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Diversity and cellulolytic activity of culturable bacteria isolated from the gut of higher termites (*Odontotermes* sp.) in eastern Thailand

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Abstract. *Boontanom P, Chantarasiri A. 2021. Diversity and cellulolytic activity of culturable bacteria isolated from the gut of higher termites (*Odontotermes *sp.) in eastern Thailand. Biodiversitas 22: 3349-3357.* Cellulolytic bacteria are vital symbionts associated with the gut of all higher termites. *Odontotermes* termites are a higher termite widely found in Thailand. However, information concerning the diversity of cellulolytic bacteria in this termite gut remains inadequate. The aim of this study is to isolate and identify the culturable cellulolytic bacteria from the *Odontotermes* gut collected from eastern Thailand. The crude cellulases produced from the most active cellulolytic bacterium were further characterized. Thirty-two cellulolytic bacteria were isolated and subsequently classified by PCR-RFLP of the 16S rRNA gene. A total of 10 different RFLP patterns were obtained belonging to five bacterial genera, namely *Acinetobacter*, *Bacillus*, *Citrobacter*, *Paenibacillus*, and *Serratia*. The *B. cereus* strain TWV503 was considered to be the most active cellulolytic bacterium based on the CMC agar method. *B. cereus* strain TWV503 showed CMCase activity at 2.190 ± 0.063 U/mL of CMCase and 0.276 ± 0.031 U/mL of FPase. The optimum temperature and pH for CMCase activity were 50°C and the neutral pH ranging from 7.0 to 8.0, respectively. CMCase activity remained stable at up to 70°C and neutral pH ranging from 7.0 to 8.0 for 24 hours of incubation. This study revealed novel information related to cellulolytic bacteria isolated from the gut of *Odontotermes* termites collected from Thailand.

Keywords: *Bacillus cereus*, cellulase, cellulolytic bacteria, *Odontotermes*, termite

INTRODUCTION

Termites are small eusocial insects at the taxonomic rank of Order Isoptera, Phylum Arthropoda that are highly abundant in tropical and subtropical regions (Brune 2014). They are commonly classified into two major groups involving lower and higher termites. The lower termites are phylogenetically basal and comprise six families including Mastotermitidae, Termopsidae, Hodotermitidae, Kalotermitidae, Serritermitidae, and Rhinotermitidae (Hongoh 2011; Korb 2018). The higher termites are phylogenetically apical and comprise only the family Termitidae (Hongoh 2011; Korb 2018). Almost all families of lower termites are wood feeders, except for the members of family Hodotermitidae, which are dead grass feeders (Hongoh 2011). The higher termites forage a wide variety of lignocellulosic biomass and materials such as wood, dry grass, herbivore dung, lichen, plant litter, organic matter, and soil (Hongoh 2011; Brune 2014; Mikaelyan et al. 2015). Therefore, they play a vital role in general plant decomposition, carbon cycling of organic matters, and recycling nutrients of ecosystems (König et al. 2013; Otani et al. 2014).

Microbial diversity associated with termite gut has been widely studied by both culture-dependent and independent methods (Manjula et al. 2016). These microbes show a mutualistic beneficial relationship with termites and typically convert complex polysaccharides of lignocellulosic biomass into simpler saccharides by hydrolytic enzymes comprising cellulases, cellubiases, hemicellulases, glucosidases, and gluconases (Maurice and Erdei 2018). Lower termites predominantly harbor archaea, bacteria, and flagellated protists within their digestive tracts, whereas higher termites are mainly associated with bacteria (Brune 2014; Peterson and Scharf 2016). However, gut microbiota varies markedly among termite species (Maurice and Erdei 2018).

Previous studies reported that the total number of bacteria in the termite gut is within the range of 10^7 to 10^{11} per milliliter (König et al. 2013), which mainly belong to Phyla Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria (Manjula et al. 2016). Many strains of bacterial symbionts are considered cellulolytic bacteria and have been isolated (Ferbiyanto et al. 2015; Sharma et al. 2015; Sreena et al. 2015). Cellulolytic bacteria can hydrolyze the β-1,4 glycosidic linkage of cellulose polymers producing sugar derivatives by their cellulolytic enzymes, generally called cellulases (Chantarasiri 2015; 2020). Cellulases are grouped into the glycosyl hydrolase (GH) family comprising endoglucanases (E.C. 3.2.1.4), exoglucanases (E.C. 3.2.1.91, E.C. 3.2.1.176), and β-glucosidases (E.C. 3.2.1.21) (Juturu and Wu 2014; Obeng et al. 2017). Today, cellulases comprise up to 8% of the worldwide industrial enzyme demand (Shweta 2014). They have shown their potential applications over decades in various industrial processes such as textile, food and feed, paper and pulp, biorefinery, and agricultural industries (Menendez et al. 2015). Therefore, the study of cellulolytic

bacteria in termite gut is challenging for the screening of effective cellulases.

