HAYATI Journal of Biosciences 22 (2015) 197–200

HOSTED BY

Contents lists available at ScienceDirect

HAYATI Journal of Biosciences

journal homepage: http://www.journals.elsevier.com/hayati-journal-of-biosciences

Short communication

Characterization and Identification of Cellulolytic Bacteria from gut of Worker *Macrotermes gilvus*

Andri Ferbiyanto, Iman Rusmana,* Rika Raffiudin

Department of Biology, Faculty of Mathematic & Natural Sciences, Bogor Agricultural University, Dramaga Campus, Bogor 16880, Indonesia.

ARTICLE INFO

Article history:
Received 7 July 2015
Accepted 22 September 2015
Available online 11 December 2015

KEYWORDS:
16S rRNA,
cellulolytic activity,
gut bacteria,
Macrotermes gilvus,
Paracoccus yeei

ABSTRACT

As a social insect, termite colony consists of three castes, i.e. reproductive, soldier, and worker castes. In their role of cellulose digestion, the worker termites use two sources of cellulolytic enzyme that include cellulases produced by the termite and the gut symbions. *Macrotermes gilvus* classified in mound builder termite, mostly depend on cellulolytic bacteria for cellulose digestion. This study aims to characterize cellulolytic bacteria of termite gut symbionts of worker *M. gilvus* and to identify the cellulolytic bacteria based on sequences of 16S ribosomal RNA (rRNA) gene. Cellulolytic bacteria of termite gut were isolated and cultured in CMC (Carboxymethyl cellulose) media. The biochemical characters of bacterial isolates were assayed using Microbact 12A and 12B. Cellulolytic activity was determined based on formation of clear zone and cellulolytic index on CMC plate media. The bacterial isolate that has the highest cellulolytic index was analyzed for its 16S rRNA gene sequences. Four isolates of cellulolytic bacteria were successfully isolated from gut of *M. gilvus* with aerobic and anaerobic conditions. The highest formation of cellulolytic index (2.5) was revealed by RA2. BLAST-N (Basic Local Alignment Search Tool for Nucleotides) result of 16S rRNA gene sequences of RU4 and RA2 isolates showed that the isolate has similarity with *Bacillus megaterium* and *Paracoccus yeei*, respectively. This result indicated that RA2 isolate was *P. yeei*, a cellulolytic bacterium of a termite gut of *M. gilvus*.

1. Introduction

Termites are known as social insects that have various morphologies. Based on its reproduction ability, they are classified into two types: reproductive (queen) and non-reproductive (worker and soldier castes). Queen's job is to produce and lay eggs, soldier's job is to guard the colony, and worker's jobs are to nurse, repair the colony, and food gathering (Eggleton 2011).

Although termites are considered as a pest, they have ecological functions. They devour the cellulose material such as litter and wood (Rosengaus *et al.* 2011). To digest cellulose, termites have to provide cellulolytic enzymes, i.e. cellulase produced by the termite itself and by the termite symbionts (Nakashima *et al.* 2002). Cellulose is a linear polymer of glucose linked through β-1,4-glycosidic linkages. These linkages are hydrolyzed by cellulase, which also plays a role in recycling the polysaccharides. Cellulase consists of various enzymes such as endoglucanase (EC 3.2.1.4), exoglucanase (EC 3.2.1.74), and β-glucosidase (EC 3.2.1.21) (Morana *et al.* 2011).

Termites contain diverse microbes in their gut and they are classified into lower and higher termites. Lower termites have protists and bacteria in their gut, although there is less information about the bacteria. However higher termites lack protists and contain only prokaryotes (Ohkuma and Brune 2011).

Termite gut bacteria can be classified into bacteriodales, clostridiales, cyanobacteria, mycoplasmatales, firmicutes, actinobacteria, proteobacteria, and bacillales (Shinzato *et al.* 2005; Warnecke *et al.* 2007). Some gut bacteria from terminidae have been identified. Those bacteria had similarity with *Clostridium* genus, *Anaerovorax odorimutans*, *Erysipelothrix rhusiopathie*, *Eubacterium seraeum*, and *Sporobacter termitidis* (Schmitt-Wagner *et al.* 2003).

