

Original Research Paper

# Diversity of Cellulolytic Bacteria from the Digestive Tract of *Oryctes rhinoceros* Larvae

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**Abstract:** Lignocellulose is a ubiquitous biopolymer that, once degraded into monomeric products, could be used to produce a wide range of products. Bioconversion using indigenous microbes could be a solution to the large array of cellulosic waste and it could be sourced from exotic organisms such as Asiatic *rhinoceros* beetle (*Oryctes rhinoceros* L). This study aimed to cellulolytic bacteria from the digestive tract of *Oryctes rhinoceros* larvae verify their cellulolytic abilities and identify the species based on 16S rRNA. Seven isolates were purified from the larvae and all were able to hydrolyze cellulose with the highest cellulolytic index displayed by isolate B01L (1.28), while the lowest by B06L (0.15). The highest cellulase activity assayed using the 3,5-Dinitrosalicylic acid (DNS) method was produced by B01L at 0.010 U/mL. Molecular identification using 16S rRNA assigned seven species as *Bacillus tequilensis* strain B01L, *Bacillus pacificus* strain B02L, *Serratia marcescens* strain B03L, *Lysinibacillus fusiformis* strain B04L, *Lactococcus geraviae* strain B05L, *Pseudomonas monteilii* strain B06L and *Priestia aryabhatai* strain B07L. All bacterial genera from the digestive tract had the ability to degrade cellulose. Then the result suggests that all bacteria have an important role in cellulose degradation and may have mutualistic traits with the host, such as cellulose bioconversion.

**Keywords:** *Oryctes rhinoceros* L., Cellulolytic, 3,5-Dinitrosalicylic Acid (DNS), Enzyme Activity

## Introduction

Lignocellulose is a fairly common organic substance made up of the polymer's cellulose, hemicellulose, and lignin. Cellulose and lignin are important sources for producing useful products such as sugars from fermentation processes, chemicals, and liquid fuels. Microorganisms known as cellulolytic and ligninolytic bacteria produce the enzymes cellulase and ligninase, which may hydrolyze cellulose and lignin into less complex compounds (Ahmad *et al.*, 2013; Dubey *et al.*, 2014).

Cellulose is a carbohydrate that plays an important role in life, namely as one of the main energy sources for living things. Bacteria cause most of the degradation of cellulose and lignin occurring in nature. Bacteria use multi-enzyme systems to overcome this obstacle. Numerous extracellular enzymes having binding modules with cellulose and lignin conformations are produced by

aerobic bacteria. Unique extracellular multienzyme complexes known as cellulase are seen in anaerobic bacteria. Binding to a non-catalytic structural protein (scaffold) encourages a particular component's activity in the direction of the crystalline substrate. *Clostridium thermocellum*, a thermophilic bacterium produces the most intricate and thoroughly researched cellulosomes. Cellulase preparations are capable of decomposing natural cellulose (e.g., filter paper) as well as modified cellulose such as carboxymethyl cellulose or hydroxyethyl cellulose. Carbohydrates consist of monosaccharides, disaccharides, and polysaccharides, the most abundant carbohydrates found in plants, especially wood, which are polysaccharides composed and lignocellulose of starch. The lignocellulosic component consists of D-glucose monomer units bonded to 1,4-glycosidic bonds.

Some insects that are believed to be the source of cellulolytic and ligninolytic bacteria are *rhinoceros*

beetles (*O. rhinoceros* L.). The digestive tract of these insects can degrade cellulose and lignin. This refers to the habitat of the adult *rhinoceros* beetle and larvae of *O. rhinoceros* which are commonly found in empty fruit bunches of oil palm and the composition of their contents which are a food source for *O. rhinoceros*. In insects that consume wood as food, microorganisms frequently assist in a variety of ways, including the initial acquisition of enzymes produced by microorganisms on digestible substrates, pre-digestion of the substrate before digestion, and enrichment of nutrients in the form of microbial cells and metabolites. The intestine may contain 105-109 bacterial cells associated with 26 phyla, which are nonpathogenic microorganisms. About 65% of insects have symbiotic bacteria, which are crucial for their normal growth and development. Bacteria and insects can have symbiotic relationships that range from mutualistic to commensal. According to the role of the symbiotic bacteria, endosymbionts' main and secondary forms are the two types of intracellular symbionts found in insects. Frond borer from the genus *Oryctes* is considered a very detrimental pest. Endosymbionts exist in the *Oryctes* genus, and an insect-bacterial association in this particular case may be useful to investigate (El-Sayed and Ibrahim, 2015). Insect organs can harbor a variety of microorganisms, from homogenous to complex bacterial communities. Several studies have indicated the important contributions of gut-associated microbes to the physiological attributes of the insect hosts and their life cycle. However, there are still few reports on the diversity, physiology, and ecology of insect-associated microbes, especially the ones from beetles that develop within the wood and bark of trees.

