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ORIGINAL ARTICLE

In-situ oxygen profiling and lignin modification in guts of wood-feeding termites

Jing Ke[1], Jian-Zhong Sun[2,3], Hung D. Nguyen[4], Deepak Singh[1], Karmen C. Lee[2], Haluk Beyenal[4] and Shu-Lin Chen[1]

[1]*Department of Biological Systems Engineering, Washington State University, Pullman, Washington,* [2]*Coastal Research and Extension Center, Mississippi State University, Poplarville, Mississippi, USA,* [3]*School of the Environment, Jiangsu University, Zhenjiang, Jiangsu Province, China,* [4]*The Gene and Linda Voiland School of Chemical Engineering and Bioengineering, Washington State University, Pullman, Washington, USA*

Abstract Reports on the capability of wood-feeding termites (WFTs) in degrading wood particles and on the existence of aerobic environment in the localized guts suggest that their high efficiency of cellulose utilization is not only caused by cellulase, but also by biochemical factors that pretreat lignin. We thus extend the hypothesis that for highly efficient accessibility of cellulose, there should be direct evidence of lignin modification before the hindgut. The lignin degradation/modification is facilitated by the oxygenated environment in intestinal microhabitats. To test our hypothesis, we conducted experiments using a dissolved oxygen microelectrode with a tip diameter < 10 μm to measure oxygen profiles in intestinal microhabitats of both *Coptotermes formosanus* (Shiraki) and *Reticulitermes flavipes* (Kollar). Lignin modification during passage through their three gut segments was also analyzed with pyrolysis gas chromatography/mass spectrometry. The data showed relatively high levels of oxygen in the midgut that could have promoted lignin oxidation. Consistent with the oxygen measurements, lignin modifications were also detected. In support of previously proposed hypotheses, these results demonstrate that lignin disruption, which pretreats wood for cellulose utilization, is initiated in the foregut, and continues in the midgut in both termites.

Key words *Coptotermes formosanus*, lignin modification, oxygen profile, pretreatment, *Reticulitermes flavipes*, wood-feeding termites (WFTs)

Introduction

Lignin, the second most abundant organic polymer in nature, is covalently linked to hemicellulose and crosslinks different plant polysaccharides to confer mechanical strength to plant cell walls and protect other structures from being damaged by external factors (Boerjan *et al.*, 2003; Chabannes *et al.*, 2001). On the other hand, lignin is heterogeneously and irregularly constructed of cross-linked phenylpropanoid monomers of cinnamyl alcohol derivatives (Goodell *et al.*, 2003). The phenylpropanoid polymer resists chemical, enzymatic and microbial degradation (Tuomela *et al.*, 2000), and is covalently linked at various points with hemicellulose to form a matrix surrounding the orderly cellulose microfibrils (Jeffries, 1990). This arrangement results in resistance to saccharification, thus severely limiting biochemical conversion of lignocellulosic biomass to ethanol (Chen & Dixon, 2007) and other biofuels. Realization of commercial

Correspondence: Shu-Lin Chen, Department of Biological Systems Engineering, PO Box 646120, Washington State University, Pullman, WA 99164-6120, USA. Tel: +1 509 335 3743; fax: +1 509 335 2722; email: chens@wsu.edu

Jian-Zhong Sun, School of the Environment, Jiangsu University, Zhenjiang, 212013, China. Tel: +86 0511 88796122; e-mail: jzsun1002@hotmail.com

success for lignocellulosic ethanol production will require advanced pretreatment methods that efficiently remove protective lignin from cellulose (Mosier *et al.*, 2005). Current chemical and physical pretreatment methods suffer from high costs and undesirable environmental impacts. Alternative biological pretreatments, such as that by white- and brown-rot fungi are often too slow to be economically feasible (Filley *et al.*, 2000; Martinez *et al.*, 2008). However, lignocellulose-feeding insects, especially wood-feeding termites (WFTs), have proven to be a promising model organism for biological pretreatment of biomass due to their highly efficient digestion of wood (65%–99% wood-cellulose and hemicellulose utilization within 24 h) under natural conditions (Esenther & Kirk, 1974; Wood, 1978; Brune, 2007). The plant cell wall degradation processes in such systems may provide another potential alternative for biomass processing.