Odontotermes is a genus of fungus-growing termites in the family Termitidae (higher termites) that reside throughout the tropical and subtropical regions of Africa and Asia (Chiu et al. 2018). *Odontotermes* termites are agricultural pests that attack crops and trees, and are commonly found in Thailand (Wititsiri 2011; Chiu et al. 2018). Information about cellulolytic bacteria isolated from the *Odontotermes* termites is scarce. Therefore, this study aimed to isolate and screen cellulolytic bacteria from the gut of *Odontotermes* termites in the eastern region of Thailand. The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and nucleotide sequencing analysis of 16S rRNA genes were used to describe the diversity of isolated cellulolytic bacteria.

MATERIALS AND METHODS

Study area and sampling of termites

The study area of this study includes three provinces located in the eastern region of Thailand comprising Rayong Province (12° 49′ N, 101° 13′ E), Chachoengsao Province (13° 37′ N, 101° 18′ E) and Prachin Buri Province (14° 6′ N, 101° 19′ E). *Odontotermes* termites were collected randomly from the groves and grasslands of the study area. The collected termites were morphologically identified based on their unique heads and mandibles. Higher termites in the genus *Odontotermes* have symmetrical mandibles curved at the tips, with minute or nonexistent teeth at the right mandible. The left mandible has one or two teeth or a serrated cutting edge. Fifty termites were collected during the winter season in January 2017 and kept in sterilized plastic bags at 4°C. Bacterial isolation was performed within 48 hours of collection.

Procedures

Isolation of bacteria from termite gut samples

The isolation of bacteria from collected termites was conducted according to Sharma et al. (2015) with minor modifications. Termite samples were surface sterilized by dipping into 70% (v/v) ethanol for 10 seconds and washed in sterilized 0.85% (w/v) NaCl solution for 30 seconds. Each entire gut of a sterilized termite was picked out by an inoculum needle under an SMZ445 stereomicroscope (Nikon, Japan). The dissection procedures were performed under aseptic conditions in a modified NU-440 biosafety cabinet (NuAire, USA). The termite gut was transferred to 1 mL of Tryptone soy broth (TSB) (HiMedia, India) and incubated at 30°C for 24 hours to enrich the culturable bacterial symbionts. The enriched cultures were serially diluted with sterilized 0.85% (w/v) NaCl solution to obtain 1:10,000 dilutions and spread plated on Tryptone soya agar (TSA) (HiMedia, India). All culture plates were incubated at 30°C for 24 hours. The bacterial isolates were selected based on the morphology of colony, and the colony was subsequently purified by streak plated on TSA.

Screening of cellulolytic bacteria

The bacterial isolates were cultured in 1 mL of TSB at 30°C for 24 hours. One drop (5 µL) of each isolated bacterium was placed on CMC agar for screening of cellulolytic bacteria. The CMC agar contained carboxymethyl cellulose (CMC) as a sole carbon source for bacterial growth which was previously described by Chantarasiri (2015). All culture plates were incubated at 30°C for 48 hours and then flooded with iodine solution for 15 minutes. The iodine solution comprised 0.33 (w/v) I_2 and 0.67% (w/v) KI as described by Chantarasiri et al. (2015). The cellulolytic bacteria produce the clear zones around the colonies on CMC agar by the hydrolysis mechanisms of their producing cellulases. These clear zones were visualized after staining by iodine solution. The hydrolysis capacity (HC) value of cellulolytic bacteria was calculated from the ratio between the diameter of the clear zone and the diameter of the bacterial colony (Chantarasiri 2014). All the experiments were performed in triplicates.

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of 16S rRNA genes

The genomic DNA of each cellulolytic bacterium was extracted using a Genomic DNA isolation kit (Bio-Helix, Taiwan). Polymerase chain reaction (PCR) amplification of the 16S rRNA genes was carried out using an OnePCR reaction mixture (Bio-Helix, Taiwan) with a universal 27F primer (5′-AGAGTTTGATCMTGGCTCAG-3′) and a universal 1492R primer (5′-TACGGYTACCTTGTTACG ACTT-3′) for 35 amplification cycles. The amplification parameters were conducted according to Boontanom and Chantarasiri (2020) in a Master cycler Nexus Gradient thermal cycler (Eppendorf, Germany), which involved a preheating step at 94°C for 4 minutes, a denaturation step at 94°C for 40 seconds, an annealing step at 55°C for 1 minute, an extension step at 72°C for 1 minute 10 seconds, and a final extension step at 72°C for 10 minutes.

The restriction fragment length polymorphism (RFLP) analysis of the PCR products was performed by two restriction enzymes of *Msp*I and *Alu*I (New England Biolabs, UK) in a CutSmart buffer (New England Biolabs, UK) following the previous protocol described by Chantarasiri (2020). The PCR products were cleaved with *Msp*I and *Alu*I at 37°C for 12 hours then the reaction was terminated by heating the reaction mixtures at 80°C for 15 minutes. The resulting DNA fragments were electrophoresed on a 3% (w/v) OmniPur agarose gel (Calbiochem, Germany) and visualized by Novel Juice staining (Bio-Helix, Taiwan) on an MD-25/HD-25 UV-transilluminator (Wealtec, Taiwan). The PCR marker used was OneMark 100 RTU DNA ladder (Bio-Helix, Taiwan).

