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Research

Elucidating The Lignocellulose Digestion Mechanism *Coptotermes curvignathus* Based on Carbohydrate-Active Enzymes Profile Using The Meta-Transcriptomic Approach

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ABSTRACT

Termites are efficient lignocellulose decomposers that thrive on woody materials and contribute to carbon mineralization in both tropical and subtropical regions. Due to hydrolytic stability and crosslinking between the polysaccharides (cellulose & hemicellulose) and the lignin via ester and ether linkages, termites would require a large variety of enzymes to degrade lignocellulose. *Coptotermes curvignathus*, an endemic species of termite from Southeast Asia, has been classified as an urban pest in the region and is known as the largest and most aggressive among the oriental *Coptotermes* spp. Its Carbohydrate-Active enzymes (CAZymes) are the main interest of this study. RNA of *C. curvignathus* was extracted and sequenced using Illumina Hiseq 2000 sequencing platform, and *de novo* assembled with Trinity pipeline. There were 101 CAZymes families in *C. curvignathus* digestome. CAZymes break down complex carbohydrates and glycoconjugates for a large body of biological roles and perform their function, usually with high specificity. Enzymes coding for glycosyl hydrolase (GH) families had the highest transcript abundance, accounting for about 93% of the total CAZymes reads. This was followed by CBM (≈1%), GT family (≈4%), CE family (<1%), AA family (<2%), and PL family (<1%). Due to the carbohydrate diversity exceeding the number of protein folds, CAZymes have evolved from a limited number of progenitors by acquiring novel specificities at substrate and product levels. Such a dizzying array of substrates and enzymes makes *C. curvignathus* a high-performance lignocellulose degrader.

Key words: Lignocellulose degradation, RNA sequencing, termite gut, wood-feeding termite

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INTRODUCTION

As one of the significant drivers in the global carbon cycle, termites are studied to understand what makes them highly efficient in extracting energy from lignocellulosic materials (Ni & Tokuda, 2013). Lignocellulose, a highly abundant and recalcitrant carbon source, comprises cellulose, hemicellulose, and lignin. Cellulose and hemicellulose are polysaccharides that are a primary structural component of the plant cell wall and are bound together by lignin. The molecular architecture of lignin is heterogeneous, phenolic, aromatic, highly branched, and polydisperse, and makes the entire structure more hydrophobic and resistant to degradation.

Complete biodegradation of lignocellulose requires a combination of multiple enzymes that attack the different moieties of the polymer. This can be completed by termites and their consortium of gut microorganisms that work to co-metabolize lignocellulose. Ke *et al*. (2012) reported that lower wood-feeding termites could accomplish the wood degradation process in hours instead of weeks or months in

a fungal system. Termites can selectively modify and decompose the lignin by ~25% and accomplish a maximized utilization of cellulose at >90% and various hemicellulose components at ~60% (Ke *et al*., 2011).

Coptotermes curvignathus Holmgren, an indigenous lower wood termite commonly found in the Indo-Malayan region, is regarded as one of the most voracious wood-feeding termites in oil palm estates (Chan *et al*., 2011). Their digestive strategies in decomposing lignocellulosic material are of great interest to scientists. This study aims to decipher the lignocellulose degradation mechanisms deployed by *C. curvignathus* through a meta-transcriptomics approach.

MATERIALS AND METHODS

Sample collection and total RNA isolation

Termites were collected from Bintulu, Central Sarawak, Malaysia, and identified as *C. curvignathus* using soldier morphometric measurements based on Tho (1992) and Thapa (1981). The *C. curvignathus* soldier is distinctive in being large as they are the largest among all oriental *Coptotermes* spp. They possess strong incurved mandibles, head length (from the head to the side of the mandible) of 1.45 mm – 1.85 mm, and head width of 1.25 – 1.57 mm.

The digestome of *C. curvignathus*, which included the salivary gland, foregut, midgut, hindgut, rectum, and gut content, was extracted and immediately flash-frozen in liquid nitrogen. Total RNA isolation was performed using Sepasol RNA1 Super G (Nacalai Tesque, Japan) according to the manufacturer protocol with minor modifications where the separation phase using chloroform was repeated twice.

