Isolation and identification of cellulolytic bacteria from termites gut (Cryptotermes sp.)

Peristiwati*, Y S Natamihardja and H Herlini

Department of Biology, Universitas Pendidikan Indonesia, Bandung, Indonesia

*Corresponding author’s email: peristiwatidj@upi.edu

Abstract. The energy and environmental crises developed due to a huge amount of cellulosic materials are disposed of as “waste.” Cellulose is the most abundant biopolymer on Earth. The hydrolysis of cellulose to glucose and soluble sugars has thus become a subject of intense research. Termites are one of the most important soil insects that efficiently decompose lignocelluloses with the aid of their associated microbial symbionts to a simpler form of sugars. The steps of this study consisted of cellulose isolation, cellulolytic bacteria isolation and identification. Cellulose degrading bacteria from termite (Cryptotermes sp.) gut flora were isolated, screened and their identification was studied which showed halo zones due to CMC agar. Among 12 isolates of bacteria, six isolates were cellulolytic. MLC-A isolate had shown a maximum in a cellulolytic index (1.32). Each isolate was identified based on standard physical and biochemical tests. Three isolates were identified in the genus of Clostridium, one isolate be placed in the group of Mycobacteriaceae, Lactobacillaceae or Coryneform and the last one in the genus Proteus.

1. Introduction
Nowadays in the techno-economic era, faced environmental crises due to a huge amount of disposal materials as “waste.” There are composed of a municipal solid with containing of 40–50% cellulose, 9–12% hemicelluloses, and 10–15% lignin on a dry weight basis [1,2]. Furthermore, cellulose, a crystalline polymer composed of β-1, 4 linked D-glucose molecule is the most abundant and renewable biopolymer on earth [3]. The hydrolysis of cellulose to glucose and soluble sugars has thus become a subject of intense research and industrial interest [4]. Complete hydrolysis of cellulose to glucose requires the synergistic action of three enzymes. Cellulase system consists of three classes of soluble extracellular enzymes, i.e 1,4-β-endoglucanases, 1,4-β-exoglucanases, and β-glucosidases (β-D glucoside glucohydrolases or cellobiases). Cellulolytic is a biological process which controlled and processed with cellulase system [5].

One of the best sources for a cellulolytic system is symbiotic microorganisms in the intestinal tract of an organism with cellulose as a source of metabolizable sugar (glucose) [6]. Insects have evolved effective strategies to utilize lignocellulosic substrates as sources of energy, which makes them an optimal resource for prospecting for novel cellulolytic enzymes. Extensive efforts have characterized lignocellulose degradation in termites [7-10].

Termites are one of the most important creatures to decompose lignocelluloses with the aid of their associated microbial symbionts to a simpler form of sugars [11]. The symbiotic relationship between the protistan community within the termite gut seems to endow termites with the ability to degrade
cellulose from complex natural lignocellulose that is composed of lignin, hemicellulose, and cellulose [12-14].

The anaerobic cellulolytic bacteria from termite gut is needed to explore, although these insects are very efficient lignocellulose-decomposers. Recent studies (Brauman et al., submitted for publication) have indicated that their gut microbiota may provide a suitable model system to understand the electron flow involved during biopolymer degradation by anaerobic microbial communities [15].

Limitations of these cellulolytic enzymes were in ability to withstand high temperatures, low specific activity, generation of inhibitory agents during hydrolysis, and susceptibility to pH changes [16]. In this research, we were investigated on cellulolytic bacteria isolated from the gut of a wood-feeding termite, Cryptotermes sp.

2. Methods

2.1. Termites collection
Termites were collected from Ciwaruga Subdistrict, West Java, Indonesia. They live in woody materials (Cryptotermes sp., Kalotermitidae). These termites were transfer migrated into the container and were identified at Research Laboratorium, Department of Biology Education, Indonesia University of Education.

2.2. Extraction from termites
Termites sterilized by washing with 70% alcohol. Each termite was separated into its head and body after removing the heads with forceps, the bodies were crushed with the help of glass rods. The paste obtained from the termites’ gut was used for isolation of the bacteria with a series of sequential dilutions used to reduce a dense culture of cells to a more usable concentration before inoculated to media.

2.3. Media used for culture
Media that used for inoculation of the bacteria were, Nutrient Broth agar (Nutrient Broth 9 g/l and agar 15 g/l) and CMC media (MgSO4 .7H2O 0.05 g/100 mL, NaCl 0.23 g/100 ml, Na2HPO4.2H2O 0.5 g/100 ml, Yeast extract 0.2 g/100 ml, CMC 1 g/100 ml and with or without Agar 1.5 g/100 ml). Nutrient agar was used for the isolation of general bacteria. CMC media was used because it preferably supports the growth of cellulolytic bacteria.