Nucleotide sequencing and phylogenetic analysis of amplified 16S rRNA genes

The 16S rRNA genes of each cellulolytic bacterium were PCR amplified according to the aforementioned method. They were preliminary verified on a 1.5% (w/v) OmniPur agarose gel and visualized by Novel Juice staining. The 1,500 bp-PCR products were purified and sequenced by the services of Macrogen Inc. (Korea). The

sequenced 16S rRNA genes were aligned for similarity analysis by the BLASTn program based on the nucleotide collection (nr/nt) database and a megablast algorithm from the National Center for Biotechnology Information (NCBI). The phylogenetic tree of cellulolytic bacteria was analyzed by SeaView software version 5.0.1 and FigTree software version 1.4.4 with the neighbor-joining (NJ) method for 100,000 bootstrap replications. All the resulting nucleotide sequences were deposited in the GenBank database of NCBI under the accession numbers MW713798, MW713799, MW713800, MW713801, MW713802, MW713926, MW713927, MW713936, MW713979, and MW713980.

Preparation of the crude cellulases from the most effective cellulolytic bacterium

The most effective cellulolytic bacterium based on the HC value was *B. cereus* strain TWV503. It was subsequently cultured for preparation of the crude cellulases in a CMC liquid medium (Chantarasiri 2015). The bacterial cultures were shaken under an aeration condition at 150 rpm for 48 hours at 30°C. Crude cellulases were harvested from the liquid medium as the cell-free supernatant by centrifugation at 4,500 ×g for 30 minutes. The crude enzyme solution was concentrated by 10-kDa Amicon ultra centrifugal filter units (Millipore, Ireland) and kept at 4°C.

Cellulolytic activity assays of the crude cellulases from the most effective cellulolytic bacterium

The cellulolytic activity assays of the crude cellulases were conducted as previously described study (Chantarasiri et al. 2015). Endoglucanase activity (CMCase activity) was determined by incubating 0.5 mL of crude cellulases with 0.5 mL of 2% (w/v) CMC sodium salt as a substrate in an assay buffer at 50°C for 30 minutes. Total cellulase activity (FPase activity) was determined by incubating 0.5 mL of crude cellulases with 50 mg of grade 1 qualitative filter paper (Whatman, Germany) as a substrate in an assay buffer at 50°C for 1 hour. The reducing sugars released from the substrates were spectrophotometrically determined by a standard 3,5-dinitrosalicylic acid (DNS) method at 540 nm. The cellulolytic activity values were calculated by a glucose standard curve. One unit (U) of cellulolytic activity was defined as the amount of enzyme required to release 1 μmol of the reducing sugars as glucose equivalent per minute under the assay conditions. The assay buffer used was 50 mM sodium phosphate buffer at pH 7.0 according to the previous study (Chantarasiri 2020). Spectrophotometric analysis was performed using an AccuReader microplate reader (Metertech, Taiwan). All the experiments were performed in triplicates.

Enzymatic characterization of the crude cellulases from the most effective cellulolytic bacterium

The experiment focused on the temperature and pH values that affected the CMCase activity of the crude cellulase producing from *B. cereus* strain TWV503. The CMCase assay was measured accordingly, as mentioned in the previous experiment. All the experiments were performed in triplicates.

The optimum temperature of the cellulolytic activities was characterized at temperatures ranging from 25°C to 80°C in an assay buffer. Thermal stability was characterized by pre-incubating the crude cellulases at temperatures ranging from 25°C to 80°C for 24 hours in an assay buffer, and the relative activity of cellulases was monitored. The assay buffer used was 50 mM sodium phosphate buffer at pH of 7.0.

The effect of pH on the cellulolytic activities was characterized in the pH-varied buffers at 50°C. The optimum pH of the cellulolytic activities was determined in assay buffers comprising 50 mM citrate buffer (pH 4.0-6.0), 50 mM sodium phosphate buffer (pH 6.0-8.0), 50 mM Tris-HCl buffer (pH 8.0-9.0), and 50 mM glycine-NaOH buffer (pH 9.0-10.0). The pH stability was measured by pre-incubating the crude cellulases at 50°C for 24 hours in the above-mentioned buffers, and the relative activity of cellulases was monitored.

Data analysis

The statistical analysis was achieved by one-way ANOVA followed by Tukey's test with a 95% confidence interval using R software version 4.0.3 (R Core Team 2020).