In a previous study, Sari *et al.* (2016) reported eleven bacterial isolates from *O. rhinoceros* larvae with a majority of cellulolytic bacteria (63.6%) and dominated by *Bacillus* spp. and *Citrobacter*. Another study by Dini *et al.* (2018) recovered 24 bacteria found in *O. rhinoceros* larvae's gastrointestinal tract with a similar emblage of *Bacillus* and *Citrobacter* as ligninolytic strains. Verification of the potential ability of cellulolytic bacteria can provide solutions to industrial problems. This study reports on the cellulolytic diversity of local bacteria in North Sumatra and can be used as an isolated source for ethanol and glucose production processes.

## Materials and Methods

### Isolation of Bacteria from the Digestive Tract

Larvae of *O. rhinoceros* were collected from PT Perkebunan Nusantara II Kabupaten Deli Serdang, North Sumatra Province, Indonesia. Larvae of instar III were dissected using a sterile dissection kit. The digestive tract was removed and suspended in a sterile physiological saline solution. Ten-fold serial dilution was

employed to the suspension and diluted until  $10^8$  CFU/mL (Dini and Munifah, 2014). Approximately 0.1 mL of each dilution was spread onto Nutrient Agar (NA) medium and incubated at 37°C for 48 h. Any visible bacterial colonies were purified into a new NA medium and characterized based on morphological appearances including shape, elevation, edge, and colony color. Biochemical tests were employed based on the physiological traits including amyolytic activity, gelatin hydrolysis, citrate and sugar utilization, and motility test (Liyanage and Manage, 2015).

### Screening of Cellulolytic Bacteria

Preliminary screening of cellulolytic bacteria was based on extracellular enzyme activity on solid carboxymethyl cellulose (CMC, Sigma Aldrich) medium. The solid medium was prepared (g/L) as follows: 1 g CMC; 0.02 g  $MgSO_4 \cdot 7H_2O$ ; 0.075 g  $KNO_3$ ; 0.05 g  $K_2HPO_4$ ; 0.002 g  $FeSO_4 \cdot 7H_2O$ ; 0.004 g  $CaCl_2 \cdot 2H_2O$ ; 0.2 g yeast extract; 1.5 g agar and 0.1 g glucose. Each bacterial isolate was inoculated on top of a CMC medium and incubated at 37°C for 48 h. After incubation, a Congo red solution was poured on top of bacterial colonies and left for 30 min. The solution was removed and rinsed with 0.2 M of NaCl thrice for 15 min. Halo zones around bacterial colonies indicate the positive results of the cellulolytic activity. The cellulolytic index was calculated using the formula as follows (Meryandini *et al.*, 2009; Coronado-Ruiz *et al.*, 2018).

### Cellulase Production

Two to three loopfuls of bacterial colonies were suspended in a sterile physiological saline solution to obtain an optical density (OD600) of 0.5. Ten milliliters (mL) of bacterial suspensions were inoculated into 90 mL of liquid CMC medium and incubated at 37°C for 24 h under agitation (120 rpm). After incubation, five mL of culture medium were centrifuged at 6,000 rpm for 10 min. The supernatants containing extracellular cellulase were collected and used for the next experiment.

### Cellulase Assay

The procedure for determining cellulase activity was based on the release of reducing sugar using 3,5-Dinitrosalicylic Acid (DNS) (Murtiyaningsih and Hazmi, 2017). One unit of cellulase activity is equivalent to the amount of glucose (mg/mL) per enzyme reaction at 50°C within 30 min. One mL of cellulase was reacted with one mL of 1% of CMC in one mL of 20 mM phosphate buffer (pH 7.0) and incubated as described previously. Two mL of DNS were then added, vortexed, and incubated at 50°C for 10 min in a water bath. The resulting solution was read in a UV-Vis spectrophotometer at 540 nm for 30 min (Lim *et al.*, 2015; Lokapirnasari *et al.*, 2015; Jannah *et al.*, 2018).

### Molecular Identification

DNA was isolated from bacterial isolates using the “freeze and thaw” method and pure bacterial isolates were grown on NA medium. One loopful of the isolate was suspended in a 2 mL Eppendorf tube containing 100  $\mu$ L of sterile double distilled water. The cell suspension was heated at 90°C for 10 min after being frozen at -10°C for 10 min. The “freeze and thaw” was repeated twice for efficient cell breakdown. The solutions were centrifuged at 10,000 $\times$  g for 10 min. The supernatant containing crude DNA was kept at 4°C for the amplification.