Sequencing and *de novo* assembly and transcript quantifications

The cDNA library and sequencing were constructed using the TruSeq RNA Kit and Illumina Hiseq 2000 sequencing platform (Illumina, USA). Sample libraries were sequenced independently for 100 bp pair-end cycles, and raw sequenced data were pre-processed with the SolexaQA package to obtain high-quality paired-end reads. These reads were concatenated into pool data for *de novo* assembly using the Trinity assembly software (Grabherr *et al*., 2011).

Quantification of the Trinity assembled transcripts and unigenes was estimated using alignment-based methods, RSEM (Li & Dewey, 2011) and alignment-free methods, Kallisto (Bray *et al*., 2016) and Salmon (Patro *et al*., 2017), where the minimum threshold criteria set were all transcripts should have at least one estimated read count in each method to eliminate false positives.

Sequence analysis and CAZymes assignment

All assembled transcripts and unigenes were annotated by searching against the Swissprot (The Uniprot Consortium, 2023) and RefSeq (O'Leary *et al*., 2016) database using BLASTx with an *e*-value of 0.00001, as well as submitted to online search using dBCAN2 for CAZymes annotation where only annotations present in at least two tools were accepted as results (Zhang *et al*., 2018).

RESULTS AND DISCUSSION

Overview of transcriptome profile and carbohydrate active enzymes profile of *Coptotermes curvignathus* digestome

A total of 326011 unigenes were successfully assembled (Table 1), and 714 unigenes were predicted as carbohydrate-active enzymes, namely 435 glycosyl hydrolases (GH), 200 glycosyl transferases (GT), 30 auxiliary activities (AA), 27 carbohydrate-binding modules (CBM), 13 carbohydrate esterases (CE) and ten polysaccharide lyases (PL) (Figure 1). These CAZymes were assigned to 101 CAZymes families, most of which belonged to GH and GT, each with 45 families assigned. Families such as AA, CBM, CE, and PL had 3, 5, 4, and 1 families assigned to them, respectively. Despite having the same number of families assigned to them, GH unigenes accounted for approximately 93% of the total CAZymes reads, indicating their predominant role in the degradation of lignocellulose. However, it should be noted that the resource reservoir for glycosyl hydrolases is far more comprehensive compared to the other CAZymes family, thus leading to the overrepresentation of this family (Drula *et al*., 2022).

Cellulases and hemicellulases

Glycosyl hydrolases were the predominant CAZymes group, with 45 members assigned. Cellulases were predominantly expressed in the digestome of *C. curvignathus* (Figure 2). Among them, the most abundant cellulases included GH9 endoglucanases from host origins, GH5 endoglucanases with host and symbiotic origins, where the host owned had higher enzyme abundance compared to the symbiotic, and GH7 cellobiohydrolases/exoglucanases with symbiotic origins (Table 2). Generally, the host-owned enzymes had a higher abundance compared to the symbiotic. However, the situation for enzyme diversity was different, where the GH family with symbiotic origins owned more diverse enzymes than the host. For instance, GH7 with symbiotic origins had 78 unigenes, while GH9 with host

origins had only 3 unigenes (Supplementary data: Table 1). This situation was similar to other lower termites like *C. formosanus* and *Reticulitermes flavipes*, where members from GH7 and GH5 with protist origins were more diverse than the host-owned cellulases (Tartar *et al*., 2009; Geng *et al*., 2018).

Table 1. Summary of RNA sequencing data used in the construction and *de novo* assembly data *of Coptotermes curvignathus* baseline transcriptome set

Raw data	
Sequence reads	404,346,236
Sequence read length (bp)	40,838,969,836
High-quality filtered data	
Sequence reads	289,378,647
Sequence read length (bp)	23,775,284,769
% Reads	72
% Read length (bp)	58
***De novo* assembly**	
Total “unigenes”	326,011
Total transcripts	373,777
Total transcripts size (bp)	256,761,320
Mean transcript size (bp)	687
Median transcript size (bp)	362
Max transcript size (bp)	14,785
Min transcript size (bp)	200
N50 length (bp)	1,122
Number of 'transcript' (≥1 kb)	61,780
% 'Transcripts’ (≥1 kb)	17
Transcript size (bp) (≥1 kb)	136,263,743
% 'Transcripts size' (≥1 kb)	53
Number of 'transcripts' (≥10 kb)	85
% 'Transcripts’ (≥10 kb)	<1