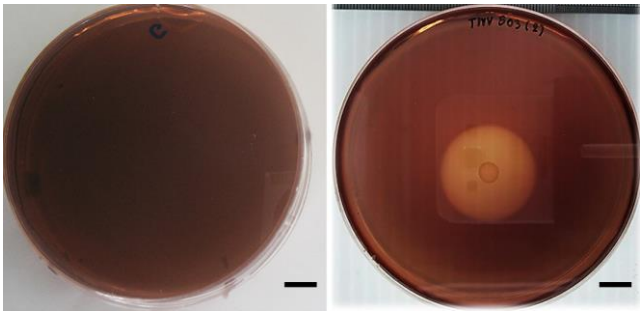
RESULTS AND DISCUSSION

Isolation and screening of cellulolytic bacteria from the termite gut samples

Result revealed that 72 bacterial isolates were isolated on TSA medium from the gut of fifty *Odontotermes* termites under laboratory conditions. Thirty-two bacterial isolates were considered as active cellulolytic bacteria (44.4% of the isolated bacteria) based on the CMC agar method. These cellulolytic bacteria were obtained from 16 isolates of Rayong Province (named with the isolate codes of TWV and PJE), 3 isolates of Chachoengsao Province (named with the isolate codes of EHK), and 13 isolates of Prachin Buri Province (named with the isolate codes of BEI and IKN). The HC values of the cellulolytic bacteria ranged from 1.07 ± 0.04 to 4.44 ± 0.10, as shown in Table 1. Isolate TWV503 from a termite gut in Rayong Province showed maximum HC of 4.44 ± 0.10 and was designated as the most effective cellulolytic bacterium in this study. The cellulolytic zone of TWV503 on CMC agar after iodine solution staining is shown in Figure 1.

Table 1. HC values of thirty-two cellulolytic bacteria isolated from termite gut in three provinces of eastern Thailand.

HC value	Bacterial isolates		
	Rayong Province	**Chachoengsao Province**	**Prachin Buri Province**
1.00-1.99	TWV101, TWV102, TWV301, TWV502	EHK105, EHK401	IKN501
2.00-2.99	PJE201	EHK503	BEI104, BEI203, BEI305, BEI502, IKN102, IKN201
3.00-3.99	TWV103, TWV202, TWV302, TWV402, TWV404, PJE101, PJE202, PJE401, PJE402, PJE501		BEI102, BEI201, BEI304, BEI308, BEI402, IKN103
> 4.00	TWV503		



A **B**

Figure 1. The cellulolytic zone around the bacterial colonies on CMC agar after iodine solution staining. A. Non-cellulolytic bacterium as negative control, B. Cellulolytic bacterium isolate TWV503. Bar = 1 cm

PCR-RFLP analysis of the 16S rRNA genes amplified from the cellulolytic bacteria

The 16S rRNA genes of the isolated cellulolytic bacteria were amplified by the PCR method, cleaved by the restriction enzymes, and electrophorized on agarose gel. The resulting RFLP profiles electrophorized on agarose gel are shown in Figure 2. Ten different patterns of the RFLP profiles were obtained from 32 isolates of cellulolytic bacteria. The different patterns of the RFLP profiles are summarized in Table 2. All RFLP patterns were definite arrangements and practicable for bacterial categorization.

The RFLP profiles revealed that the cellulolytic bacteria isolated from the termite gut in Rayong Province had the highest biodiversity with 5 different patterns. The RFLP-TWV-03, RFLP-PJE-01, and RFLP-BEI-01 patterns were the most commonly found patterns in the RFLP profiles. Interestingly, there were 5 RFLP patterns from different provinces that were closely similar comprising RFLP-TWV-03, RFLP-PJE-01, RFLP-EHK-01, RFLP-BEI-01, and RFLP-IKN-01. A pair of patterns RFLP-TWV-01 and RFLP-EHK-02 also showed a similar pattern. The similar RFLP patterns were possibly believed to be the same species of cellulolytic bacteria associated with the gut of *Odontotermes* termites.

Identification of the cellulolytic bacteria by nucleotide sequencing and phylogenetic analysis

The genomic DNA was extracted from 10 different bacteria based on the RFLP patterns and amplified by the universal primers, 27F and 1492R. The alignment results exhibited that the cellulolytic bacteria from termite gut belonged to 5 genera including *Acinetobacter*, *Bacillus*, *Citrobacter*, *Paenibacillus,* and *Serratia* (Table 3). The *Acinetobacter*, *Citrobacter*, and *Serratia* genera were classified in the Phylum Proteobacteria, whereas the *Bacillus* and *Paenibacillus* genera were classified in the Phylum Firmicutes.

The cellulolytic bacteria isolated from the termite gut in Rayong Province (RFLP-TWV and PJE-patterns) were closely similar to *Acinetobacter baumannii*, *Bacillus cereus*, *Citrobacter farmeri*, and *Serratia marcescens* with 99% identity and 99% query coverage. The cellulolytic bacteria isolated from termite gut in Chachoengsao Province (RFLP-EHK-patterns) were closely similar to *B. cereus* and *Citrobacter* sp. with 99% identity and 99% query coverage. Lastly, the cellulolytic bacteria isolated from termite gut in Prachin Buri Province (RFLP-BEI and IKN-patterns) were closely similar to *B. cereus* and *Paenibacillus polymyxa* with 99% identity and 98-99% query coverage. All E values obtained from the alignment were zero. The phylogenetic tree of the isolated cellulolytic bacteria with 100,000 bootstrap replications is shown in Figure 3. There were 6 RFLP patterns that fell into the phylogenetic clade of Firmicutes, such as RFLP-TWV-03, RFLP-PJE-01, RFLP-EHK-01, RFLP-BEI-01, RFLP-INK-01 and RFLP-INK-02 with a bootstrap value of 60-100, while the RFLP-TWV-01, RFLP-TWV-02, RFLP-TWV-04, and RFLP-EHK-02 were clustered in the phylogenetic clade of Proteobacteria with a bootstrap value of 40-100. The alignment and phylogenetic tree results showed the explicit identification of cellulolytic bacteria isolated from the termite gut. The alignment and phylogenetic tree results confirmed the hypothesized identification of the same RFLP patterns, as mentioned above. The group of RFLP patterns (RFLP-TWV-03, RFLP-PJE-01, RFLP-EHK-01, RFLP-BEI-01, and RFLP-IKN-01) was identified as *B. cereus*, while a pair of RFLP patterns (RFLP-TWV-01 and RFLP-EHK-02) was identified as *Citrobacter* species.