The 16S rRNA gene was amplified using a Polymerase Chain Reaction (PCR) apparatus utilizing the extracted DNA. Primer 63f (5'-CAGGCCTAACACATGCAAGTC-3') and Primer 1387r (5'-GGGCGWGTGTACAAGGC-3'), which are universal primers for numerous strains of bacteria, were employed for 16S rRNA gene amplification. (Ferbianto *et al.*, 2015; Marchesi *et al.*, 1998). A total of 25  $\mu$ L is made up of 2  $\mu$ L of DNA template, 12,5  $\mu$ L of Master Mix 2 $\times$  GoTaqGreen, 1  $\mu$ L (10 pmol) of each primer, and 8,5  $\mu$ L of nuclease-free water. The PCR was then programmed to run at pre-denatured conditions 94°C for 2 min, denatured 92°C for 30 sec, annealing 55°C for 30 sec, primer elongation or extension 72°C for 1 min, and post-PCR 72°C for 5 min and carried out for 40 cycles for 1 h.

The 1% agarose gel (1 g agarose in 100 mL Tris Acetate EDTA (TAE) 1 $\times$ ) was used to display the PCR results, heated with a hotplate and stirred until dissolved, cooled for 5 min then added 1 $\mu$ L of Ethidium Bromide (EtBr) and homogenized and then poured into in a gel mold. At the time of electrophoresis, a 1 kb DNA molecule marker was given. After 60 min of electrophoresis at 80 volts and 400 mA, the results were observed using a UV-transilluminator. The amplified DNA was subsequently purified and commercially sequenced (Macrogen Inc., Singapore) to determine the DNA base order.

### Phylogenetic Analysis

Raw sequence from each bacterial isolate was checked for its similarity to the online database using the Basic Local Alignment Search Tool (BLAST) feature in GenBank. The sequence accessions were retrieved from the site and used for multiple sequence alignment in MEGA 11. The phylogenetic tree was constructed based on the best-predicted DNA model using the Kimura-2 parameter and 1000 $\times$  bootstrapping, with gaps taken as data for divergence (Tamura *et al.*, 2013). All isolates were classified into species but with distinct strains ([https://www.ncbi.nlm.nih.gov/nuccore/?term=OQ359422:OQ359428\[accn\]](https://www.ncbi.nlm.nih.gov/nuccore/?term=OQ359422:OQ359428[accn])).

## Results

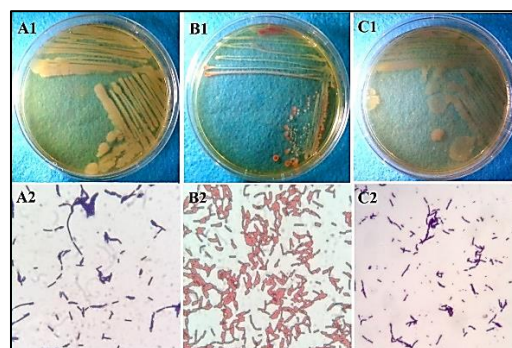
### Morphological and Biochemical Characteristics of Bacteria

The digestive system of *O. rhinoceros* larvae was used as an isolation source for indigenous cellulolytic bacteria. Seven bacterial isolates were obtained through the isolation process, all of which had unique morphological and biochemical traits (Table 1). Seven isolates were rounded, flat and had cream-colored colonies (B01L, B02L, B04L, B05L, B06L, and B07L) while the B03L colony was red in color (Fig. 1). Based on gram staining, two isolates had gram-negative bacteria (B03L and B06L), while five isolates had gram-positive bacteria.

All bacterial isolates showed negative reactions in amylase, hydrogen sulfide production, and motility. They showed positive reactions in gelatin hydrolysis. In terms of citrate utilization, only four isolates, i.e., B03L, B04L, B05L, and B06L showed positive reactions.

### Cellulolytic Activity of Bacterial Isolates

Seven bacterial isolates were tested for their extracellular cellulolytic activity on a solid CMC medium as shown in Fig. 2. These isolates were immersed in Congo red solution and were subjected to the cellulolytic screening procedure. The cellulolytic index of strain B01L (1.28) was the highest among other bacterial isolates. In addition, isolate B06L displayed the lowest activity (0.15). The clearing zone of enzymatic activity by Congo Red will be visible around the emerging colonies. The dye is eluted by the NaCl solution in the clearing zone, where cellulose has been converted into simple sugars by enzymatic hydrolysis. Cellulolytic microorganisms utilize cellulose in various forms including CMC as a preferred medium for their growth. Behera *et al.* (2014); Artiningsih (2006) stated that a species producing a zone diameter that is relatively large compared to its colony diameter may indicate its high production of extracellular enzymes.