Macrotermes is found in South Africa, Arabian Peninsular, Thailand, Malaysia Peninsular, and Indonesia (Kalshoven 1956; Cowie 1989: Meyer *et al.* 1999; Yamada *et al.* 2007; Bakhtiari *et al.* 2010). Major soldiers of *Macrotermes* have a large body, reddish-brown head, rounded and widened meso- and metanotal, 17 segment in antenna, and a few hair in the body (Ahmad 1958). *Macrotermes gilvus* (Isoptera: macrotermitinae) is a mound builder termite that lives in Malaya and Indo-China to Indonesia and Philippines (Roonwal and Chhotani 1961). As a higher termite, *M.*

* Corresponding author.
E-mail address: irusmana@ipb.ac.id (I. Rusmana).
Peer review under responsibility of Institut Pertanian Bogor.

http://dx.doi.org/10.1016/j.hjb.2015.07.001

gilvus posseses bacteria in its gut. These bacteria function as a second source of cellulolytic enzyme. They are indigenous bacteria in termite guts and had never been studied before. The aim of this study was to characterize cellulolytic bacteria from gut termites and to identify selected bacteria based on 16S ribosomal RNA (rRNA) gene sequences.

2. Materials and Methods

2.1. Bacterial isolation

Termites were collected from the forest backyard of Marine and Science Faculty, Bogor Agricultural University, Indonesia. Termites were surface sterilized using 70% ethanol for 30 seconds. The rectum of termites was stabbed by an inoculation needle, then striked on CMC plate medium. The plate was incubated at room temperature for 3 days with both aerobic and anaerobic condition. Grown bacteria were purified on CMC plate medium. Morphological determination of bacterial colony was based on Microbial Application Laboratory Manual in General Microbiology 8th Ed (Benson 2008).

2.2. Biochemical and cellulolytic activity assay

Microbact 12A and 12B were used to determine biochemical reactions of isolated bacteria. Oxidase reaction was tested using oxidase paper and motility test was conducted using hanging drop method. Cellulolytic activity was measured as a diameter of clear zone after the CMC plate was poured by 1% congo red. Cellulolytic index was calculated using formula as follow:

$$\text{Cellulolytic index} = \frac{(\text{Diameter of zone} - \text{Diameter of bacterial colony})}{\text{Diameter of bacterial colony}}$$

2.3. DNA isolation

DNA isolation was done using XPrep DNA Soil mini kit (PhileKorea, Korea), according to the manufacturer's instructions.

2.4. Amplification of 16S rRNA gene

The 16S rRNA gene was amplified using universal primer, 20F (5′- AGAGTTTGATCATGGCTCAG -3′) and 1500R (5′-GGTTACCTTGTTACGACTT -3′) (Weisburg *et al.* 1991). Polymerase chain reaction (PCR) was performed in a Thermocycler GeneAmp PCR System 2400 (Perkin Elmer). Takara LaTaq GC Buffer was used for PCR amplification. Each reaction mixture (20 μL) contained 0.5 μL primers (10 pmol/μL), 0.2 μL 10 × Long Polymerase Taq (5 U/μL), 10 μL GC buffer (5 mM), 3.2 μL dNTP (2.5 mM), 2 μL isolated DNA, and nuclease free water until final volume reaches 20 μL. Thermocycling conditions were set up as follows: initial denaturation 94°C for 4 minutes, followed by 35 cycles of 94°C for 40 seconds, 55°C for 1 minute, 72°C for 1 minute 10 seconds, and final elongation at 72°C for 10 minutes. After amplification, 3 μL PCR product was migrated in 1.5% agarose gel and visualized using SyberGreen staining on ultraviolet transilluminator.

2.5. Bioinformatics analysis and phylogenetic tree construction

PCR product was directly sequenced using a DNA sequencer (ABI PRISM 3100). The data of 16S rRNA gene sequences were compared with database at GenBank using BLAST-N search program in National Center Biotechnology Information (http://www.ncbi.nlm.nih.gov). The 16S rRNA gene sequences were aligned and phylogenetic tree was constructed using MEGA 5.05 software with neighbor-joining method at 1000X bootstraps (Tamura *et al.* 2011).