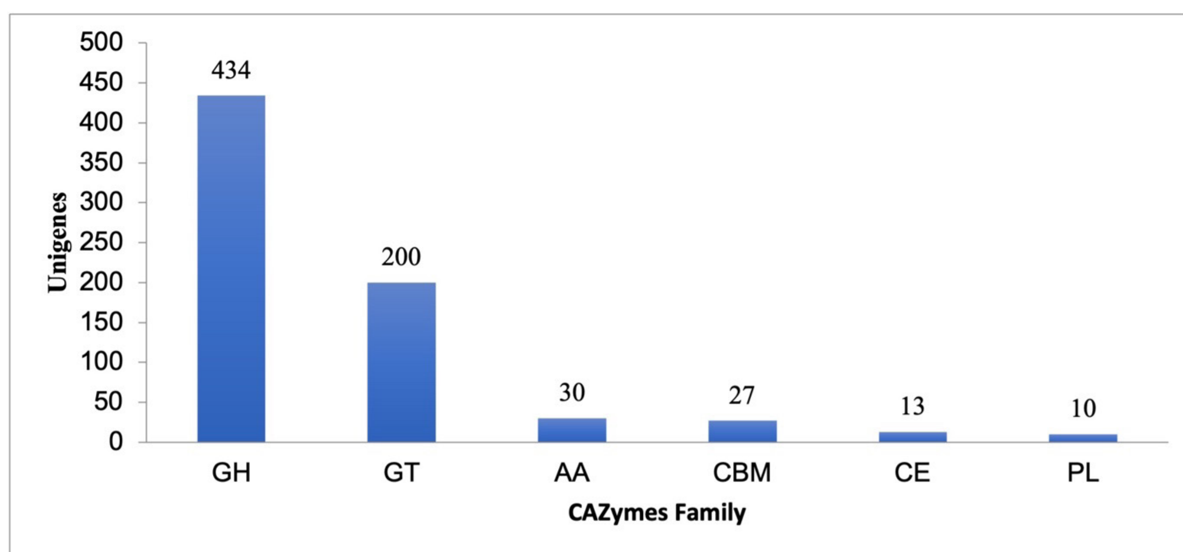

Fig. 1. The number of CAZymes unigenes defined in the transcriptome of *Coptotermes curvignathus* digestome; GH glycosyl hydrolase, GT glycosyl transferase, AA auxiliary activities, CBM carbohydrate-binding module, CE carbohydrate esterase, PL polysaccharide lyase.