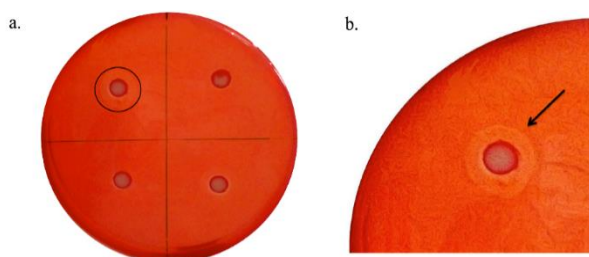


**Fig. 1:** Bacterial isolates from the digestive tract of *O. rhinoceros* larvae on NA medium (top view); (A1) B01L, (B1) B03L, and (C1) bacterial isolate B04L. Bottom view are gram staining microscopic observation; (A2) B01L, (B2) B03L and (C2) B04L

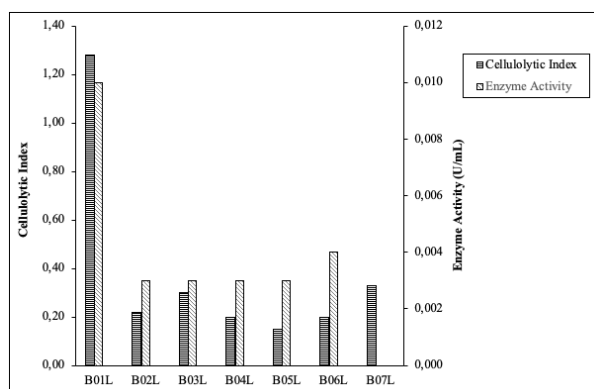
**Table 1:** Morphological and biochemical characteristics of bacterial isolates

Isolates code	Form	Colony color	Colony elevation	Gram	Biochemical test				
					Starch hydrolysis	Gelatin	Citrate	Hydrogen sulfide	Motility
B01L	Round	Cream	Flat	+	-	+	-	-	-
B02L	Round	Cream	Flat	+	-	+	-	-	-
B03L	Round	Red	Flat	-	-	+	+	-	-
B04L	Round	Cream	Flat	+	-	+	+	-	-
B05L	Round	Cream	Flat	+	-	+	+	-	-
B06L	Round	Cream	Flat	-	-	+	+	-	-
B07L	Round	Cream	Flat	+	-	+	-	-	-

Notes: (+) = positive reaction; (-) = negative reaction



**Fig. 2:** (a) Screening of cellulolytic bacteria on 1% CMC medium with Congo red; (b) a colony of isolate B01L showing clear zone



**Fig. 3:** Graph of cellulolytic index and enzyme activity (CMCase) of bacterial isolates from the digestive tract of *O. rhinoceros*

According to the findings of the CMCase activity test (Fig. 3), of the seven bacterial isolates that were taken from the *O. rhinoceros* larvae's digestive tract, all bacterial isolates showed the ability to hydrolyze cellulose. Bacterial test for CMCase activity using 1% CMC (w/v) as the sole carbon source and evaluated for their CMCase activity by the DNS method is used to measure the amount of reducing sugar. Based on the results (Fig. 3) showed that the bacterial isolate with the isolate B01L has the highest activity of 0.010 U/mL. B07L isolate, on the other hand, showed no activity. In our investigation, the results revealed that there could be no