3. Results

3.1. Morphological and biochemical characteristics of gut cellulolytic bacteria

Four bacteria were isolated from the gut of worker *M. gilvus*, i.e. RU1, RU3, RU4, and RA2. The RU isolates were isolated from aerobic condition, and RA isolate was isolated from anaerobic condition. RU1 isolate is an aerobic bacterium with coccus cell morphology and is Gram negative. Its bacterial colony is rounded, smooth, and convex. RU3 isolate is a facultative bacterium with bacilli cell and is Gram positive. Its bacterial colony is smooth and convex. RU4 is a facultative bacterium with bacilli and is Gram positive. Its bacterial colony is round and flat. RA2 isolate is facultative bacterium with coccus cell and Gram negative. Its bacterial colony is round and convex (Figure 1).

All bacterial isolates showed positive reactions to biochemical assay of protease and β-galactosidase. They also showed negative reactions to biochemical assay of ornithine, hydrogen sulfide reduction, indole, citrate, malonate, sorbitol, rhamnose, adonitol, raffinose, and arginine. However, RU1 isolate showed positive reaction to biochemical assay of glucose and xylose. RU3 isolate showed positive reaction to biochemical assay of lysine and sucrose, whereas RU4 isolate showed positive reaction to biochemical assay of urease and arabinose. The anaerobic RA2 isolate showed positive reaction for biochemical assay of inositol and arabinose.

3.2. Cellulolytic activity of bacterial isolates

Cellulolytic activity of bacterial isolates was based on clear zone of degraded CMC area around the colony. Cellulolytic activity test showed that RA2 isolate has the largest cellulolytic index (2.5) and RU3 isolate has the smallest cellulolytic index (0.75) (Table 1).

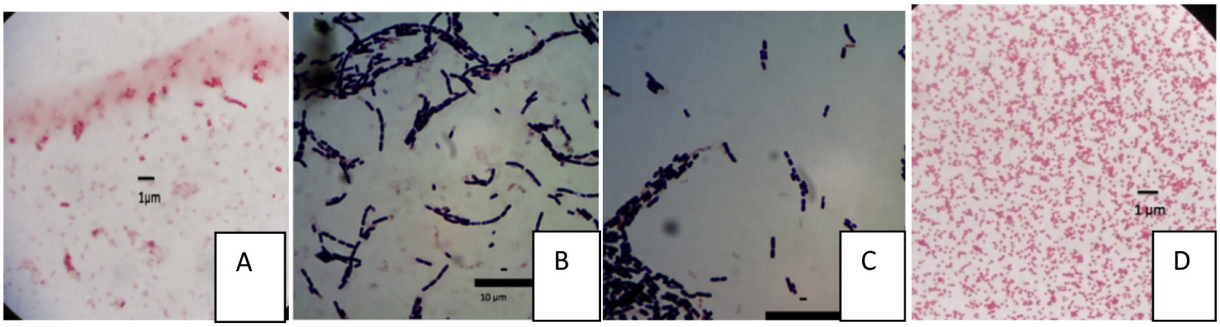

Figure 1. Gram staining of isolated cellulolytic gut bacteria. (A) RU1, (B) RU3, (C) RU4, and (D) RA2 bacterial isolates.

Table 1. Cellulolytic index of isolated cellulolytic gut bacteria

Isolate	Diameter of colony (mm)	Diameter of cellulolytic zone (mm)	Cellulolytic index
RU1	3.5	6.5	0.87
RU3	6.0	10.5	0.75
RU4	8.0	14.5	0.81
RA2	2.0	5.0	2.50

Based on cellulolytic index and growth, RU4 and RA2 isolates were potential isolates.