2.4. Test for cellulolytic activity
Agar plates were prepared with 1% CMC. Strains were streaked and petriplates were incubated at 37°C for 48 hours. Petriplates were flooded with 0.1% Congo red reagent and left for 20 minutes. Then the plates were washed with 1M NaCl. Clearance zones called halo zones are seen against the red color of Congo red for the positive test. Enzyme activity was indexed as the diameter of the colony plus the surrounding clear zone divided by the diameter of the colon and only the isolates that produced a clear zone around the colony were chosen for further study.

2.5. Identification of cellulolytic isolates
Each isolate was identified based on standard physical and biochemical tests [17], including motility, Gram and Endospore staining, the methyl red (MR) test, the Voges-Proskauer (VP) test, the activities of catalase, oxidase, urease, the production of indole and gas production from glucose. Different carbon sources (D-Lactose, D-Dextrose and D-Sucrose) were used to evaluate carbon utilization. Except for the gelatinase activity test (which was performed at 20 °C), all of the tests were performed at 28 °C in the appropriate medium and were conducted according to standard methods and compared with Bergey’s Manual of Systematic Bacteriology [18]. 24-48 hours old cultures were used for all the tests.
3. Results and Discussion

3.1. Isolation bacteria
Isolation of strains on plates containing Nutrient Broth Agar resulted in 12 isolates with different shape, elevation, margin and color characteristics. The colony characteristics of separated mix culture on media following to the method for basic identification described by Cappuccino and Sherman [19].

| No | Colony   | Shape | Elevation | Margin  | Colour        |
|----|----------|-------|-----------|---------|---------------|
| 1  | Colony (1) | Irraguler | Convex   | Undulate | White        |
| 2  | Colony (2) | Irraguler | Raised   | Undulate | White       |
| 3  | Colony (3) | Circular  | Flat      | Undulate | White-transparant |
| 4  | Colony (4) | Circular | Convex   | Entire   | White |
| 5  | Colony (5) | Irraguler | Umbonate | Curl     | White |
| 6  | Colony (6) | Irraguler | Flat      | Curl     | White |
| 7  | Colony (7) | Circular | Umbonate | Lobate   | White |
| 8  | Colony (A) | Filiform | Convex   | Lobate   | White-Yellowish |
| 9  | Colony (B) | Irraguler | Flat      | Lobate   | White |
| 10 | Colony (C) | Circular  | Umbonate | Undulate | White-transparant |
| 11 | Colony (D) | Circular  | Umbonate | Entire   | White-Yellowish |
| 12 | Colony (E) | Circular  | Convex    | Siliate  | Yellowish |

3.2. Screening of cellulose degrading bacteria
All the isolates were tested for the production of cellulolytic enzyme on CMC Agar plates. Congo red 0.1% staining of these plates along with 0.1% NaCl revealed 6 isolates producing clear zones on the plates around the colonies indicating cellulose hydrolysis. Congo red provides the basis for a rapid and sensitive screening test for cellulolytic bacteria. All these isolates were selected for further investigation on the basis of the area of clear zone.

![Figure 1. Clear zones of Cl-1 (a), Cl-3 (b), MLC-A (c), Cl-4 (d), Pr-C (c) and Cl-D (d) isolates on CMC agar after Congo red staining.](image)
Table 2. Cellulolytic index of each isolate

| No | Isolate  | Colony Diameter | Clear Zones Diameter | Cellulolytic Index |
|----|----------|-----------------|----------------------|-------------------|
| 1  | Isolate X-4 | Non Cellulolytic | -  | - |
| 2  | Isolate X-5 | Non Cellulolytic | -  | - |
| 3  | Isolate X-6 | Non Cellulolytic | -  | - |
| 4  | Isolate Pr-C | 1 cm            | 0.2 cm               | 1.20              |
| 5  | Isolate Cl-D | 2.05 cm         | 0.4 cm               | 1.19              |
| 6  | Isolate X-E | Non Cellulolytic | -  | - |
| 7  | Isolate MLC-A | 1.55 cm         | 0.5 cm               | 1.32              |
| 8  | Isolate Cl-B | 2.75 cm         | 0.2 cm               | 1.07              |
| 9  | Isolate Cl-2 | 1.95 cm         | 0.4 cm               | 1.20              |
| 10 | Isolate Cl-3 | 3.8 cm          | 0.4 cm               | 1.10              |
| 11 | Isolate X-1 | Non Cellulolytic | -  | - |
| 12 | Isolate X-7 | Non Cellulolytic | -  | - |

Cellulolytic activity test showed that the diameter of clear zones from these 6 cellulolytic bacteria varied from organism to organism. The data present in Table 1 revealed that Cl-2, Cl-3, MLC-A, Cl-B, Pr-C and Cl-D isolates were active cellulose producers. MLC-A isolate had shown maximum in cellulolytic index (1.32). On the other hand, non cellulolytic isolates were not shown colony growth and its clear zones.