consistent link between plate assay and enzyme assay. These findings are consistent with a study by Febria *et al.* (2021) that evaluated bacterial cellulases from the forest floor. Indigenous cellulolytic bacteria were isolated from sago waste in a different study by Faizah *et al.* (2020) and it was also discovered that the isolate with the highest cellulolytic index actually declined in the enzyme assay. This could be affected by the inoculum's form, which is planktonic during cellulase synthesis. Because the inoculum was in the form of a biofilm, isolates displayed increased cellulolytic activity on solid media. Since cellulose is not only degraded by cellulase but also by other components including Extracellular Polysaccharides (EPS) and protein, Deng and Wang (2022) claimed that cellulose breakdown activity is ideal when bacteria form biofilms. This is in line with a study by Ali *et al.* (2019) that reported using cellulose traps, 33 distinct bacterial isolates from the termite *P. hypostoma* intestines. The sole source of carbon used to recover the strains was carboxymethyl cellulose. In two different types of cellulose medium, cellulolytic strains were identified as Mineral Salt Medium (MSM) containing CMC as the sole carbon source. A Congo red assay that produces a clear zone revealed that five isolates had high cellulolytic activity. *Paenibacillus lactis* (AFC1) was identified as the species of these isolates based on biochemical analyses and 16S rRNA gene sequencing ( $1.47 \pm 0.1$  U/mL), *Lysinibacillus macrolides* (AFC4)  $1.93 \pm 0.1$  U/mL, *Stenotrophomonas maltophilia* (AFC3)  $2.28 \pm 0.1$ , *Lysinibacillus fusiformis* (AFC2)  $0.22 \pm 0.1$  U/mL and *Bacillus cereus* (AFC5)  $0.23 \pm 0.1$  U/mL.

### Molecular Identification of Bacterial Isolate

Prokaryotes' 16S ribosomal subunit contains the 16S rRNA gene, which is frequently utilized for molecular characterization and determining the prokaryotic phylogenetic tree. Contig region for the 16S rRNA gene in the isolates comparing these contig regions with NCBI National Center for Biotechnology Information (NCBI) GenBank entries using the BLAST technique ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). Molecular 16S rRNA sequence identification and use of BLAST NCBI revealed a

resemblance between the isolated B01L of 100% similarity to *B. tequilensis* strain 10b (NR\_104919.1), B02L had the highest similarity of 100% to *B. pacificus* strain MCCC 1A06182 (NR\_157733.1), B03L isolate to *S. marcescens* strain NBRC 102204 (NR\_114043.1) with the similarity 99%, B05L isolate to *L. garaviae* strain JCM 10343 (NR\_113268.1) and B07L to *P. aryabhatai* B8W22 (NR\_118442.1). Other closely related strains were B04L (97%) to *L. fusiformis* strain DSM 2898 (NR\_042072.1) and B06L to *P. monteilii* strain NBRC 103158 (NR\_114224.1), with a similarity of 92% in database GeneBank (Table 2). Similarity-based on the GenBank database based on a 16S rRNA gene BLAST-N comparison to each of the two isolates that are closest to it. *B. tequilensis* strain B01L adjacent to *B. subtilis* and *B. mojavensis*; *B. cereus* strain B02L to *B. pacificus*; *S. marcescens* strain B03L adjacent to *S. nematodiphila* and *E. coli*; *L. fusiformis* strain B04L with *L. mangiferihumi*; *L. garaviae* strain B05L to *L. farmosinensis*; *P. monteilii* strain B06L to *P. entomophila*; and *P. aryabhatai* strain B07L adjacent to *B. zanthoxyli* and *P. megatarium*.

The genomic DNA PCR was then visualized by electrophoresis. The results from genomic analysis of the

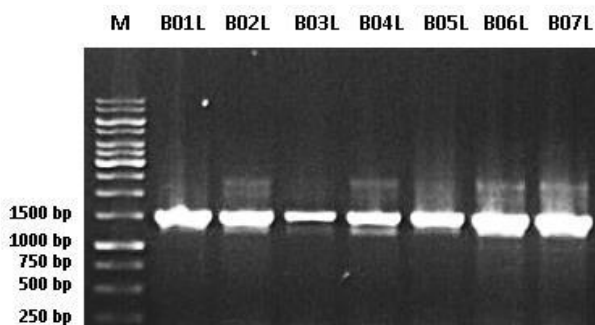
PCR reaction showed that the 16S rRNA gene fragment was at 1500 bp. Marchesi *et al.* (1998), stated that primers 63f and 1387r were good for bacterial identification. The primer in the PCR process serves as a barrier to the target DNA fragment being amplified. PCR amplicon results were visualized with 1% agarose gel using a 1 kb DNA marker (Fig. 4). The bacterial isolates were then extracted their DNA for further molecular identification using the 16S rRNA gene.

The identification of certain genes that are markers of a taxon level in cellulolytic and ligninolytic bacteria has been widely developed. The identification of small and large subunit DNA and multilocus genes. Analysis of the 16S rRNA gene is the most commonly used method of identification of cellulolytic bacteria (Embley and Stackebrandt, 1994). The identification of the 16S rRNA gene is based on many factors. The 16S rRNA gene is multi-copy because there are about 150-300 copies in the genome. This makes it easier to find them in the genome. In the 16S rRNA gene, variable and conservative regions can be used to distinguish between species. The 16S rRNA gene is generally a non-functional gene and is more conservative due to slow evolution (Avisé, 1994; Palys *et al.*, 1997).