Table 2. Different RFLP patterns and numbers of the thirty-two isolated cellulolytic bacteria from termite gut

RFLP patterns	Bacterial isolates	Total number of bacterial isolate
RFLP-TWV-01	TWV101, TWV502	2
RFLP-TWV-02	TWV102	1
RFLP-TWV-03	TWV103, TWV202, TWV302, TWV402, TWV404, TWV503	6
RFLP-TWV-04	TWV301	1
RFLP-PJE-01	PJE101, PJE201, PJE202, PJE401, PJE402, PJE501	6
RFLP-EHK-01	EHK105, EHK503	2
RFLP-EHK-02	EHK401	1
RFLP-BEI-01	BEI102, BEI104, BEI201, BEI203, BEI304, BEI305, BEI308, BEI402, BEI502	9
RFLP-IKN-01	IKN102, IKN103, IKN501	3
RFLP-IKN-02	IKN201	1

Table 3. Identity percentage of the 16S rRNA gene sequences for the isolated cellulolytic bacteria

RFLP patterns	Closely related bacteria	GenBank Accession No. (Database)	Identity (%) *	GenBank Accession No. (Deposited)
RFLP-TWV-01	*Citrobacter farmeri* strain 33-5	MN400096.1	99.93	MW713798
RFLP-TWV-02	*Acinetobacter baumannii* strain st10	MF102141.1	99.86	MW713799
RFLP-TWV-03	*Bacillus cereus* strain MBL13	GQ148914.1	99.72	MW713802
RFLP-TWV-04	*Serratia marcescens* strain SerEW01	MK961214.1	99.93	MW713800
RFLP-PJE-01	*Bacillus cereus* strain P14	JN700160.1	99.86	MW713801
RFLP-EHK-01	*Bacillus cereus* strain 165PP	KM349191.1	99.38	MW713926
RFLP-EHK-02	*Citrobacter* sp. strain XT-15	KR063546.1	99.65	MW713927
RFLP-BEI-01	*Bacillus cereus* strain D21	KC441762.1	99.51	MW713979
RFLP-IKN-01	*Bacillus cereus* strain LXJ77	MN746190.1	99.17	MW713980
RFLP-IKN-02	*Paenibacillus polymyxa* strain KCTC 3627 clone 6	HE981771.1	99.24	MW713936

Note: * The identity results were analyzed on March 8, 2021

Remarkably, *B. cereus* were the predominant bacteria found in the gut of *Odontotermes* termites in this study. All the resulting nucleotide sequences were deposited in the GenBank database of NCBI under the accession numbers MW713798, MW713799, MW713800, MW713801, MW713802, MW713926, MW713927, MW713936, MW713979, and MW713980.

Cellulolytic activity and enzymatic characterization of the crude cellulases from *B. cereus* strain TWV503

The bacterium isolate TWV503 was considered the most effective bacteria based on its HC value from CMC agar. This bacterium was then designated as *B. cereus* strain TWV503 according to the alignment and phylogenetic tree results of the 16S rRNA gene. The result of cellulolytic activity assays showed that *B. cererus* strain TWV503 could produce crude cellulases 2.190 ± 0.063 U/mL of CMCase and 0.276 ± 0.031 U/mL of FPase under experimental conditions. It was also noted that *B. cereus* strain TWV503 produced cellulases with satisfactory CMCase performance.

Crude cellulases from *B. cereus* strain TWV503 were characterized for CMCase activity at different temperatures and pH values. The optimum temperature and pH for CMCase activity were a moderate temperature at 50°C and a neutral pH ranging from 7.0 to 8.0 of sodium phosphate buffer ($p < 0.01$). The optimum temperature and pH for the enzyme are shown in Figures 4.A and 5.A, respectively. The crude cellulases of *B. cereus* strain TWV503 remained stable at up to 70°C and pH range of 7.0 to 8.0 after 24 hours of experimental incubation ($p < 0.01$). Thermal and pH stability for the enzymes is shown in Figures 4.B and 5.B, respectively. The buffer types barely affected the activity and stability of CMCase at the same pH value, as shown in Figure 5.

Discussion

Termites are major lignocellulosic biomass feeding insects that have ecological importance in the global carbon cycle (König et al. 2013). Termites can digest the ingested lignocellulosic biomass not only by their cellulases but also by cellulases from the microbial symbionts in their gut. Higher termites in the family Termitidae mainly harbor cellulolytic bacteria, which show a mutualistic beneficial relationship in their gut. However, few studies in recent decades have reported on cellulolytic bacteria isolated from the higher termites in Thailand. A report of cellulolytic bacteria isolated from the *Microcerotermes* higher termites collected from Thailand found that the cellulolytic *B. subtilis* isolate M 015 provided the highest endoglucanase activity, whereas *B. subtilis* isolate M018 showed the highest FPase activity (Taechapoempol et al. 2011). Metagenomic analysis of microbial communities residing in the hindguts of *Microcerotermes* termites collected from Thailand showed only 2 active clones of cellulase activity closely related to the unidentified uncultured bacteria (Nimchua et al. 2012).