For a long time, little has been revealed about lignin modification in termites, but it has been thought that host-derived enzymes and/or a consortium of micro-organisms may be involved in degradation (Scharf & Tartar, 2008). Recently, WFTs' high efficiency in wood digestion has attracted researchers' attentions (Sun & Zhou, 2010); however, most research has focused on the cellulase system. To fully understand wood degradation in termites, the pretreatment system must also be addressed. Kyou *et al.* (1996) pointed out that wood lignin plays a role in maintenance of health in workers of *Coptotermes formosanus* Shiraki. French and Bland (1975) inferred lignolysis for *Coptotermies lacteus* and *Nasutitermies exitiosus* by measuring incorporation of ^{14}C-label into tissues of termite fed wood previously grown with [3-^{14}C] cinnamic acid. Butler and Buckerfield (1979) reported that lignin degradation occurs within termites' bodies, and not in voided fecal pellets, although the site of degradation was not identified, and the relevance of gut organisms in the process was not addressed (Breznak, 1982). Katsumata *et al.* (2007) suggested there were some changes in the structural features of lignin during digestion by termites. Later, Geib *et al.* (2008) proved that there were significant levels of propyl side-chain oxidation (depolymerization), demethylation of ring methoxyl group, and ring hydroxylation on lignin after passage through the gut of a Pacific dampwood termite (*Zootermopsis angusticollis*). The author demonstrated the lignin degradation/modification process occurs in the whole gut environment. Scharf and Tartar (2008) summarized that significant lignin degradation, which should be the first step in the process of lignocellulose degradation, can take place in the termite gut; and Tartar *et al.* (2009) recently confirmed this statement by identifying lignase gene candidates in the *Reticulitermes* gut. In all likelihood, the success of WFTs in wood-cellulose digestion can not only be attributed to cellulase, but also to pretreatment factors that modify lignin and increase accessibility of wood-cellulose. Lignin degradation by WFTs is intriguing but may be ambiguous in some way, because the termite gut used to be considered as anaerobic on the basis of presence of strictly anaerobic micro-organisms in termite hindgut (Eutick *et al.*, 1976; Breznak, 1982; Katsumata *et al.*, 2007), but high oxygen tension is required for lignin degradation (Ten Have & Teunissen, 2001; Scharf & Tartar, 2008). In an anaerobic environment, polymeric lignin will not be degraded, and wood may persist in non-degraded form for several hundreds or thousands years (Blanchette, 1995). The presence of molecular oxygen is a prerequisite for the initiation of lignin depolymerization since oxygen acts as a co-substrate in the modification/disruption of lignin by oxidative enzymes (Breznak & Brune, 1994; Scharf & Tartar, 2008). There is no conclusive evidence that the inter-monomeric linkages in insoluble, highly polymeric lignins are attacked in the absence of oxygen (Colberg, 1988; Young & Frazer, 1987).

For this study, we hypothesized that WFTs have evolved exactly the right distribution of oxygen in gut microhabitats to facilitate a process of enzymatic modification on less recalcitrant lignin linkage, and thus increase accessibility of cellulose. To test the hypothesis, we studied two species of lower subterranean WFTs within the same family of Rhinotermitidae, *Coptotermes formosanus* (Shiraki) and *Reticulitermes flavipes* (Kollar). These two WFTs have been the subjects of much research on termite control due to their highly efficient digestion capability of cellulose, assisted by the cellulolytic protozoa in the hindgut (Tokuda *et al.*, 1999; Shelton & Grace, 2003). Of these two well-known termite species, *C. formosanus*, just possesses three species of cellulolytic protozoa for cellulose digestion (Yoshimura *et al.*, 1993; Hu & Zhu, 2003); while the other one, eastern subterranean termite, *R. flavipes*, holds more than 10 types of cellulolytic protozoa to assist wood degradation. These two termite genera have world-wide distribution and are important structural pests. Thus, we exploited the advances in microelectrode technology (Revsbech & Jørgensen, 1986; Revsbech, 1989; Gross *et al.*, 2008) to obtain oxygen profiles of the guts from these two well-known termite species, and investigated where the lignin oxidation might occur. We then correlated these oxygen profiles with actual lignin modification during passage through WFT gut segments measured with pyrolysis gas chromatography/mass spectrometry (Py-GC/MS).