**Table 2:** BLAST-N of 16S rRNA genes of isolates

Isolates code	Scientific name	Max score	Total	Query coverage %	E. value	Percent identity %	Accession length	Accession number
B01L	<i>Bacillus tequilensis</i> strain 10b	2567	2567	100	0	99.86	1456	NR_104919.1
	<i>Bacillus mojavensis</i> strain IFO 15718	2556	2556	100	0	99.71	1427	NR_118290.1
B02L	<i>Bacillus subtilis</i> strain NCDO	2479	2479	98	0	99.13	1407	NR_118972.1
	<i>Bacillus pacificus</i> strain MCCC 1A06182	2298	2298	100	0	100.00	1509	NR_157733.1
	<i>Bacillus paranthracis</i> strain MCCC 1A00395	2298	2298	100	0	100.00	1509	NR_157728.1
B03L	<i>Bacillus cereus</i> strain IAM 12605	2298	2298	100	0	100.00	1486	NR_115526.1
	<i>Serratia marcescens</i> strain NBRC 102204	1210	2412	99	0	100.00	1467	NR_114043.1
	<i>Serratia nematodiphila</i> DZ0503SBS1	1205	2399	99	0	99.85	1500	NR_044385.1
	<i>Enterobacter coli</i> ATCC BAA-2102 s train LF7	1195	2273	99	0	99.54	1535	NR_117547.1
B04L	<i>Lycimibacillus fusiformis</i> strain DSM 2898	1790	1790	97	0	96.60	1515	NR_042072.1
	<i>Lysinibacillus mangiferihumi</i> strain M-GX18	1727	1727	97	0	95.59	1452	NR_118146.1
	<i>Lysinibacillus sephaericus</i> strain DSM 28	1724	1724	97	0	95.50	1515	NR_042073.1
B05L	<i>Lactococcus garaviae</i> strain JCM 10343	2314	2314	100	0	100.00	1510	NR_113268.1
	<i>Lactococcus formosinensis subs. bovis</i> strain BSN307	2292	2292	100	0	99.68	1404	NR_152050.1
	<i>Lactococcus taiwanensis</i> strain 0905C15	1905	1905	99	0	94.25	1463	NR_114327.1
B06L	<i>Pseudomonas monteilii</i> strain NBRC 103158	1434	1434	92	0	94.35	1462	NR_114224.1
	<i>Pseudomonas plecoglossicida</i> strain NBRC 103162	1428	1428	92	0	94.24	1462	NR_114226.1
	<i>Pseudomonas taiwanensis</i> DSM 21245	1428	1428	92	0	94.24	1469	NR_116172.1
B07L	<i>Priestia aryabhatai</i> B8W22	2309	2309	100	0	100.00	1291	NR_118442.1
	<i>Bacillus zanthoxyli</i> strain 1433	2303	2303	100	0	99.92	1438	NR_164882.1
	<i>Priestia megatarium</i> strain NBRC 15308	2298	2298	100	0	99.84	1477	NR_112636.1





**Fig. 4:** PCR results for the gene encoding 16S rRNA. Row (1) DNA Marker 1 kb; Rows 2- 11) DNA encoding genes for 16S rRNA isolates bacterial from the digestive tract of *O. rhinoceros*

## Discussion

The objective of this study was to identify isolates based on 16S rRNA and obtain possible cellulolytic bacteria from *O. rhinoceros* larvae. Cellulases that were isolated from microorganisms were widely used. Cellulase enzymes from microbes can be used to reduce the amount of cellulose waste, such as leaf piles in the final disposal area, agricultural waste, and seaweed on the beach, and can add value to the use of waste into processed organic fertilizers, in addition to the environmental and industrial fields. This is due to the fact that organic waste is a component of an environmental issue that, if not controlled and managed properly, has the potential to pollute the environment. Cellulase enzymes can be used to modify all of these materials to create a variety of useful products for farming and biogas production. Some applications of organic products that are beneficial for agriculture include local Microorganisms, liquid organic fertilizer, solid organic fertilizers, and bokashi as well as organic pesticides.

