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Isolation and Identification Cellulolytic Bacteria from Termite Gut Obtained from Indralaya Peatland area

D Oktiarni^{1,2}, Hermansyah^{2*}, Hasanudin², Miksusanti², E Nofyan³, G Kasmiarti⁴

¹Mathematics and Natural Sciences Study Program, Graduate School Sriwijaya University, Palembang, Indonesia

²Chemistry Department, Faculty of Mathematics and Natural Sciences, Bengkulu University, Bengkulu, Indonesia

³Biology Department, Faculty of Mathematics and Natural Sciences, Sriwijaya University, Palembang, Indonesia

⁴Environmental Management Study Program, Graduate School Sriwijaya University, Palembang, Indonesia

*Email: hermansyah@unsri.ac.id

Abstract. The production of second-generation bioethanol as renewable energy has developed very rapidly and has become a promising alternative energy source. Bioethanol production using biomass can be obtained alternatively from cellulose in wood, sawdust, organic waste, and agricultural waste. This research used termites obtained from Indralaya peatland area as organisms that can decompose cellulose into glucose with the cellulase enzymes produced by bacteria in the digestive tract. Cellulases are enzymes capable of hydrolyzing lignocellulose into glucose. The study aimed to isolate and identify of cellulolytic bacteria from termite gut obtained from Indralaya peatland area. The bacterial isolates were classified by using morphological and biochemical standard methods, and identification based on Bergey's Manual of Determinative Bacteriology. Cellulolytic bacteria of termite gut were isolated and cultured on CMC (Carboxymethyl cellulose) agar medium. The activity of cellulolytic bacterial was conducted based on halo area and cellulolytic index on CMC agar medium. Among 64 isolates of bacteria, 24 isolates were identified as cellulolytic bacteria. Furthermore, our isolates with higher cellulolytic index were identified as the *Staphylococcus*, *Microbacterium*, *Bacillus*, and *Brevibacterium* genus.

1. Introduction

Cellulose is the main component of lignocellulose. Lignocellulose consists of cellulose (40-50%), hemicellulose (25-35%), and lignin (15-20%). Cellulose has β -1,4 glycosidic bonds, that difficult to hydrolyze into monomers/monosaccharides. Hemicelluloses, like xylan, has β -1,4 glycosidic bonds linked to mannose, galactose, arabinose and glucuronic acid. Hemicellulose is more easily hydrolyzed into monosaccharides. Lignin is a heterogeneous polymer found in lignocellulose, usually having three aromatic alcohols in the form of coniferyl alcohol, sinapyl, and p-coumaryl [1].

Several countries have developed technology to hydrolyze cellulosic biomass to produce bioethanol. Bioethanol is an energy source that has the potential to be developed as renewable and eco-friendly energy [2-5]. Bioethanol needs higher cost and produces chemical compounds that are harmful to the environment [3, 6-8]. One of the technologies used to produce bioethanol from cellulosic biomass is by enzymatic process [3]. Hydrolysis of cellulose using enzymes is an effective step, environmentally



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friendly and does not produce harmful chemical compounds [6]. Some organisms take cellulose as a main food, like termites. Bacteria in the digestive tract of termites have enzymes that are able to hydrolyze cellulose into glucose.

Termites are insects that can degrade cellulose into simple monosaccharides, which is cellulolytic enzymes present in the digestive tract of termites. Related study reported that in the digestive tract of termites found various bacteria and fungi that have cellulolytic enzyme activity, converting cellulose into monomers. Three groups of cellulase enzymes are capable of hydrolyzing cellulose, endoglucanase, exoglucanase, and β -glucosidase enzymes [1, 9].

In South Sumatra Province, especially in Indralaya-Palembang area, most of peatlands are planted with oil palm/rubber plantations and without plantations. In this research, termite samples were obtained from peatland in Indralaya area, so the aim of this research is to identify the termite species on this peatland area. Furthermore, the isolation and identification of bacterial species in the termite digestive tract determined by morphological and biochemical characteristics.

2. Materials and Methods

2.1. Collected termite and preparation

The termites were collected from Tanjung Senai peatland in Indralaya. The samples were sampled from mound and were kept in a plastic container with mound soil and fungus comb.