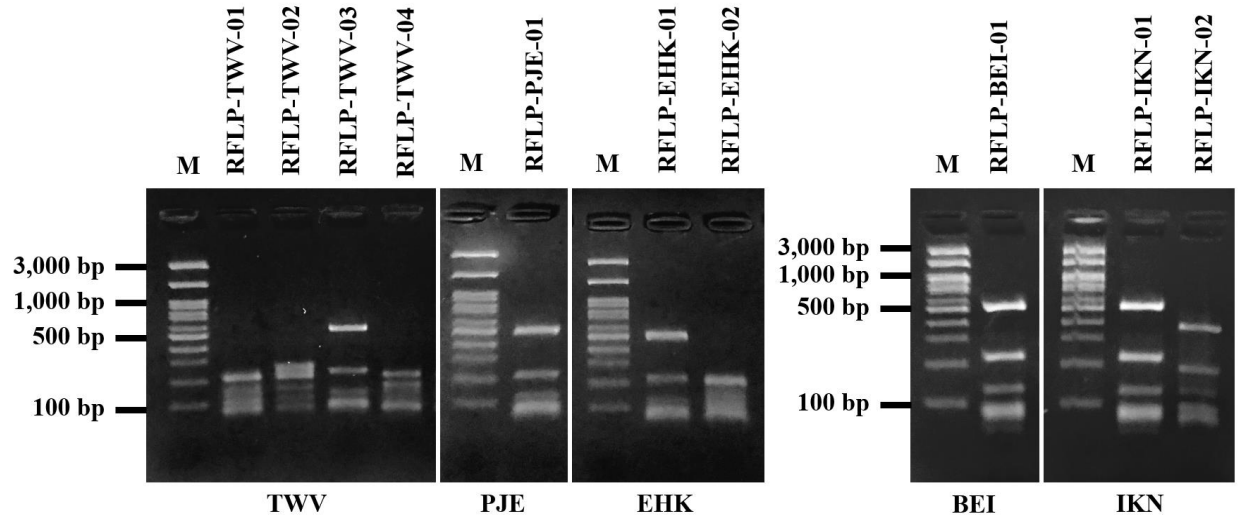


Figure 2. RFLP profiles resulting from the PCR-RFLP analysis of the thirty-two isolated cellulolytic bacteria. M denotes OneMark 100 RTU DNA ladder

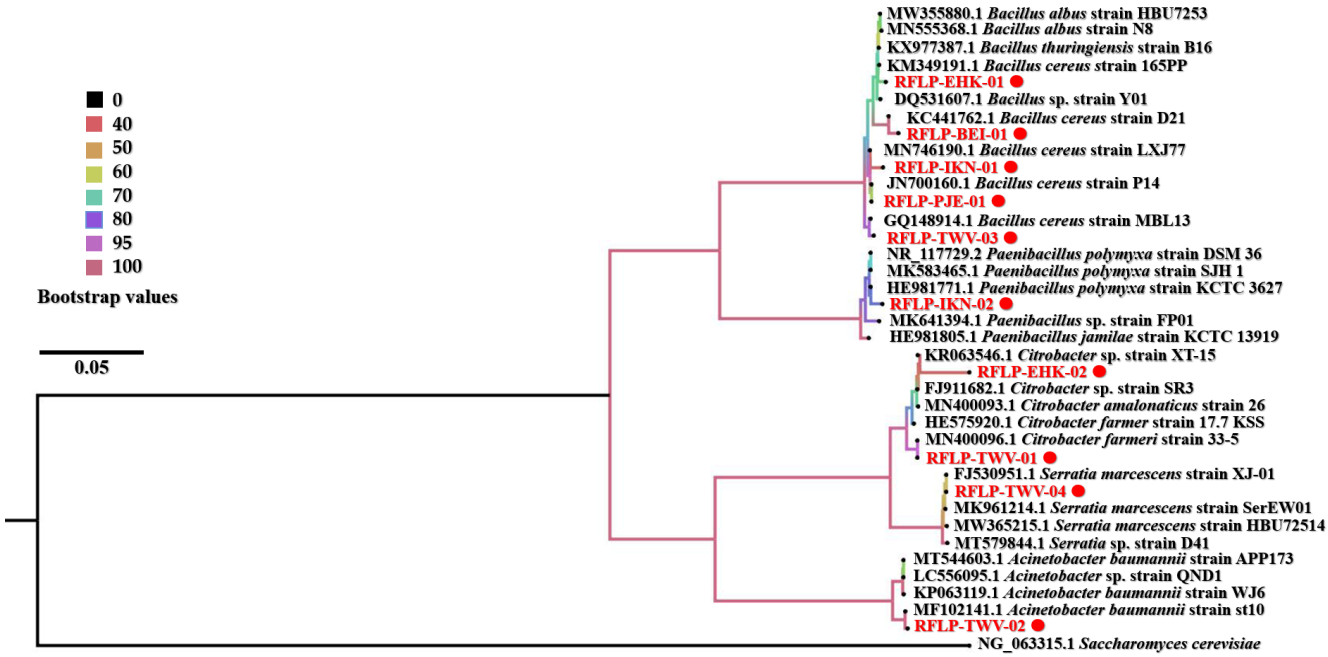


Figure 3. Phylogenetic tree of isolated cellulolytic bacteria using the NJ algorithm with 100,000 bootstrap replications. The phylogenetic tree was generated by SeaView software version 5.0.1 and FigTree software version 1.4.4

