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Isolation and Identification of Cellulolytic Bacteria from Gut of Horn Beetle Larvae (*Oryctes rhinoceros* L.)

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ABSTRACT. The horn beetle larvae (*Oryctes rhinoceros* L.) contain symbiotic bacteria that are used to digest and degrade cellulose as food so that it has the potential to produce cellulase enzymes. This study aims to isolate, characterize and identify microbial symbionts from horn beetle larvae that have the potential to produce cellulase enzymes.The methods in this study include morphology and physiology identification of bacteria, qualitative and quantitative activity tests and species determination using 16S rRNA sequencing technique. Based on the results of morphological observations, five bacterial isolates were taken which has the potential as a cellulase producer is indicated by the presence of a clear zone that is produced when a qualitative test is carried out using congo red staining with different cellulolytic indices. Based on the quantitative bacterial activity test using UV-Vis, the highest activity was found in PES3 isolates at 1.62 x 10-2 and PES5 at 1.61 x 10-2. Species determination results found that PES3 isolates belonged to the genus Acinetobacter and PES5 belonged to Pseudomonas. In addition to the isolates obtained for the environment and the industrial sector, cellulolytic bacteria can provide added value such as hydrolyze cellulose waste into alternative fuels.

Keywords: Acinotebacter junii, cellulose, cellulolytic, *O. rhinoceros*, pseudomonas guguanensis

INTRODUCTION

Cellulase is one of the enzymes that is very much needed and has the potential for biotechnology applications in various fields in the industrial sector, so that the demand for cellulase is increasing (Pramanik et al., 2021). Cellulase can be used to assist the production process of paper, cloth, food, and medicine, while in the environmental field, enzymes are able to decompose plant waste to produce fermented sugar (Sriariyanun et al., 2016). Cellulase can also be applied to the paper industry, namely to smooth paper pulp, while in the textile industry it is used to maintain the color and brightness of the fabric (Okal et al., 2020). Cellulase enzymes are produced from microorganisms capable of producing cellulase, namely cellulolytic microorganisms.

Cellulolytic bacteria are types of bacteria that have the ability to degrade substrates containing cellulose. Isolation and production of enzymes from Aspergillus niger using peanut shells as a carbon source (Sulyman et al., 2020), production of cellulase enzymes from Bacillus sp. isolated from rice fields (Sriariyanun et al., 2016). These bacteria are also found in various ecosystems in nature, such as agricultural land, peat soil, digestive tract of ruminants, body cells and digestive tract of invertebrate animals. Isolation of bacteria that produce cellulase enzymes from the intestines of the digestive tract of insects or pests such as termites (Ali et al., 2019), Cossus-cossus (Baharuddin et al., 2014), *Bombyx mori* (Anand et al., 2010), and *Oryctes rinhoceros* (Pradeep at al., 2019) has been carried out by finding various types of bacteria that are applied as decomposers, hydrolysis of cellulose and other applications.

Horn beetle larvae (*Oryctes rhinoceros* L.) are insect pests that can cause damage to crops and even cause a decrease in oil palm production (Sasauw et al., 2017). These insects also consume coconut stalks, sugarcane residues, organic waste and can be found in coconut trunks and rotting sawdust. The food source of horn beetle larvae is material that contains cellulose,according to (Barbosa et al., 2020) that these larvae contain cellulolytic bacteria in the back intestine. The process of digestion of food that takes place in the

hind intestine, in this section there are a number of symbiotic bacteria that secrete the cellulase enzyme to decompose cellulose. Therefore, this study will isolate and identify bacteria from horn beetle larvae that have the potential to produce cellulase enzymes.

EXPERIMENTAL SECTION

Materials and Instruments

Horn beetle larvae (taken from the Bulu Taba oil palm plantation, North Mamuju Regency, 70% alcohol (C_2H_5OH), aluminum foil, aquades (H_2O), methyl-red, 3,5-dinitrosalysilic acid (DNS) (Merck), all materials for bacterial growth media were purchased from Merck. The instruments used are spectrophotometer UV-Vis Genesys 20, polymerase chain reaction (PCR) machine Bio-Rad, and microscope Binocular/ Olympus CX23LED.

Sampling

The horn beetle larvae were collected from decaying oil palm tree trunks in Bukit Harapan Village, West Sulawesi, Indonesia. The larvae were taken alive and kept in jars by taking powder from the decay of coconut stalks as a source of nutrition.