3.3. Molecular identification of selected gut cellulolytic isolates

The 16S rRNA gene is a component of 30S ribosomal subunit of prokaryotes, commonly used in molecular characterization and determination of phylogenetic tree among prokaryotes. The contig region of 16S rRNA gene from RU4 isolate was 850 bp and RA2 isolate was 650 bp. Comparing these contig regions with NCBI GenBank entries using BLAST algorithm (http://www.ncbi.nlm.nih.gov) showed that RU4 isolate has 98% maximum identity with a sequences of *Bacillus megaterium* (NR_043401.1) and RA2 isolate has 99% similarity with *Paracoccus yeei* (NR_029038.1) (Figure 2) (Tables 2 and 3).

4. Discussion

Bacteria isolated from termite gut have characteristics of either facultative anaerobe or microaerophile (Wenzel *et al.* 2002). Based on Gram staining, RU1 and RA2 isolates are Gram negative bacteria. However RU3 and RU4 isolates are Gram positive bacteria. Cellulolytic activity measurement showed that RA6 isolate has the highest cellulolytic index (2.5). However this activity is lower than that of the study by Gupta *et al.* (2012). Their bacterial isolates had cellulolytic index in the range of 4.29−5.49.

The amplification of 16S rRNA gene sequences with primer 20F and 1500R showed 1500 bp DNA amplicons (Weisburg *et al.* 1991). Based on molecular identification of 16S rRNA gene, RU4 isolate has 98% similarity with *Bacillus megaterium* (NR_043401.1) and RA2 isolate has 99% similarity with *P. yeei* (NR_029038.1). *Bacillus megaterium* is a Gram positive bacterium with rod-cell shape, and

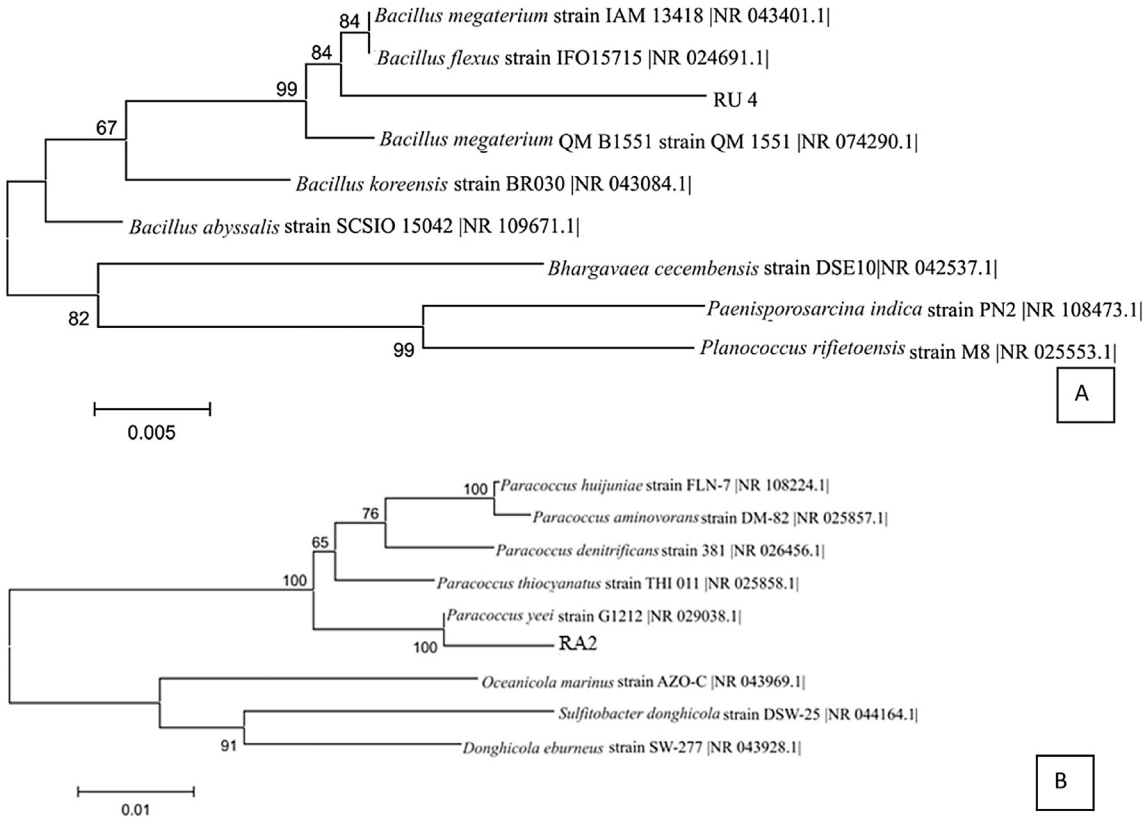

Figure 2. Phylogenetic tree of (A) RU4 and (B) RA2 bacterial isolates.