This study involved the isolation and screening of the culturable cellulolytic bacteria from the gut of *Odontotermes* higher termites collected from 3 provinces located in the eastern region of Thailand. Noticeably, information about the gut microbial diversity of the genus *Odontotermes* remains inadequate (Makonde et al. 2013). Thirty-two cellulolytic bacteria were isolated and subsequently classified by PCR-RFLP analysis. Ten different RFLP patterns were obtained and the representative bacterium from each RFLP pattern was identified. Result exhibited that 6 species of cellulolytic bacteria from 2 phyla were genetically and phylogenetically identified, including *B. cereus* and *P. polymyxa* of the Phylum Firmicutes, and *A. baumannii*, *Citrobacter* sp., *C. farmeri*, and *S. marcescens* of the Phylum Proteobacteria. According to a previous report on the bacterial diversity in the gut of *Odontotermes* sp. and *O. somaliensis* termites, it was found that 7 bacterial phyla represented such as Actinobacteria, Bacteroidetes, Firmicutes, Planctomycetes, Proteobacteria, Spirochaetes, and Synergistetes (Makonde et al. 2013).

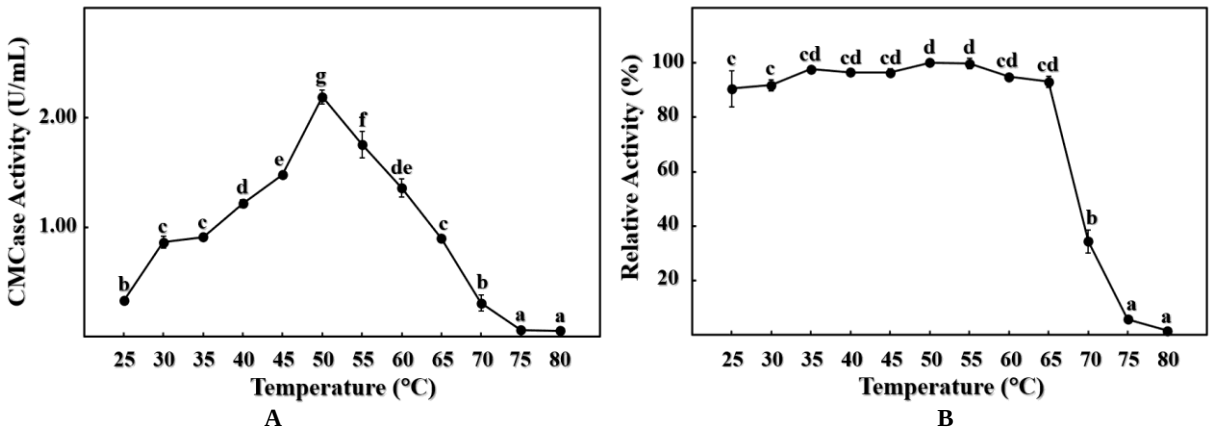


Figure 4. (A) The optimum temperature and (B) thermal stability of CMCase activity from crude cellulases of *B. cereus* strain TWV503. Error bars represent the standard deviation of the triplicates. The mean values followed by the same letter were not significantly different according to Tukey's test ($p < 0.05$) among the CMCase activity