Bacterial Isolation from the Horn Beetle Larvae

Each the horn beetle larvae was separated from the head and body after removing the head with forceps, take out the gut. The paste obtained from *O. rinhoceros* guts was used for bacterial isolation with a series of successive dilutions used to reduce the solid cell culture to a more usable concentration before being inoculated into the medium. Then put into an Erlenmeyer tube containing 9 mL of 0.9 (b/v) physiological solution. The Erlenmeyer tube was then shaken using a shaker (speed of 150 rpm, 30 minutes) to obtain a suspension which was expected to contain prospective bacterial isolates. The suspension was then diluted with serial dilutions (10-1 to 10-6) each dilution in the test tube was homogenized using a vortex mixer. The results of the dilution were then taken as much as 0.1 mL which was then spread using the scatter plate method on NA media. The isolates were incubated for 24 hours at 37 °C (Batubara et al., 2021).

Cellulolytic Qualitative Activity

Agar plates were prepared with 1% CMC. The strain was streaked and petri plates were incubated at 37 °C for 72 hours. Petri plates were flooded with 0.1% (b/v) Congo-red reagent and left for 15 minutes. Then the plates were washed with 1 M NaCl. The clear zone was an indicator that the inoculated bacterial isolates can use cellulose as a carbon source. The clear zone was formed due to the hydrolysis of cellulose into glucose in CMC media (Batubara et al., 2021).

Morphological Characteristics of Bacteria

The morphological characteristics of bacteria include the shape, edge, elevation and color of bacterial colonies.

Physiological Characteristics Of Bacteria

Physiological characteristics of bacteria were carried out in two stages, namely Gram staining and biochemical analysis. Gram staining was done to distinguish the Gram properties of bacteria. Each bacterial were stained by the Gram stain method following a standard staining protocol (Caico, 2006). Bacterial biochemical analysis was carried out using several tests, such as motility test using Sulfide Indole Motility (SIM) medium, sugar fermentation ability test, hydrogen sulfide production using Triple Sugar Iron Agar (TSIA) medium, catalase test, Methyl-Red-Voges Proskaeur (MR-VP) and Simon's Citrate Agar (SCA) test (Batubara et al., 2021).

Quantitative Activity Test-DNS Method

The cellulase enzyme activity test was based on the amount of reducing sugar (glucose) produced from the hydrolysis of cellulose using DNS method. A mixture of 1 mL of crude extract solution of cellulase enzyme, 1 mL of phosphate buffer pH 7.0 and 1 mL of 1% (b/v) CMC was incubated at 37 °C for 30 minutes. After that, 3 mL of DNS reagent was added to the mixture, then homogenized with a vortex for 10 seconds at 1200 rpm. The mixture is heated in boiling water for 5 to 10 minutes, then cooled in iced water. The results of the reaction were measured absorbance using a UV-Vis spectrophotometer at the maximum wavelength. Calculation of glucose levels by substituting the absorbance of the solution obtained in the determination of glucose levels into the regressive equation of the calibration curve of the glucose standard solution.

16S rRNA Molecular Assay

DNA extraction of potential endophytic bacteria was carried out by centrifugation of colonies of potential isolates that had been suspended in a sterile saline solution. The pellets were resuspended by adding 0.5 mL of InstaGene Matrix (Bio-Rad, USA). After that, it was incubated at 56 °C for 30 minutes, then praised at 100 °C for 10 minutes. The supernatant (which already contained bacterial DNA) was stored at 40 °C. The isolated DNA was used for amplification of the 16S rRNA gene using a PCR machine. The results of the PCR can be known qualitatively through electhrophoresis using 1% (b/v) agarose gel which is then visualized with the help of a UV transilluminator. The cycles that occur in PCR are obout 35 cycles. The primers used for 16S rRNA gene amplification were primers 63f (5'-CAG GCC TAA

CAC ATG CAA GTC-3') and 1387r (5'-GGG CGG WGT GTA CAA GGC-3'). 1 of 20 of the overall reaction's volume was added as DNA concrete. 35 cycles of DNA amplification were performed, with the initial denaturation settings being 94 °C for 45 second, 55 °C for 60 second, and 72 °C for 60 second. PCR product purification using the montage PCR clean up kit (Milipore). The PCR result were determined by the DNA base sequence using primers 518F (5'-CCAGCAGCCGCGTA ATACG-3') and 800R (5'-TACCAGGGTATCTAATCC-3') (Tamura et al., 2011), as well as big dye terminator cycle sequencing kit (Applied BioSystem) and then the analysed using Applied BioSystem model 3730XL automatic DNA sequencing system (Applied BioSystem). Then the sequence data were compared with data in GenBank using the BLAST program from The National Center for Biotechnology Information (NCBI) database, website (http://www.ncbi.nlm.nih.gov), the sequence were aligned using ClustalW with Bioedit and method for phylogenetic using MEGA 11 program (Harahap et al., 2018).