Table 2. BLAST-N results of 16S rRNA gene of RU4 isolate

Accession number	Species	Maximum score	Total score	Query cover	*e* Value	Maximum identity
NR_043401.1	*Bacillus megaterium* strain IAM 13418	1557	1557	100%	0	98%
NR_024691.1	*B. flexus* strain IFO15715	1550	1550	100%	0	98%
NR_074290.1	*B. megaterium* QM B1551	1541	1541	100%	0	98%
NR_043084.1	*B. koreansis* strain BR030	1491	1491	100%	0	97%
NR_109671.1	*B. abyssalis* strain SCSIO 25043	1469	1469	100%	0	97%

Table 3. BLAST-N results of 16S rRNA gene of RA2 isolate

Accession number	Species	Maximum score	Total score	Query cover	*e* Value	Maximum identity
NR_029038.1	*Paracoccus yeei* strain G1212	1127	1127	100%	0	99%
NR_025858.1	*P. thiocyanatus* strain THI 011	1061	1061	100%	0	98%
NR_026456.1	*P. denitrificans* strain 381	1055	1055	100%	0	97%
NR_042715.1	*P. aminophilus* strain ATCC 49673	1048	1048	100%	0	97%

has positive reaction for biochemical assay of galactosidase, citrate, VP (Voges Proskauer) test, gelatinase, glycerol, inositol and mannitol, ribose, L-arabinose, D-xylose, galactose, D-glucose, and D-fructose (Logan and Berkeley 1984). However, in this study, RU4 isolate showed negative reactions for biochemical assay of citrate, VP test, inositol, xylose, mannitol, and glucose, but showed positive reaction on gelatinase assay. *B. megaterium* was reported as a gut cellulolytic bacterium in termite *Zootermopsis anguisticollis* (Wenzel *et al.* 2002).

P. yeei is a non-motile Gram negative bacterium with coccus-bacilli cells. It shows positive reactions for biochemical assay of catalase, oxidase positive, nitrate reduction, arginine dehidrogelase, arabinosa and malate assimilation. But it shows negative reaction for biochemical assay of urease, indole, esculin hydrolysis, gelatinase, assimilisation of glucose, mannose, maltose, gluconate, caprate, and citrate (Funke *et al.* 2004). However, we found that RA2 isolate showed positive reactions for biochemical assay of arginine, arabinose, gelatinase, glucose, indole, and β-galactosidase. The differences of these biochemical assay characteristics indicate that RU4 and RA2 isolates are different bacterial strains of *B. megaterium* and *P. yeei*, respectively, due to different termite's guts as sources of isolated cellulolytic bacteria.

Conflict of interest

There is no conflict of interest.

References

Ahmad M. 1958. Key to the Indomalayan termites. *Biologia* 4:33–198.

Bakhtiari AR, Zakaria MP, Ramin M, Yaziz MI, Lajis MN, Xinhui B. 2010. Characterization of perylene in tropical environment: comparison of new and old fungus comb for identifying perylene precursor in *Macrotermes gilvus* termite nests of Peninsular Malaysia. *Environ Asia* 3(1):13–9.

Benson HJ. 2008. In: Brown AE (Ed.). *Microbiological Applications Laboratory Manual in General Microbiology*. New York (US): McGraw-Hill.

Cowie RH. 1989. The zoogeographical composition and distribution of the Arabian termite fauna. *Biol J Linn Soc* 36:157–68.

Eggleton P. 2011. An introduction of termites: biology, taxonomy, and functional morphology. In: Bignell DE, Roisin Y, Lo N (Eds.). *Biology of Termites: A Moderns Synthesis*. New York (US): Springer Dordcreht Heidelberg. pp. 1–26. http://dx.doi.org/10.1007/978-90-481-3977-4_1.