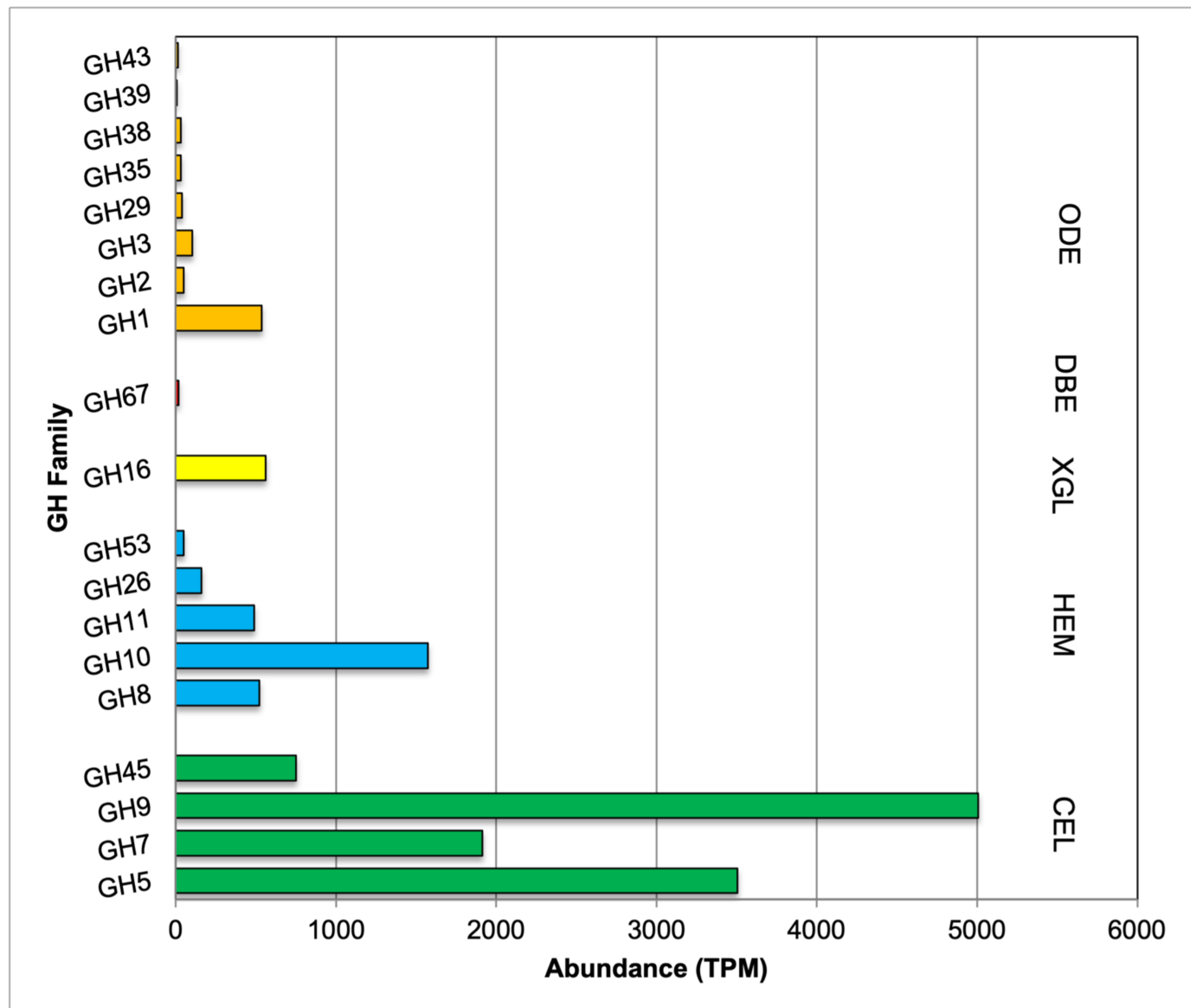

Fig. 2. Inventory of glycosyl hydrolase (GH) families for the degradation of cellulose and hemicellulose in the *Coptotermes curvignathus* digestome; CEL cellulases, HEM hemicellulases, XGL xyloglucanases, DBE debranching enzymes and ODE oligosaccharide degrading enzymes.

All the endoglucanases had putative endohydrolysis activity (EC 3.2.1.4) randomly cleft the (1,4)-β-D-glucosidic linkage in the cellulose chain. At the same time, exo-acting enzymes such as cellobiohydrolases and exoglucanases digest the cellulose chain from the chain ends (Merino & Cherry, 2007). The predominant presence of these cellulases indicated that they are the core enzymes of cellulose degradation. This can be attributed to the aggressiveness of *C. curvignathus*, attacking and consuming a vast amount of cellulolytic materials, as observed in *C. formosanus* (Tokuda *et al*., 2004).

Except for GH9 endoglucanases, enzymes from the other three GH families (GH5, GH7 & GH45) may have other specific substrate digestion abilities. For instance, in addition to their (1,4)-β-D-glucosidic linkage hydrolysis (EC 3.2.1.4) ability, GH7 cellobiohydrolases/exoglucanases may also possess other digestion abilities, such as hydrolysis of (1,4)-linkage between glucosamine residues in partly acetylate chitosan (EC 3.2.1.132) and hydrolysis of (1,4)-β-D-glucosidic linkage in cellulose and similar substrates to release cellobiose from the reducing end of the chains (EC 3.2.1.176). The GH7 was also reported to be active against soluble synthetic substrates such as 4-methylumbelliferyl-β-D-cellobioside, 4-nitrophenyl-β-D-cellobioside, and 4-nitrophenyl-β-D-lactoside (Woon *et al*. 2017). These specific abilities allowed a different type of cellulose or similar substrate to be digested, and the termite became highly adaptable in their cellulose degradation ability.