RESULTS AND DISCUSSION

After incubation and morphological observation, 5 isolates with the following characteristics were selected (**Table 1**). From **Table 1** it is known that the bacterial isolates have their respective characteristics, namely brownish white color, regular and irregular edges, raised elevation and round shape. The grouping of bacteria is then carried out microscopically with gram staining, which is to see the bacteria that have been stained under a microscope for further characteristic observations. The results can be seen in **Figure 1**.

The results of observations in **Figure 1** show that the PES1 bacterial isolates are purple rods, while the other 4 isolates are red rods. This shows that the first isolate is Gram- positive while the other four isolates are gram-negative because they can bind safranin dye, the difference in color binding in gram-negative and positive bacteria is due to the difference in the isoelectric point of the protoplasm and the permeability of the cytoplasmic membrane of the cell wall (Sukmawati, 2018).

Screening of bacterial isolates was carried out with the clear zone test to see the cellulolytic activity of the five isolates. The clear zone test used 1% (b/v) congo red staining. Cellulase producing bacteria form a clear zone with a red background as a nondegradable area. Congo red solution is dye that will diffuse into the agar medium which is absorbed by long chains of polysaccharides which have D-Glucan bonds resulting from cellulolytic activity. The ability of bacteria to grow on CMC media indicates that bacteria can utilize cellulose as a source of carbon or nutrients. The clear zone is an early indication to determine the ability of bacteria to decompose cellulose (Meryandini et al., 2010).

Table 1. Morphological characteristics of cellulolytic bacteria isolated from the horn beetle larvae

No.	Isolate Code	Color	Edge	Elevation	Shape
1	PES1	Pale yellow	Irregular	Raised	Round
2	PES2	Brown	Irregular	Raised	Round
3	PES3	Pale yellow	Regular	Raised	Round
4	PES4	Pale yellow	Irregular	Raised	Round
5	PES5	Pale brown	Irregular	Raised	Round

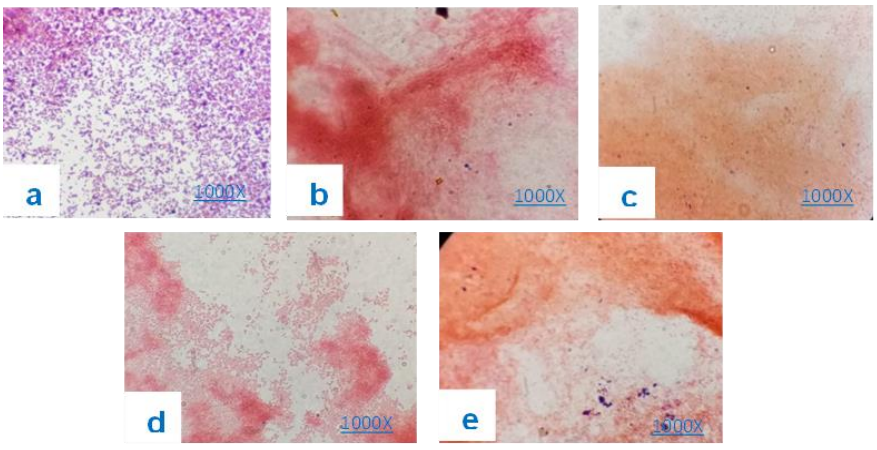

Figure 1. Gram staining results of horn beetle larvae bacterial isolates (**a**) PES1 (**b**) PES2 (**c**) PES3 (**d**) PES4 (**e**) PES5