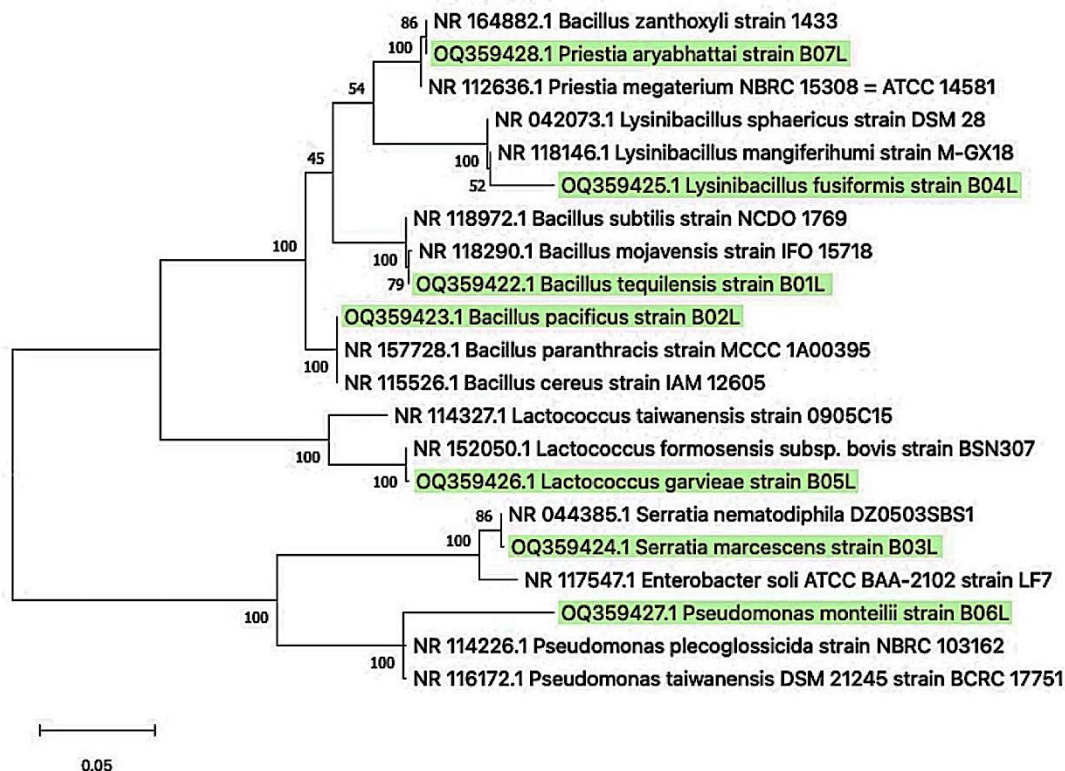
Seven isolates among them show the focus isolates ability to cellulase. Isolate B01L index of 1,28, isolates form a clear zone on CMC media after adding 0.1% Congo red. The degradation of cellulose by cellulolytic bacteria results in the presence of this clear zone with the index cellulolytic. Cellulose which is hydrolyzed on agar medium if flooded by Congo red will produce a clear zone because Congo red cannot bind to the medium without the 1,4-glycosidic linkages contained in the cellulose polymer, this is due to the presence of cellulase enzymes so the cellulose polymer bonds are hydrolyzed. Rinsing with NaCl will dissolve the Congo red, especially in the area around the colony containing hydrolyzed cellulose derivatives such as cellodextrin, cellobiose, and glucose because the Congo red is not

tightly bound so that a clear zone is seen. The CMCase activity of cellulolytic bacterial colonies on CMC agar media surrounded the colony in a clear area. Cellulase is typically recognized to be produced by bacteria. (Amraini *et al.*, 2017). Peristiwati *et al.* (2018), reported to have successfully isolated 6 bacterial cellulolytic and highest index cellulolytic of 1.32. Based on common physical and biochemical testing, each isolate was identified. Three Clostridium isolates were found, one belongs to the *Mycobacteriaceae*, *Lactobacillaceae*, or Coryneform family and the final isolate belongs to the Proteus genus.

The genomic DNA PCR was then visualized by electrophoresis. The resulting band from the PCR reaction showed that the 16S rRNA gene fragment was at 1500 bp. Marchesi *et al.* (1998), stated that primers 63f and 1387r were good for bacterial identification. The primer in the PCR process serves as a barrier to the target DNA fragment being amplified. PCR amplicon results were visualized with 1% agarose gel using a 1 kb DNA marker (Fig. 4). The bacterial isolates were then extracted their DNA for further molecular identification using the 16S rRNA gene.