Materials and methods

Sample collection and preparation

Two species of WFTs, *C. formosanus* and *R. flavipes*, collected in Poplarville, Mississippi, were fed at 28°C, 90% humidity on 6.8 × 1.5 × 0.5 inches of Southern pine (*Pinus australis* F. Michx) blocks, the lignin of which consists almost exclusively of guaiacyl propane subunits.

Isolation of termite guts was performed under a dissection microscope. The body wall of each termite was opened and pulled aside to expose the intact gut. Then, the gut was extended to allow identification of different segments.

In-situ *measurements of oxygen concentration profiles in termite gut*

Construction of microelectrode A dissolved oxygen microelectrode (DOM) is an amperometric device in which oxygen diffuses through a silicone rubber membrane, arrives at a cathodically polarized working electrode, and is reduced to water (Fig. 1). The device uses an Ag/AgCl half-cell as the counter-electrode and a noble metal of gold as the working electrode. Applying −0.8 V typically satisfies the limiting current condition, and the measured current is proportional to the dissolved oxygen concentration in the vicinity of the sensor tip (Lewandowski & Beyenal, 2007). Construction of the dissolved oxygen microelectrode consisted of the following steps: (i) making an outer case; (ii) opening the tip of the outer case; (iii) applying a silicone rubber membrane; (iv) etching the Pt wire to a tip with a diameter of a few micrometers; (v) making a shaft using Corning 8161 glass; (vi) sealing the Pt wire in the shaft; (vii) recessing the tip of the shaft; (viii) electroplating the tip of the Pt wire with gold; (ix) making the electrical connections; (x) preparing the Ag/AgCl reference electrode; (xi) assembling all of the components; (xii) filling the outer case with the electrolyte; and (xiii) calibrating the microelectrode. The entire process of the construction is given in detailed in Lewandowski & Beyenal (2007).

Measurement of oxygen profiles Each oxygen profile measurement was replicated three times using three different worker termite guts for each termite species. Each termite gut sample was used for just one measurement to prevent the diffusion of oxygen caused by gut wall damage by the tip of the microsensor. After each dissection, the isolated termite guts were placed on a dissection plate and quickly coated with a thin layer of 1% agarose in

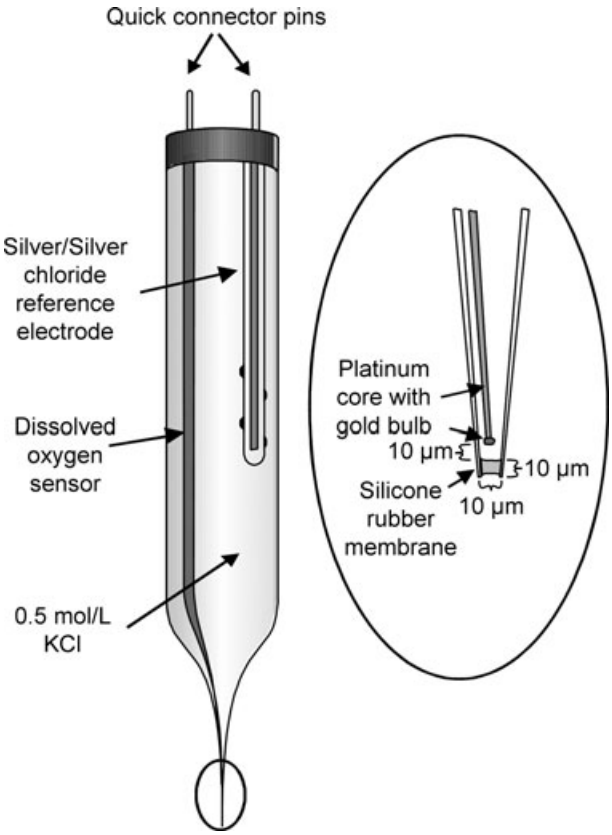


Fig. 1 Dissolved oxygen microelectrode (DOM). Reproduced from Lewandowski and Beyenal (2007).

Ringer's solution to fix the gut and restrict the diffusion of oxygen for the conservation of the oxygen state in the gut. The small amount of 65°C low melting point agarose was used in an effort to avoid the gut wall and hindgut symbiotic microbiota damage. During the measurements, the isolated gut incubated in the argrose retained normal muscular activity (peristalsis) for at least half an hour (Brune *et al.*, 1995a). All readings were completed within 10 min of dissection. We isolated the intact gut, thus acquiring the opportunity to study the foregut, which is a location of host phenol-oxidizing laccases (Tartar *et al.*, 2009).