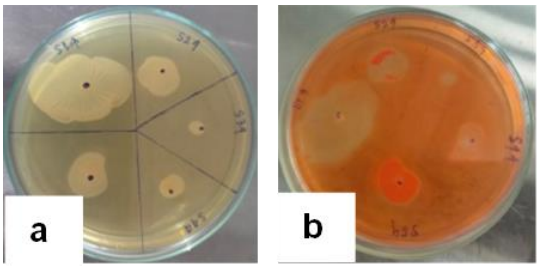

Figure 2. Clear zone of bacterial isolates (**a**) Isolates before staining (**b**) Clear zones after staining with congo red

Table 2. Cellulolytic index and cellulose enzyme activity of isolates from the horn beetle larvae

No.	Isolate Code	Cellulolytic Index (mm)	Cellulase Enzyme Activity (U/mL)
1	PES_1	0.59	1.50×10^{-2}
2	PES_2	4.43	1.30×10^{-2}
3	PES_3	0.90	1.62×10^{-2}
4	PES_4	1.10	1.50×10^{-2}
5	PES_5	2.15	1.61×10^{-2}

The highest cellulolytic index was found in PES2 isolates at 4.43 followed by PES5 at 2.15, while the lowest isolates were found in PES3 isolates at 0.90. The cellulolytic index obtained from this horn beetle larvae isolate was higher than the results of previous studies that carried out cellulolytic activity tests on *Oryctes rhinoceros*, the highest cellulase activity obtained was 0.90 of the eleven selected isolates (Nasution et al., 2021). Cellulolytic index above 2 mm is considered high (Batubara et al., 2021). Based on the criteria of (Davis & Stout, 1971) there are three categories in the diameter of the clear zone. The strong category is 10 mm-20 mm, medium category is 5 mm-10 mm, while the weak category is less than 5 mm. Therefore, to confirm the activity of the bacterial isolates, quantitative activity tests were also carried out using the DNS method. The test results can be seen in **Table 2**.

Cellulase activity quantitatively showed that PES3 and PES5 had the highest activity, therefore, to determine the cellulase activity of isolates, a quantitative confirmation test is also needed, because it can give different results, such as PES2 has a high cellulolytic index but in quantitative tests, PES3 and PES5 have higher activity, this is probably because during congo red staining, the clear zone is not visible. Further characterization was carried out by biochemical tests which included SCA test, catalase test, TSIA test, gelatinase test, motility test, methyl red-voges proskaeur (MR-VP) test, and carbohydrate fermentation test with the results as shown in **Table 3**.

Based on the table of biochemical tests, it shows that for the TSIA test there was a positive test on the PES1 isolate code, namely the formation of alkaline acid marked by a yellow color change on the top and red on the bottom, while the TSIA test on other isolates had negative results. During the TSIA test, the formation of tau H_2S gas was also observed which was marked by the formation of a black color on the scratch marks, in the five isolates no gas was formed.

Table 3. Results of biochemical tests

Biochemical Test	PES_1	PES_2	PES_3	PES_4	PES_5
TSIA	Alkaline acid	Neutral alkaline	Neutral alkaline	Neutral alkaline	Neutral alkaline
SCA	-	+	+	-	+
Urea	-	-	-	-	-
SIM (Motility)	-	-	-	-	-
Catalase test	+	+	+	+	+
MR	-	+	-	+	+
VP	-	+	-	-	-
Indo	-	-	-	-	-
H_2S	-	-	-	-	-
Glucose	+	+	+	+	+
Lactose	+	+	+	+	+
Sucrose	-	-	-	-	-
Maltose	+	-	-	-	+

The next test is SCA whose positive test is indicated by a color change in the media from green to blue. The test results showed that there were 3 positive isolates, namely PES2, PES3 and PES5. The presence of sodium carbonate will change the bromthymol blue indicator in the medium causing the medium to change color from green to dark blue (Batubara et al., 2021).

Urea test was carried out for a positive reaction characterized by a change in the medium to pink. Color changes can occur when the enzyme urease breaks the carbon and nitrogen bonds to form ammonia. The presence of ammonia causes the medium to become alkaline/base so that the phenol red indicator will turn pink in the medium, this indicates a positive reaction or its production. The test results showed that the five isolates had negative test results. The SIM test showed negative results on the five isolates, while the catalase test showed the formation of air bubbles in the five bacterial isolates, meaning that all bacterial isolates were able to produce gas or oxygen (Davis & Stout, 1971).