2.2. Isolation of bacteria from termite

Ten of workers termite were taken by using sterile tweezers and sterilized with 70% alcohol for 30 seconds. Then rinsed with sterile distilled water and dried for 1 minute. The termite was dissected and all parts of the termite digestive tract were taken and crushed to obtain a suspension. Then 0.1 mL of the suspension was taken and spread on carboxymethyl cellulose (CMC) agar media at 37°C for 2 days.

2.3. Screening of cellulolytic bacteria

Bacterial isolates from single colonies were transferred to CMC agar media for subculture and incubated at 37°C for 2 days. Bacterial isolates on CMC agar media were taken as much as 1 ose and scratched on CMC agar media aseptically and incubated at 37°C for 2 days [10]. After incubation, CMC media was stained with 1% (w/v) Congo red for 30 minutes, and destained with 1 M NaCl solution for 15 minutes. The bacterial isolates with higher clear zones further screened to determine cellulolytic of bacterial isolates. Bacterial isolates on CMC agar media were taken as much as 1 ose and scratched on CMC agar media aseptically and incubated at 37°C for 2 days [10]. After incubation, CMC media was stained with 1% (w/v) iodine for 15 minutes at room temperature and then decolorized with 1 M NaCl solution for 15 minutes.

2.4. Cellulolytic activity test

The clear zone indicates the ability of bacterial isolates to decompose cellulose. The comparison of the diameter of the clear zone and the diameter of the colony means the cellulolytic index of the enzyme activity in the bacteria. To see the cellulose index, it can be calculated by the formula [9, 11]:

$$\text{Cellulolytic index} = \frac{\text{diameter of clear zone} - \text{diameter of bacterial colonies}}{\text{diameter of bacterial colonies}}$$

2.5. Morphology and biochemical assay

Bacterial isolates were observed morphologically, including macroscopic test: texture, colour, shape, colony size, and elevation, and microscopic test: Gram staining and colony cell. Biochemical assay was carried out on bacterial isolates: indole test, motility test, triple sugar iron (TSI) test, urease test, catalase test, citrate test, methyl red (MR) test, and voges proskauer (VP) test [1, 9, 12].

3. Results and Discussion

3.1. Identification of termite

The termite that obtained from Indralaya peatland was identified as *Macrotermes gilvus* Hagen (Figure 1). It was reported that termites in rubber plantations were *Microtermes* species and *Macrotermes gilvus*, in oil palm plantations was usually from *Ondotermes* species, and in drywood construction was *Cryptotermes* species [6].



Figure 1. *Macrotermes gilvus* Hagen of (A) worker and (B) soldier.

3.2. Screening of bacterial isolates

Sixty-four bacterial were isolated from *Macrotermes gilvus* Hagen gut on CMC agar media. The screening of bacteria conducted on CMC agar media showed that among 64 isolates, 24 isolates were identified as cellulolytic bacteria (Figure 2 and Figure 3). Almost of bacterial isolates showed the cellulolytic activity, that supported by iodine staining on CMC culture plates, which showed the clear zones due to cellulose degradation. Four of isolates of 37, 39, 40 and 62 with higher cellulolytic index of 3, 4, 2.5 and 2.5, respectively (Table 1), then further characterized by morphological and biochemical assay. In agreement with our study, in the digestive tract of the worker termite *Macrotermes gilvus* Hagen, there were bacteria *Bacillus megaterium* and *Paracoccus yeei* with cellulolytic index of 0.81 and 2.5 [10].

3.3. Morphological and biochemical characteristics

The morphological and biochemical characteristics of four highest cellulolytic isolates are described in Table 2 and Table 3. Morphological characters of all the bacterial isolates were smooth texture and flat elevation, white colour with circular shape. The size of each isolate of 37, 39, 40 and 62 are 0.5-1; 1-2; 2-3; 0.5 mm, respectively. Four isolates showed Gram-positive bacteria with coco (isolates 37 and 39), cocobasil (isolate 40) and basil (isolate 62) shape.