Funke G, Frodl R, Sommer H. 2004. First comprehensively documented case of *Paracoccus yeei* infection in a human. *J Clin Microbiol* 42:3366–8. http://dx.doi.org/10.1128/JCM.42.7.3366-3388.2004.

Gupta P, Samant T, Sahu A. 2012. Isolation of cellulose-degrading bacteria and determination of their cellulolytic potential. *Int J Microbiol* 2012:1–5. http://dx.doi.org/10.1155/2012/578925.

Logan NA, Berkeley RCW. 1984. Identification of *Bacillus* strain using the API system. *J Gen Microbiol* 130:1871–82.

Kalshoven LGE. 1956. Observation on *Macrotermes gilvus* Holmgr. In Java-3 accumulations of finely cut vegetable mater in the nests. *Insect Soc* 3(3):455–61.

Meyer VW, Braack LEO, Biggs HC, Eberohn C. 1999. Distribution and density of termite mounds in the Northern Kruger National Park, with specific reference to those constructed by *Macrotermes* Holmgren (Isoptera: Terminidae). *Afr Entomol* 7(1):123–30.

Morana A, Maurelli L, Ionata E, La Cara F, Rossi M. 2011. Cellulases from fungi and bacteria and their biotechnological applications. In: Golan AE (Ed.). *Cellulase: Types and Action, Mechanisms and Uses*. New York (US): Nova Science Publisher, Inc.. pp. 1–79.

Nakashima K, Watanabe H, Saitoh H, Tokuda G, Azuma J-I. 2002. Dual cellulose digesting system of the wood-feeding termite, *Coptotermes formosanus* Shiraki. *Insect Biochem Mol Biol* 32:777–84.

Ohkuma M, Brune A. 2011. Diversity, structure, and evolution of the termite gut microbial community. In: Bignell DE, Roisin Y, Lo N (Eds.). *Biology of Termites: A Moderns Synthesis*. New York (US): Springer Dordcreht Heidelberg. pp. 1–26. http://dx.doi.org/10.1007/978-90-481-3977-4_15.

Roonwal ML, Chhotani OB. 1961. The termite *Macrotermes gilvus* malayanus (Haviland) (Termitidae) in Burma. *Proc Nat Inst Sci India* 27:308–16.

Rosengaus RB, Traniello JFA, Bulmer MS. 2011. Ecology, behavior and evolution of disease resistance in termites. In: Bignell DE, Roisin Y, Lo N (Eds.). *Biology of Termites: A Modern Synthesis*. Berlin: SpringerVerlag. pp. 165–92.

Shinzato N, Muramatsu M, Matsui T, Watanabe Y. 2005. Molecular phylogenetic diversity of the bacterial community in the gut of termite *Coptotermes formosanus*. *Biosci Biotechnol Biochem* 96(6):1145–55.

Schmitt-Wagner D, Friedrich MW, Wagner B, Brune A. 2003. Phylogenetic diversity, abundance, and axial distribution of bacteria in the intestinal tract of two soil-feeding termites (*Cubitermes* spp.). *App Environ Microbiol* 69(10):6007–17. http://dx.doi.org/10.1128/AEM.69.10.6007-6017.2003.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 10:2731–9. http://dx.doi.org/10.1093/molbev/msr121.

Warnecke F, et al. 2007. Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite. *Nature* 450:560–5. http://dx.doi.org/10.1038/nature06269.

Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 1991. 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* 173:697–703.

Wenzel M, Schönig M, Berchtold M, Kämpfer P, König H. 2002. Aerobic and facultatively anaerobic cellulolytic bacteria from the gut of termite *Zootermopsis angusticollis*. *J App Microbiol* 92:32–40.

Yamada A, Inoue T, Wiwatwitaya D, Ohkuma O. 2007. A new concept of feeding group composition of termites (Isoptera) in tropical ecosystems: carbon source competitions among fungus-growing termites, soil-feeding termites, litter-layer microbes, and fire. *Sociobiology* 50:135–53.