The MR-VP test aims to determine the ability of a bacterium to oxidize glucose by producing acid as the final product and in high concentration. The Voges Proskauer test is intended to evaluate the ability of organisms to produce non-acidic substances or neutral end products such as acetylmethyl carbonyl from organic acids as a result of glucose metabolism (Biswas et al., 2020). Based on the table of biochemical tests, isolates with positive results on the MR-VP test were found in PES2 isolates. The next test is indole test which is intended to determine the ability of microbes to degrade the amino acid tryptophan, a positive reaction is indicated by the formation of a red ring on the surface of the medium. Based on the table the test results of all isolates showed negative results, which means that the isolates obtained could not hydrolyze tryptophan. The last test carried out was the carbohydrate fermentation test, namely glucose, lactose, maltose and sucrose. In the glucose and lactose test, all isolates showed positive results, while the sucrose test all isolates showed negative results, and for the maltose test, two isolates showed positive results, namely PES1 and PES5 isolates. This shows that the bacterial isolates are not able to completely hydrolyze carbohydrates (Gergonius & Sine, 2016).

The isolates that have been found in this study are cellulase producing from symbiont of horn beetle larvae with species, namely Acinetobcater and Pseudomonas can be used in the hydrolysis process of biomass waste to produce some products such as fertilizer and alternative fuels. The result supported by the results of biochemical tests, according to research (Khairiah et al., 2013) that genus Acinetobacter is a cellulose-degrading bacteria that has the form of cocobacil and is a Gram-negative group of bacteria. Bacteria can utilize glucose, mannitol, maltose and sucrose, cells do not form spores and are not motile as shown in the results of biochemical tests (**Table 3**). The genus Pseudomonas has rod-shaped cells and is Gram-negative, the results of the biochemical test are catalase positive, motility is positive or negative, simmon citrate is positive, gas is present, and does not contain H_2S. Pseudomonas bacteria are Gram-negative bacteria, rod-shaped, aerobic, and produce the enzyme catalase.

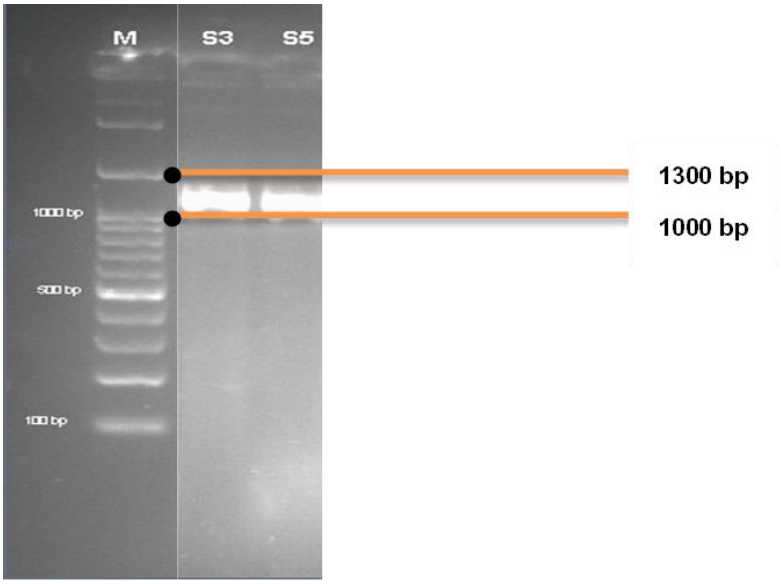

Figure 3. PCR products of PES3 and PES5 isolates using 16S rRNA primers (M = 1 kb plus ladder; S3= PES3 isolate; S5 = PES5 isolate)