The determination of motility showed positive reaction, its indicates that all bacterial isolates from genera *Enterobacteriaceae* [13]. Nevertheless, indole and urea test of four isolates showed negative result, it means that the enzyme of bacterial isolates are not hydrolyzed amino acid tryptophan and not belongs to urease enzyme [14]. From TSI test, the slant and butt showed the yellow colour which indicates that the media were acidic, it means that bacterial isolates were able to degrade lactose, sucrose, and glucose [13]. The four isolates were produced gas, but not produced H₂S gas. Data from TSI test indicates that bacterial isolates were identified as *Enterobacter* and *Bacillus* genus [14].

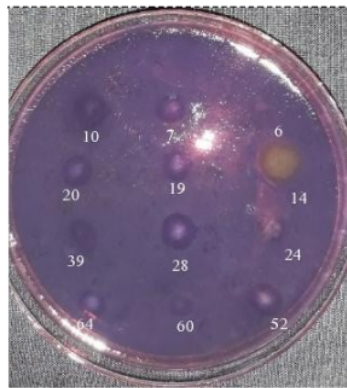


Figure 2. Cellulolytic activity of bacterial isolates (6, 7, 10, 14, 19, 20, 24, 28, 39, 52, 60, 64) on CMC agar media from *Macrotermes gilvus* Hagen (stained with iodine).

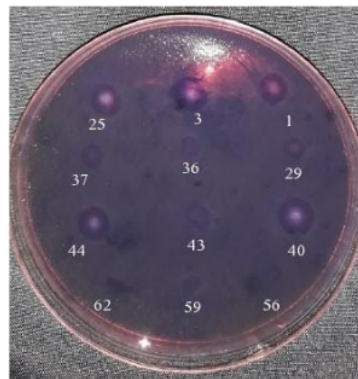


Figure 3. Cellulolytic activity of bacterial isolates (1, 3, 25, 29, 36, 37, 40, 43, 44, 56, 59, 62) on CMC agar media from *Macrotermes gilvus* Hagen (stained with iodine).

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Table 1. Cellulolytic index of cellulolytic bacteria isolated from termite gut.

Isolate	Diameter of colony (mm)	Diameter of cellulolytic zone (mm)	Cellulolytic index	Isolate	Diameter of colony (mm)	Diameter of cellulolytic zone (mm)	Cellulolytic index
1	2.0	5.0	1.5	36	1.0	3.0	2.0
3	2.0	6.0	2.0	37	1.0	4.0	3.0
6	4.0	5.0	0.3	39	1.0	5.0	4.0
7	3.0	5.5	0.8	40	2.0	7.0	2.5
10	2.0	6.0	2.0	43	2.0	4.5	1.3
14	7.0	8.0	0.1	44	2.0	5.0	1.5
19	3.0	5.0	0.7	52	3.0	6.0	1.0
20	3.0	6.0	1.0	56	1.0	2.0	1.0
24	4.0	5.0	0.3	59	1.0	3.0	2.0
25	3.0	5.5	0.8	60	2.0	5.0	1.5
28	3.0	6.0	1.0	62	1.0	3.5	2.5
29	2.0	4.0	1.0	64	3.0	5.5	0.8

The catalase test showed positive reaction, it means that the bacterial isolates producing catalase enzyme through the degradation of hydrogen peroxide carried out by the bacteria, the bacteria exclusively growth in aerobic condition. The citrate assay also showed the positive reaction, this indicates that bacteria have the ability using citrate as the only source of carbon and source of energy [13], due to raising of pH medium, so that these bacteria can growth in acidic waste.

Overall, based on Bergey's Manual of Determinative Bacteriology, the isolates 37, 39, 40 and 62 were identified as genus of Staphylococcus, Microbacterium, Bacillus, and Brevibacterium, respectively (Figure 4). Previous research reported that in rubber plantations were succeed identified bacteria of Enterobacter agglomerans, Staphylococcus hominis and Pseudomonas paucimobilis [15].

Table 2. Colony morphology of cellulolytic bacteria isolated from termite gut.