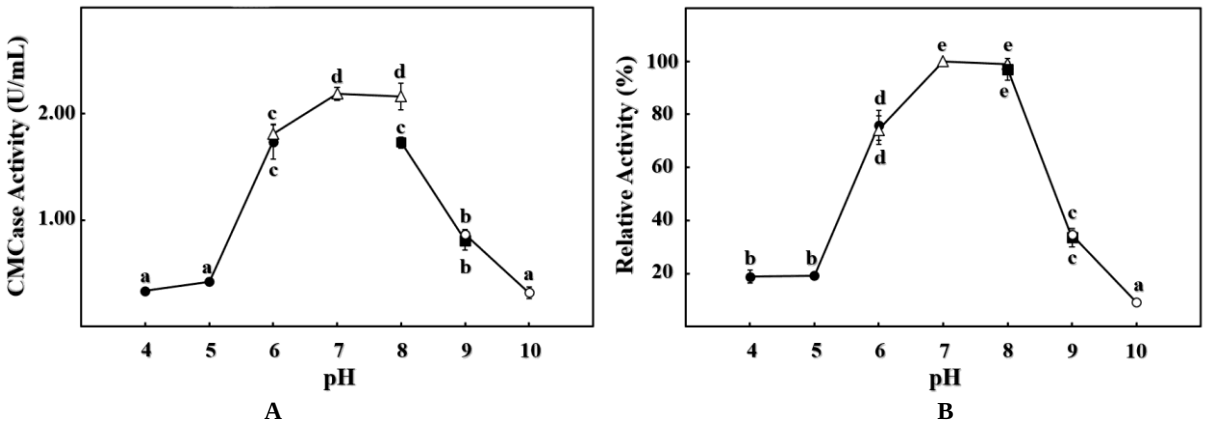


Figure 5. (A) The optimum pH and (B) the pH stability of CMCase activity from crude cellulases of *B. cereus* strain TWV503. CMCase activity was assayed in a citrate buffer (●), sodium phosphate buffer (△), Tris-HCl buffer (■), and glycine-NaOH buffer (○). Error bars represent the standard deviation of the triplicates. The mean values followed by the same letter were not significantly different according to Tukey's test ($p < 0.05$) among the CMCase activity

Bacillus cereus were the predominant cellulolytic bacteria found in the gut of *Odontotermes* termites. In this study, they were isolated from termites at all sampling sites. A previous study on the microbial community in the gut of *O. formosanus* termite collected from Taiwan reported that the predominant bacteria isolated by the culture-dependent method belonged to the genus *Bacillus* of the Phylum Firmicutes (Mathew et al. 2012). A report on the cellulolytic bacteria isolated from the gut of *Odontotermes* termites collected from India showed that *B. cereus* was the dominant cellulase producer (Sreena et al. 2015). In this study, *B. cereus* strain TWV503 was considered as the most effective bacterium based on the HC value obtained from the CMC agar method. It could produce the crude cellulases with satisfactory performance of endoglucanase (CMCase) activity but barely in total cellulase (FPase) activity. The crude cellulases showed the optimum CMCase activity at a moderate temperature of 50°C and stability at up to 70°C. In addition, its optimum pH and pH stability remained in neutral conditions. This differed from the characteristics of *B. cereus* strain ODO2 isolated from the gut of *Odontotermes* termites, which showed optimum activity at the average human body temperature (Sreena et al. 2015). The CMCase activity of *B. cereus* strain TWV503 was related to other *Bacillus* cellulases isolated from different environments (Sadhu and Maiti 2013; Chantarasiri 2015). *Bacillus* species commonly lack significant exoglucanase activity (Wu et al. 2018).

Paenibacillus polymyxa was a cellulolytic bacterium isolated and identified from the gut of termite collected from Prachin Buri Province. It is an endospore-forming bacterium that could colonize a wide range of environments (Padda et al. 2017). A recent study found that *P. polymyxa* was isolated from the gut of a higher termite (*O. hainanensis*) and was identified as a potential cellulase and hemicellulase producer (Pasari et al. 2019). A cellulolytic bacterium isolated from termite gut in Rayong Province was designated as *A. baumannii*. It is a strictly aerobic and non-motile bacterium that is reported as an opportunistic pathogen in immunocompromised individuals

(Howard et al. 2012). *A. baumannii* is one of the culturable cellulolytic bacteria with high activities of cellulases (Karthika et al. 2020). A previous study evidenced that *Acinetobacter* species could be isolated from some higher termites such as *Microcerotermes* termites (Pourramezan et al. 2012). *Citrobacter* sp. and *C. farmeri* were isolated and identified from the termites in the provinces of Rayong and Chachoengsao. The genus *Citrobacter* is a group of aerobic bacteria which widely distributed in the water, soil, food, and intestinal tracts of animals (Metri et al. 2013). *Citrobacter* species are the common symbionts of termite gut that may play an important role in nitrogen metabolism (Muwawa et al. 2016). Some species of *Citrobacter* have been isolated from the higher termites and exhibited cellulolytic activities (Kavitha et al. 2014). Moreover, *Citrobacter* species were responsible for lignocellulolytic and hemicellulolytic digestion, which have been isolated from various insects (Handique et al. 2017). The last *S. marcescens* cellulolytic bacterium was isolated from the termite gut in Rayong Province. It is a human opportunistic pathogen that naturally resides in the soil and water, producing a red pigment at room temperature (Ferreira et al. 2020). It was also reported as a cellulolytic bacterium isolated from a higher termite *O. formosanus* (Kavitha et al. 2014). However, a very recent study revealed that this bacterium had insecticidal properties on a higher termite *O. formosanus* by penetrating the insect body and destroying its digestive tract (Fu et al. 2021).

In conclusion, the gut of a higher termite, *Odontotermes* sp., is a potential source for the isolation of cellulolytic bacteria. There were 5 genera of cellulolytic bacteria isolated from the gut of *Odontotermes* termites in eastern Thailand based on the RFLP-PCR of 16S rRNA gene comprising *Acinetobacter*, *Bacillus*, *Citrobacter*, *Paenibacillus,* and *Serratia*. Cellulolytic *B. cereus* were the predominant bacteria found in the gut of the collected termites. It was revealed that *B. cereus* strain TWV503 was the most active CMCase bacterium. The prominent characteristic of its CMCase was stability under high temperature and neutral pH conditions. Therefore, *B. cereus* cellulolytic bacterium could be used in some agriculture, food, and compost industries. Further study should be done on molecular genetic engineering and enzyme purification.

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