The dominance of cellulases in termite digestion indicated their importance in lignocellulose degradation. However, more than this strategy is needed to efficiently digest the lignocellulose materials from the wood. The main diet of *C. curvignathus* is wood, composed of about 45% cellulose, 25% hemicellulose, 25% lignin, and other minor components like proteins, lipids, pectin, soluble sugar, and minerals (Rowell *et al*., 2012). The cellulose is tightly packed in a hetero lignin-hemicellulose matrix making it less accessible (Chandra *et al*., 2007).

In the digestome of *C. curvignathus*, hemicellulases were less predominant compared to cellulases. They are the second predominant group with five members, namely GH10, GH8, GH11,

GH26, and GH53 (Supplementary data, Table 1). These GH families all have symbiotic origins. Due to its abundance, GH10 xylanases with symbiotic origins were the core hemicellulase family, and their presence was more prominent compared to the other four members. Xylanases from GH10 and GH11 are the two most critical enzymes in the xylan hydrolysis system. They are crucial for initiating xylan degradation to xylose and xylo-oligosaccharide (Brennan *et al*., 2004; Honda & Kitaoka, 2004).

Table 2. Predominant putative cellulase unigenes from the digestome of *Coptotermes curvignathus*

Cellulases	TPM	Isoform	Putative enzyme	EC	Origin	Substrate
GH5						
TR72270|c1_g1	976	1	endoglucanase	3.2.1.4	B	ß-glucan
TR56928|c0_g1	720	1	endoglucanase	3.2.1.4	B	ß-glucan ß-mannan
TR165714|c0_g1	395	1	endoglucanase	3.2.1.4	B	ß-glucan
TR38408|c0_g1	307	1	endoglucanase	3.2.1.4	B	ß-glucan ß-mannan
TR160946|c0_g1	198	1	endoglucanase	3.2.1.4	B	ß-glucan ß-mannan
TR39174|c1_g1	165	1	ß-mannosidase ß-mannanase ß-glucanohydrolase exocellulase	3.2.1.25 3.2.1.78 3.2.1.73 3.2.1.74	P	ß-glucan ß-mannan cellulose
TR121262|c0_g1	113	1	endoglucanase	3.2.1.4	P	ß-glucan ß-mannan
TR132589|c0_g1	162	2	endoglucanase	3.2.1.4	P	ß-glucan ß-mannan
GH7						
TR196451|c1_g3	511	1	exoglucanase	3.2.1.176 3.2.1.132 3.2.1.4	P	cellulose chitosan
TR62136|c1_g1	321	1	cellobiohydrolase	3.2.1.4	P	cellulose chitosan
TR146211|c2_g2	170	2	cellobiohydrolase	3.2.1.4	P	cellulose
GH9						
TR84441|c1_g1	2586	4	endoglucanase	3.2.1.4	H	cellulose
TR84441|c0_g1	2417	3	endoglucanase	3.2.1.4	H	cellulose
GH45						
TR244178|c0_g2	256	1	endoglucanase	3.2.1.4 3.2.1.78	P	ß-glucan ß-mannan
TR193090|c0_g6	116	1	endoglucanase	3.2.1.4 3.2.1.78	P	ß-glucan ß-mannan
TR244178|c0_g4	113	1	endoglucanase	3.2.1.4 3.2.1.78	P	ß-glucan ß-mannan
TR244178|c0_g1	103	1	endoglucanase	3.2.1.4 3.2.1.78	P	ß-glucan ß-mannan

[#] TPM Transcript per million, EC Enzyme commission, H Host, P Protist, B Prokaryote