Isolate	Texture	Colour	Shape	Elevation	Colony size (mm)	Gram Staining	Colony cell
37	Smooth	White	Circular	Flat	0.5-1	+	Coco
39	Smooth	White	Circular	Flat	1-2	+	Coco
40	Smooth	White	Circular	Flat	2-3	+	Cocobasil
62	smooth	White	Circular	Flat	0.5	+	Basil

(+): positive result

(-): negative result

The bacteria in the digestive tract of termites *Coptotermes curvignatus* Holmgren and *Macrotermes gilvus* Hagen are *Enterobacter*, *Flavobacterium*, *Sporocythophaga*, *Staphylococcus* bacteria [12]. In the digestive tract of *Odontotermes* termites there are bacteria *Microbacterium*, *Bacillus*, *Eubacterium* [16], whereas in the digestive tract of other types of termites there are bacteria *Salmonella* spp., *Streptococcus* spp., *Staphylococcus* spp., *Bacillus subtilis* [17].

Table 3. Biochemical tests of cellulolytic bacteria isolated from termite gut.

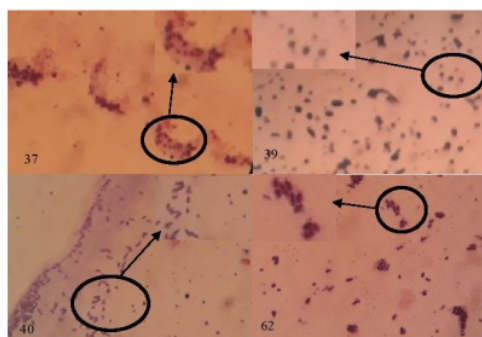
Isolate	Indole	Motility	TSI			Urease	Catalase	Citrate	MR	VP	Identification
			Ferm	H ₂ S	Gas						
37	-	+	+	-	+	-	+	+	-	+	<i>Staphylococcus</i>
39	-	+	+	-	+	-	+	+	-	+	<i>Microbacterium</i>
40	-	+	+	-	+	-	+	+	-	+	<i>Bacillus</i>
62	-	+	+	-	+	-	+	+	+	-	<i>Brevibacterium</i>

TSI: triple sugar ion test.

Ferm: fermentation result.

MR: methyl red test.

VP: Voger Proskauer test.

**Figure 4.** Bacterial isolates after Gram staining under microscope (1000 X).

The bacteria in the digestive tract of other types of termites are reported contains the bacteria *Bacillus* sp., *Cellulomonas* sp., *Enterobacter* sp. [15]. In addition, in the digestive tract of termites *Coptotermes* sp. contains the bacteria *Pseudomonas alcaligenes*, *Brevibacillus parabrevis* which has amylase activity. The termite *Cryptotermes* sp. in the digestive tract there are bacteria from the genus *Clostridium*, familia of *Mycobacteriaceae*, *Lactobacillaceae* and *Proteus* [11]. Meanwhile, in the digestive tract of the termite *Microtermes obesi* on the *Acacia nilotica* tree, there is the bacterium *Cellulomonas* sp [7].

¹² In this study all the four bacterial isolates were able growing on CMC agar media and showed the higher cellulolytic index. The bacterial isolates have the capability to produces cellulases, that can be act as biocatalyst in enzymatic hydrolysis process, hydrolyse lignocellulosic biomass into glucose. This becomes a great prospect for further research to study the role of bacteria, to isolate the enzyme in these bacteria, especially cellulolytic enzyme. In addition, the enzyme cellulolytic of these bacteria needs to developed, as a biocatalyst, converting cellulose into glucose, to produce bioethanol from cellulosic biomass by enzymatic process, especially in bioethanol production.

4. Conclusions

24 isolates bacteria from *Macrotermes gilvus* Hagen gut from Indralaya peatlands were identified as cellulolytic bacteria. Four of isolates 37, 39, 40 and 62 have higher cellulolytic index of 3, 4, 2.5 and 2.5, respectively. The isolates 37, 39, 40 and 62 were showed negative reaction to biochemical assay for indol and urease, whereas showed positive reaction for motility, catalase, and citrate. The isolates 37, 39, 40 and 62 were Gram positive and identified as genus of *Staphylococcus*, *Microbacterium*, *Bacillus*, and *Brevibacterium*, respectively.

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