3.3. Identification of cellulose degrading bacteria

A total of six positive cellulolytic isolates were selected for identification. Based on gram staining, Cl-2, Cl-3, MLC-A, Cl-B and Cl-D isolates are Gram positive bacteria with basil shaped, however Pr-C isolate is Gram Negative with coccus shaped. In endospore staining, Cl-2, Cl-3, Cl-B and Cl-D isolates can form endospore.

![Figure 2. Magnification view of Cl-2, Cl-3, Cl-B, and Cl-D endospores](image)
Biochemistry tests of each isolate

| No. | Characteristic | Cl-2 | Cl-3 | MLC-A | Cl-B | Pr-C | Cl-D |
|-----|---------------|------|------|-------|------|------|------|
| 1   | Sucrose       | (-)  | (-)  | (-)   | (+)  | (+)  | (+)  |
| 2   | Dextrose      | (+)  | (+)  | (-)   | (+)  | (+)  | (+)  |
| 3   | Lactose       | (-)  | (-)  | (-)   | (-)  | (-)  | (-)  |
| 4   | Lipid         | (-)  | (-)  | (-)   | (-)  | (-)  | (-)  |
| 5   | Starch        | (-)  | (-)  | (-)   | (-)  | (-)  | (-)  |
| 6   | Casein        | (+)  | (+)  | (+)   | (-)  | (+)  | (-)  |
| 7   | Gelatin       | (+)  | (-)  | (+)   | (+)  | (+)  | (-)  |
| 8   | Motility      | (-)  | (+)  | (-)   | (-)  | (-)  | (-)  |
| 9   | Catalase      | (-)  | (-)  | (-)   | (-)  | (-)  | (-)  |
| 10  | H2s           | (-)  | (-)  | (-)   | (-)  | (-)  | (-)  |
| 11  | Mr test       | (-)  | (-)  | (-)   | (-)  | (-)  | (-)  |
| 12  | Vp test       | (-)  | (-)  | (-)   | (-)  | (-)  | (-)  |
| 13  | Indol test    | (-)  | (-)  | (-)   | (-)  | (-)  | (-)  |

Based on the result, Cl-2 isolate positive using dextrose, gelatin and casein hydrolysis. Cl-3 isolate positive using dextrose, casein hydrolysis, and motile. MLC-A isolate negative for carbohydrate fermentation test but positive in casein and gelatin hydrolysis. Cl-B isolate positive using sucrose and dextrose and gelatin hydrolysis. Pr-C isolate positive using sucrose and casein and gelatin hydrolysis. Cl-D isolate only positive using sucrose and dextrose. We propose that the Cl-2, Cl-3, Cl-B and Cl-D isolates be placed in the genus Clostridium in a different type of species. MLC-A isolate is placed in the group of Mycobacteriaceae, Lactobacillaceae or Coryneform. Also, Pr-C isolate in the genus Proteus.

Although 12 isolates were screened in this study only 6 of the isolates showed cellulolytic activity on Crystalline cellulose media. All common termite cellulolytic bacteria species were found in the digestive tract but differ in the proportion of each individual species. These isolate strains produced cellulase was supported by Congo red staining on the culture plates which showed halo zones due to cellulose or carboxymethylcellulose degradation. The diameters of the halo zones produced by different colonies were used as a basis for comparison between various isolated strains. MLC-A isolate showed higher activity on swollen cellulose with cellulolytic index (1.32). On the other hand, Cl-B isolate was the minimum cellulolytic index (1.07). Those colonies that produced the largest diameter of halo zones were considered to have the highest cellulolytic activity [17]. This difference could probably be attributed to the difference in cellulase enzymes components produced by different isolates. The production of cellulase by these bacteria is the type of extracellular enzyme. This enzyme is produced to assist in the process of breaking down complex cellulosic molecules into simpler molecules in order to be digested in bacterial metabolism.