Both GH10 and GH11 xylanases had symbiotic origins with similar predicted 1,4-ß-D xylosidic linkage hydrolysis activity (EC 3.2.1.8) towards xylan since their sequences are phylogenetically near (Davies & Sinnott, 2008; Busk & Lange, 2013). GH10 xylanases with four unigenes have a higher abundance, but GH11 xylanases with seven unigenes owned more diverse enzymes (Table 3). This differed from several Rhinotermids (*C. formosanus*, *R. flavipes*, & *R. speratus)* metatranscriptomic studies where GH11 xylanases were more abundant instead (Todaka *et al*., 2007; Tartar *et al*., 2009; Xie *et al*., 2012). Generally, GH10 xylanases have higher accessibility toward the xylan backbone and can yield smaller xylan fragments than GH11 xylanases (Beaugrand *et al*., 2004; Hu & Saddler, 2018; Yagi *et al*., 2019). Nevertheless, this does not discount the supporting role of GH11 xylanases are

reported to be more efficient in catalyzing xylan degradation during interaction with cellulose and lignin (Yagi *et al*., 2019).

Another exciting group of xylanases is the two GH8 unigenes with putative-reducing end xylose-releasing exo-oligoxylanase activity (EC 3.2.1.156). The uniqueness of this group of xylanases is their specificity and abilities, such as to hydrolyze xylooligosaccharide but not xylan, releasing xylose from the reducing end of a xylooligosaccharide in an exo-splitting manner strictly recognizes the beta-anomeric hydroxyl group at the reducing end of the substrate (Honda & Kitaoka, 2004), which allow them to enhance the degradation of hemicellulose.

Generally, hemicellulose is easier to degrade enzymatically than cellulose. They have oligomeric structures with complex branching and acetylation patterns that make the whole structure recalcitrant (Agger *et al*., 2010). Thus, the degradation of hemicellulose required the aid of other less prominent enzymes such as GH26 ß-mannanase (EC 3.2.1.78), GH53 arabino endo-1,4-ß-galactosidase (EC 3.2.1.89), GH67 and GH115 α-glucuronidase (EC 3.2.1.131, EC 3.2.1.139) for efficient hemicellulose degradation. With side chains removed by α-arabinofuranosidases and α-glucuronidases, the reducing and non-reducing end of the xylan can be further hydrolyzed by GH8 oligoxylanases and GH3 ß-xylosidases.

Table 3. Predominant putative hemicellulase unigenes from the digestome of *Coptotermes curvignathus*

Hemicellulases	TPM	Isoform	Putative enzyme	EC	Origin	Substrate
GH8						
TR205556|c0_g1	245	1	oligoxylanase	3.2.1.156	B	xylan
TR47166|c0_g1	277	1	oligoxylanase	3.2.1.156	B	xylan
GH10						
TR112761|c0_g1	559	1	xylanase	3.2.1.8	P	xylan
TR230604|c0_g1	1009	1	xylanase	3.2.1.8	P	xylan
GH11						
TR105252|c0_g1	348	1	xylanase	3.2.1.8	P	xylan
TR181061|c0_g1	125	1	xylanase	3.2.1.8	P	xylan

[#] TPM Transcript per million, EC Enzyme commission, H Host, P Protist, B Prokaryote

Compared to cellulases and hemicellulases, degrading oligosaccharide enzymes, debranching enzymes, and xyloglucanases are less abundant (Table 4; Supplementary Data, Table 1). The GH16 xyloglucanases had putative ß-1,3-glucanase activity (EC 3.2.1.39) that facilitated the hydrolysis of (1,3)-ß-D-glucosidic linkages in the (1,3)-ß-D-glucans. They aided in the depolymerization of cellulose-like backbone that carries xylose and galactosyl-xylose substituents that the symbiotics can further digest in the termite gut (Baumann *et al*., 2007).

Table 4. Predominant putative oligosaccharide degrading enzyme and xyloglucanase unigenes from the digestome of *Coptotermes curvignathus*

GH family	TPM	Isoform	Putative enzyme	EC	Origin	Substrate
Oligosaccharide degrading enzyme						
GH1						
TR36603|c0_g1	446	1	ß-glucosidase	3.2.1.21	H	ß-glucan
				3.2.1.147		polyphenol
				3.2.1.37		xylan
				3.2.1.38		ß-fucoside
Xyloglucanases						
GH16						
TR81201|c0_g2	380	1	ß-glucanase	3.2.1.39	H	ß-glucan

[#] TPM Transcript per million, EC Enzyme commission, H Host, P Protist, B Prokaryote