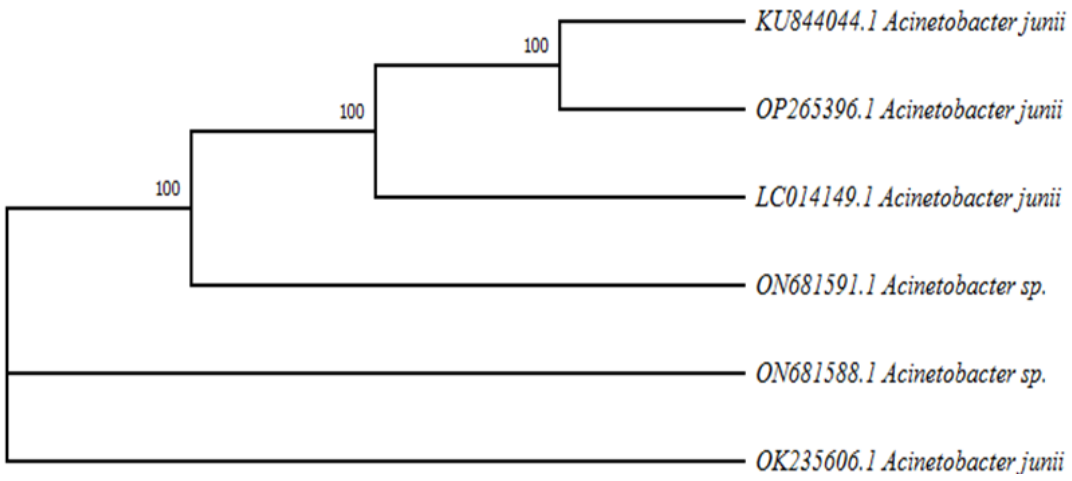

Figure 4. Phylogenetic tree of PES3 isolate obtained from NCBI database according to 16S rRNA gene sequence

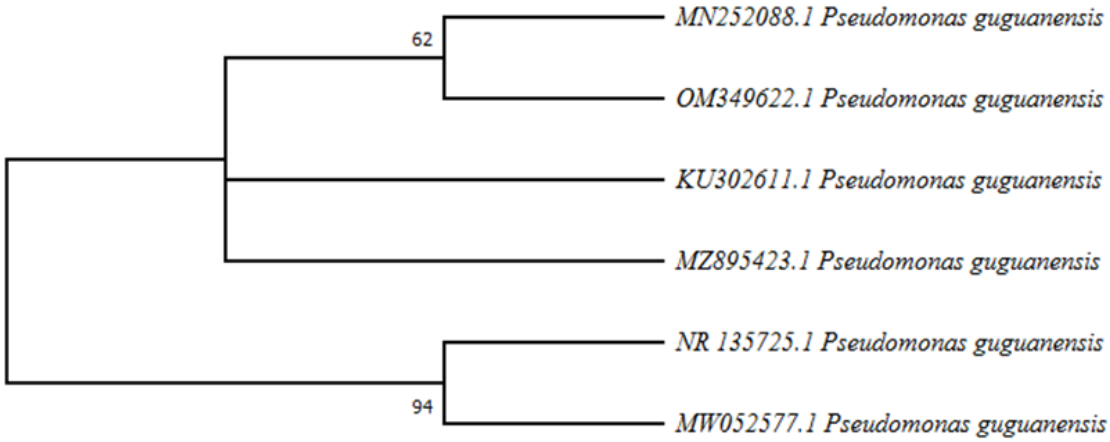

Figure 5. Phylogenetic tree of PES5 isolate obtained from NCBI database according to 16S rRNA gene sequence

Based on the highest activity of the five isolates, two isolates were selected which were continued for identification with PCR and the DNA sequencing. The result of PCR obtained that PES3 and PES5 PCR results showed that both isolates produced amplicons measuring about 1300 bp. The sequencing result was aligned online in the nucleotide BLAST tool through the (NCBI) database to identify the possible genera of the isolates base on homology. Based on the analysis of the BLAST-N program, it is known that homology of PES3 and PES5 isolate species is shown in the **Figure 4** and **Figure 5**.

The phylogenetic tree show that PES3 isolate has the highest value/similarity with Acinobacter junii, while PES5 has similarity with Pseudomonas guguanensis. Acinetobacter and pseudomonas have been identified from other types of larvae. Some bacterials isolated from Cossus-cossus larvae were found able to produce cellulase and were then identified as Acinetobacter, Peudomonas and Bacillus (Baharuddin et al., 2014).

CONCLUSIONS

Based on the data obtained, the results of morphological characterization obtained five isolates which were then physiologically, biochemically, qualitatively and quantitatively showing the potential of the isolates as cellulose producing bacteria. The highest activity was found in PES3 isolates at 1.62 x 10-2 and PES5 at 1.61 x 10-2. The isolates with the highest activity were continued for identification using DNA sequencing and the results showed that PES3 belongs to the genus Acinetobacter while PES5 belongs to Pseudomonas. The resulting isolates can be used in the decomposition of biomass waste by degrading cellulose into alternative fuels such as bioethanol.

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