The identification of certain genes that are markers of a taxon level in cellulolytic and ligninolytic bacteria has been widely developed. The identification of small subunit DNA, large subunit DNA, and multilocus gene. Analysis of the 16S rRNA gene is the most commonly used method of identification of cellulolytic bacteria (Embley and Stackebrandt, 1994). The identification of the 16S rRNA gene is based on many factors. The 16S rRNA gene is multi-copy because there are about 150-300 copies in the genome. This makes it easier to find them in the genome. In the 16S rRNA gene, variable and conservative regions can be used to distinguish between species. The 16S rRNA gene is generally a non-functional gene and is more conservative due to slow evolution (Avisé, 1994; Palys *et al.*, 1997).

The phylogenetic trees of the bacterial isolates from the *O. rhinoceros* larvae's digestive system were created using the maximum likelihood statistical method and the Kimura-2 parameter model (Fig. 5). Here, we constructed a phylogenetic to divide between *B. tequilensis* strain 10b, *B. pacificus* strain MCCC 1A06182, *S. marcescens* strain NBRC 102204, *L. fusiformis* strain DSM 2898, *L. garaviae* strain JCM 10343, *P. monteilii* strain NBRC 103158 and *P. aryabhatai* B8W22. Bacteria among prokaryotes are the only ones that can degrade cellulose. *Micrococcus* spp., *Bacillus* spp., *Pseudomonas* spp., and *Xanthomonas* spp. are cellulose-degrading bacteria (Behera *et al.* 2014).



**Fig. 5:** Phylogenetic tree of isolates based on 16S rRNA gene sequence in the GeneBank database. As shown at the nodes, the phylogram was inferred from the Kimura-2 parameter model using bootstrap percentages of 1000 replicates. Nucleotide positions are substituted by 0.05 in the bar

Index cellulolytic is an important method to determine the cellulolytic ability of microorganisms in the degradation of cellulose materials. The low index value indicates that the isolate produces low enzyme activity. Oktiarni *et al.* (2021) reported, that 64 cellulolytic bacteria were discovered in the intestines of *Macrotermes gilvus* and the 16S rRNA gene sequences revealed that they belonged to a novel species that was named *E. cloacae*, *K. pneumoniae*, *K. quasipneumoniae*, *K. varicolla*, *E. roggkampii*, and *E. asburiae*. While Huang *et al.* (2012) from the intestine of *H. parallela* larvae, 207 strains cellulolytic bacteria were identified. According to, Proteobacteria (70.05%), Actinobacteria (24.15%), Firmicutes (4.35%), and Bacteroidetes (1.45%) are the most prevalent bacterial groups in the cellulolytic bacterial community.

*Bacillus* isolates (*B. tequilensis*, *B. cereus*, and *Lysinibacillus*), *Enterobacter*, *Serratia*, *Pseudomonas*, *Lactococcus* and *Priestia* obtained in this study have been widely several earlier studies have claimed that it can degrade materials made of cellulolytic and lignin (Kassim *et al.*, 2016; Oktiarni *et al.*, 2021; Khotimah *et al.*, 2020; Arimurti *et al.*, 2017). Since the majority of *Bacillus* species are non-pathogenic and there are

numerous different *Bacillus* genera, they can be used directly in a wide range of industries (Felix *et al.*, 2019).

It was concluded that there were 10 cellulolytic bacteria in the gastrointestinal tract of *O. rhinoceros* larvae. Which were identified based on the 16s rRNA sequence, B01L was identified as *B. tequilensis* which had the highest cellulolytic index and had the potential to degrade cellulose.

## Conclusion

A total of seven isolates were successfully isolated from the digestive tract of *O. rhinoceros* larvae and identified based on the 16s rRNA gene and showed the greatest cellulolytic activity and CMCase activity in isolate B01 L with a cellulolytic index of 1.28 and an enzyme activity of 0.010 U/mL which were identified as *Bacillus tequilensis* based on the 16s rRNA encoding gene. This research provides various confirmations of the potential skills of neighboring cellulolytic bacteria from the lignocellulosic material-eating *O. rhinoceros* larvae that can provide solutions to industrial and environmental problems. For this reason, it is necessary to carry out further research on the purification and characterization studies of cellulose.

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## Author's Contributions

**Ahmad Faisal Nasution:** Data analysis and experimental development, wrote manuscript.

**Erman Munir:** Conceived the original idea, designed the study and reviewed and approved manuscript.

**Dwi Suryanto:** Designed research methodology, conducted field sampling and data interpretation.

**Yurnaliza:** Materials and equipment engagement, literature search, monitored research.

**Anthoni Agustien:** Research designed and analyses.

## Ethics

This article is entirely original and it contains never-before-seen material. The corresponding author certifies that all other authors have read and accepted the work and that there are no ethical contradictions.

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