Meanwhile, host-owned debranching enzymes such as GH1 ß-glucosidase (EC 3.2.1.21) played a crucial role in the breakdown of cellulose fragments to release the ß-D-glucose in the foregut and allowed the glucose units to be absorbed in the termite midgut. The remaining cellulose fragments are further depolymerized in the hindgut of the termite by the symbiotics (Tartar *et al*., 2009; Geng *et al*., 2018). These GH1 ß-glucosidase are also predicted to have putative minor activities such as

thioglucosidase (EC 3.2.1.147), ß-xylosidase (EC 3.2.1.37) and ß-fucosidase (EC 3.2.1.38). These abilities can allow the termite to break down components of the lignocellulose matrix and absorb the simple sugar.

Lignin and auxiliary activities components

The lignin component is the rate-limiting component of complete lignocellulose degradation for both natural and industrial processes (Veluchamy & Kalamdhad, 2017; Zhao *et al*., 2022). Many organisms avoid degrading the lignin and change its properties to access the cellulose inside, which is assumably more energy-conserving.

This wood-feeding termite also deployed this strategy from the metatranscriptomic analysis on the *C. curvignathus* digestome. Lignin modification enzymes such as AA1 laccases (EC 1.10.3.2), lignin-degrading auxiliary enzymes like AA3 glucose dehydrogenases and AA15 lytic polysaccharide monooxygenase (LMPO) (EC 1.14.99.53, EC 1.14.99.54), were found in the termite digestome (Table 5). All these putative enzymes had host origins. *C. curvignathus* expressed a variety of laccase genes that were closely related to other termites but distantly related to those of fungi or bacteria phylogenetically (Hoe *et al*. 2019).

Table 5. Inventory of auxiliary activities (AA) enzyme unigenes from the digestome of *Coptotermes curvignathus*

AA	Putative enzyme	EC	Substrate	Origin	Unigene	Isoform	TPM
AA1	Laccase	1.10.3.2	lignin	Host	8	10	36
AA3	Glucose dehydrogenase (FAD, quinone)	NA	NA	Host	17	20	203
AA15	Lytic polysaccharide monooxygenase	1.14.99.53, 1.14.99.54	chitin, cellulose	Host	5	5	104
Total					30	35	343

[#] TPM Transcript per million, EC Enzyme commission

These auxiliary activities of redox enzymes attack the bonds of the lignin structure of the lignocellulose materials that are not accessible by other enzymes and modify the structure to enhance the accessibility of cellulolytic and hemicellulolytic enzymes to the cellulose and hemicellulose. This conforms to findings by Ke *et al*. (2011), which reported that much of the lignin consumed by termites remained undigested and excreted through their feces.

Deciphering the lignocellulose degradation mechanisms in *Coptotermes curvignathus* digestome

From the metatranscriptomic analysis and CAZymes profile, the wood-feeding termite, *C. curvignathus,* adopted several strategies for efficient lignocellulose degradation (Figure 3). The process began when the termite feed on the lignocellulose materials. The chewing process breaks down the lignocellulose materials into smaller lignocellulose fragments (Fujita *et al*., 2010). Multiple enzymes forming an enzyme cocktail of endoglucanases, xyloglucanases, ß-glucosidase, laccases, and lytic polysaccharide monooxygenase are secreted to break the lignocellulose matrix and enzymatically digest the fragments. The goal here is to degrade as a breakdown and modify the lignocellulose matrix to release as much free cellulose as possible, where they are quickly digested into simple glucose units. The termite would have absorbed these accessible glucose units before they reached the hindgut where the symbiotics reside (Fujita *et al*., 2010).

Upon reaching the hindgut, the role of host-owned enzymes was likely reduced, and the lignocellulose degradation process was passed on to the symbionts (Tartar *et al*., 2009; Fujita *et al*., 2010). Here remaining cellulose predominantly consists of crystalline cellulose, and other lignocellulose components such as xylan, mannan, arabinan, galactan, and other disaccharides were further degraded and utilized, thus completing the lignocellulose degradation process. The proposed mechanisms shed light on the intricate processes involved in lignocellulose digestion, and this finding represents a significant contribution to our understanding of the biology of *C. curvignathus,* and the bioconversion of lignocellulosic materials. The digestome of this termite species harbor a reservoir of lignocellulose-degrading enzymes that can open up exciting possibilities for applications in many biotechnology and bioenergy industries.

