORL 24 are included in the genus *Pseudomonas* sp. Further identification is required on these 9 isolates to molecular identification potential bacterial species as cellulolytic and lignolytic bacteria.

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