Bacteria isolates from termite gut have different morphological and biochemistry characteristics. Most of the fiber-digesting bacteria isolated from the gut of termites showed Gram-positive [20], there are 5 out of 6 isolates were gram-positive with basil shaped. Three cellulolytic genera were identified from six isolates. Four isolates (Cl-2, Cl-3, Cl-B and Cl-D) were in the genus Clostridium with the main characteristics of this genus are Gram-positive, basil shaped, endospore formed, negative catalase and anaerobic. MLC-A isolate is placed in the group of Mycobacteriaceae, Lactobacillaceae or Coryneform with the main characteristics near to Clostridium in Bacillaceae family but cannot form endospore. Pr-C isolate was in the genus Proteus with the main characteristics are Gram-negative, facultative anaerobic, negative lactose fermentation, urea hydrolysis, and negative indol test.
4. Conclusion
Cellulose degrading bacteria successfully isolated from the gut of termites Cryptotermes sp. Six isolates showed halo zones due to cellulose or carboxymethylcellulose degradation. MLC-A isolate had shown maximum cellulolytic index. Three isolates were identified in the genus of Clostridium, one isolate be placed in the group of Mycobacteriaceae, Lactobacillaceae or Coryneform and the last one in the genus Proteus.

5. References
[1] Rani D S and Nand K 2000 Production of thermostable cellulase-free xylanase by Clostridium absonum CFR-702 Process Biochemistry 36 4 355–362
[2] Gautam S P, Bundela P S, Pandey A K, Awasthi M K and Sarsaiya S 2010 Composting of municipal solid waste of Jabalpur City Global Journal of Environmental Research 41 43–46
[3] Bhat M K and Bhat S 1997 Cellulose degrading enzymes and their potential industrial applications Biotechnology Advances 15 3-4 583-620
[4] Bhat M K 2000 Cellulases and related enzymes in biotechnology Biotechnol Adv 18 5 355-383
[5] Shewale J G 1982 Glucosidase: its role in cellulase synthesis and hydrolysis of cellulose International Journal of Biochemistry 14 6 435-443
[6] Saxena S, Bahadur J and Varma A 1993 Cellulose and hemicellulose degrading bacteria from termite gut and mould soils of India The Indian Journal of Microbiology 33 55-60
[7] Cleveland L R 1924 The physiology and symbiotic relationships between the intestinal protozoa of termites and their host, with special reference to Reticulitermes flavipes The Biological Bulletin 46 4 117-227
[8] Watanabe H, Noda H, Tokuda G and Lo N 1998 A cellulase gene of termite origin Nature 394 6691 330.
[9] Khademi S, Guarino L A, Watanabe H, Tokuda G and Meyer E F 2002 Structure of an endoglucanase from termite, Nasutitermes takasagoensis Acta Crystallographica Section D: Biological Crystallography 58 4 653-659
[10] Scharf M E and Tartar A 2008 Termite digestomes as sources for novel lignocellulases (Biofuels, Bioproducts & Biorefining)
[11] Ohkuma M 2003 Termite symbiotic systems: efficient biorecycling of lignocelluloses Appl Microbiol Biotechnol 61 1–9
[12] Watanabe H and Tokuda G 2010 Cellulolytic System in Insect Annu Rev Entomol 55 609–632 (Advance first posted online)
[13] Ohkuma M 2008 Symbioses of flagellates and prokaryotes in the gut of lower termites Trends Microbiol 16 345–352
[14] Ohkuma M 2003 Termite symbiotic system: efficient bio-recycling of lignocellulose Appl Microbiol Biotechnol 61 1–9
[15] Braunzan A, Kam M D, Labat M and Breznak J A 2017 H2/C02 acetogenesis by gut bacteria vary with termite feeding guild (Submitted to Sciences)
[16] Mousdale D M 2008 Biofuels: biotechnology, chemistry, and sustainable development (Boca, Raton Florida: CRC Press)
[17] Upadhyaya S K, Manandhar A. Mainali H, Pokhrel A R, Rijal A, Pradhan B and Koirala B 2012 Isolation and characterization of cellulolytic bacteria from gut of termite. Rentech Symposium Compendium 1 4 14-18
[18] Holt J G, Krieg R N, Sneath P H A, Staley J T and Williams S T 1994 Bergey’s Manual of Determinative Bacteriology, 9th ed. (Baltimore : Williams and Wilkins)
[19] Cappuccino J and Sherman N 2005 Microbiology A Laboratory Manual (San Fransisco Boston: New York)
[20] Matteotti C et al. 2012 Identification and characterization of a new xylanase from gram-positive bacteria isolated from termite gut (Reticulitermes santonensis) J. Protein and Purification 83 117-127