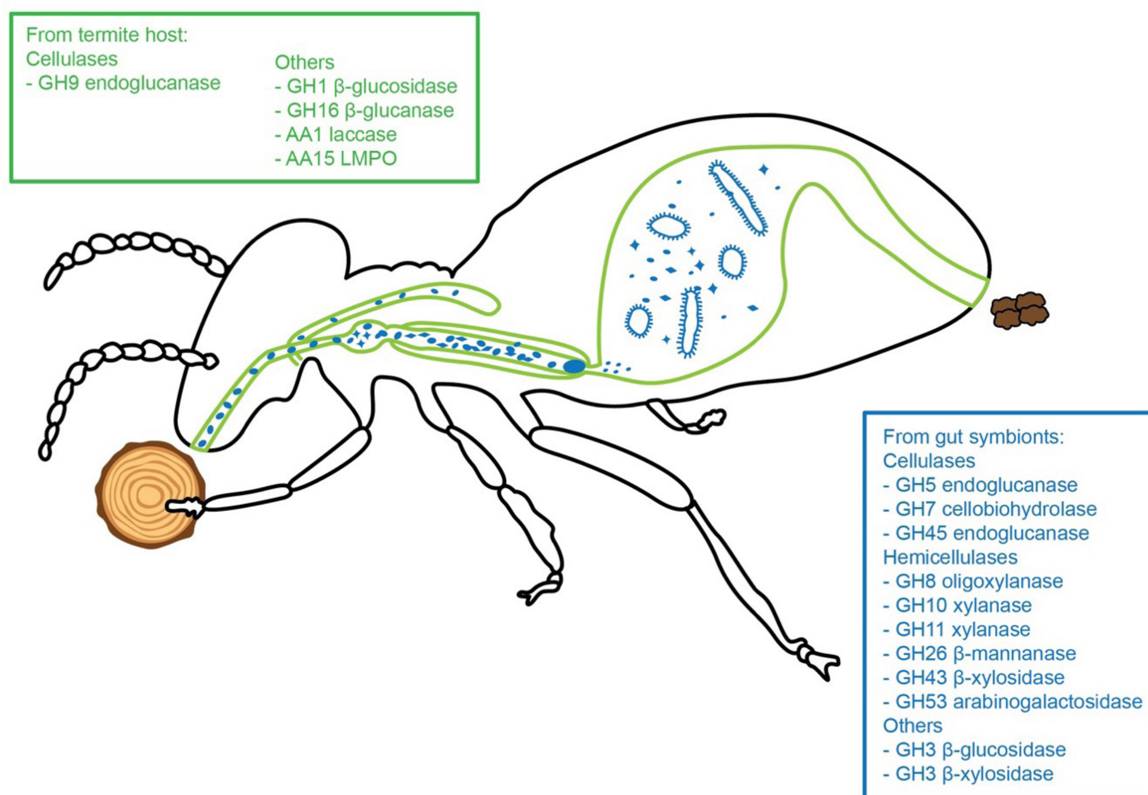

Fig. 3. Key enzymes and proposed mechanism for lignocellulose degradation by wood-feeding termite, *Coptotermes curvignathus*.

CONCLUSION

This study found that the main classes of enzymes used by *C. curvignathus* to accomplish lignocellulosic degradation include carbohydrate-active enzymes (CAZymes) and various redox enzymes that were owned by the host and protists. The host played a major role in initiating the lignocellulose enzymatic degradation process secreting redox enzymes to modify the lignin and large amount of endoglucanases to initiate the cellulose hydrolysis process. More enzymatic reactions to digest and absorb the remaining cellulose and hemicellulose that occurred in the hindgut were contributed by the symbionts to achieve maximum efficiency in lignocellulose degradation. The tactic of deploying multi-enzyme cocktails used by *C. curvignathus* could be one strategy being explored for future industrial processing. Further studies on the termite gut compartment and characterization of their specific enzymes can unveil thrilling prospects for utilization across various biotechnology and bioenergy sectors.

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ETHICAL STATEMENT

Not Applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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