The DOM had a tip diameter $< 10\ \mu$m, response times of 1–3 s, and stirring sensitivities of 0–1%. Prior to use, the microelectrodes were calibrated using two-point calibration: in the air (oxygen saturation) and in saturated Na_2SO_3 solution (zero oxygen concentration). The working electrode was polarized by applying −0.8 V against Ag/AgCl electrode using a picoammeter (HP 4140B). To determine the spatial distribution of oxygen, one point of penetration was measured in the foregut, and three points each in the midgut and hindgut (paunch and colon). The

scan of oxygen concentrations during each penetration began from a little above the surface of the intestinal wall, continued through the gut, and ended with emergence of the DOM on the opposite side of the agarose layer. Microelectrode movements were controlled by a mercury-step stepper motor (PI M-230.10S) with 10 ± 0.04 μm step size using custom-made software Microprofiler® (The Gene and Voilland School of Chemical Engineering, Washington State University, Pullman, WA, USA). Data was recorded in the computer using a data logger (Measurement COMPUTING(tm) USB-1608FS: Measurement Computing Corporation, Norton, MA, USA). All measurements were carried out at ambient temperature (28°C).

Pyrolysis and GC/MS analysis of lignin modification during passage through different gut segments of termites

Modifications of lignin during passage through each gut segment were determined from lignin chemical structure. Pyrolysis gas chromatography/mass spectrometry (Py-GC/MS) was used for this purpose. Py-GC/MS is a rapid and highly sensitive method for characterizing the chemical structure of lignin, which allows the analysis of very small amounts of sample without prior manipulation and isolation (Río *et al.*, 2004). Pyrolysis of pinewood lignin yields a range of products, of which the most characteristic are those solely based on guaiacol and its derivatives. Although there is plenty of information on the analytical pyrolysis of different wood types (Yokoi *et al.*, 2001; Schwarzinger *et al.*, 2008), reports on biologically modified wood are scarce.

The wood particle samples used in this study were obtained from different gut segments: foregut, midgut and hindgut, separated by scalpel; each piece of feces was collected at the anus of the termite before each dissection. Three foreguts, three midguts, two hindguts, and three pieces of feces were used for each sample, respectively. Sizes of the three gut segments are scaled. The guts of termites that fed on filter paper (*C. formosanus*) or mixture of α-cellulose and avicel (*R. flavipes*) for 1 month were employed as lignin-free guts. The powder of undigested pinewood was employed as control. Samples were quickly frozen in liquid N_2 to halt lignin digestion, and then put directly into a quartz tube (sample tube). The pyrolysis processes were performed with a CDS 5000 pyrolysis autosampler (CDS Analytical, Inc., Oxford, PA, USA) attached to a Thermo Trace GC 6890N/MSD 5975B gas chromatography/mass spectrometry system Agilent Technologies, Inc., Bellevue, WA, USA). The samples were first pretreated at 210°C for 3 min, and then pyrolyzed at a temperature of 500°C for 1 min, and finally kept in the pyrolysis zone for 56 min. The volatile products were separated on a 30 m × 0.25 μm inner diameter (5% phenyl)-methylpolysiloxane non-polar column with helium 4.6 as carrier gas (17.3 mL/min) and identified by interpretation of their electron-impact (EI) mass spectra and comparison to National Institute of Standards and Technology (NIST) Mass Spectral (MS) Search 2.0 electronic libraries. The pyrolysis interface was kept at 210°C, the GC/MS interface at 280°C; the GC/MS was programmed from 40°C (1 min) to 280°C (15 min) at a rate of 6°C/min. The mass spectrometer was operated in EI mode (70 eV) at a source temperature of 230°C.

Results

Distribution of oxygen in termite gut

Oxygen concentrations in each region of the termite guts measured demonstrates that the morpho-anatomical differentiation of the intestinal tract corresponds to different oxygen concentrations, which will most likely create different physicochemical microenvironments.

Radial distribution of oxygen in different segments of termite guts Figures 2 and 3 show radial oxygen concentration profiles with three times replication in three different worker termite guts for each species in two wood-feeding termites (WFTs) *C. formosanus* and *R. flavipes*. However, the data are just based on a single replicate since the measurement started from an arbitrary selected location near the gut surface and the distance of the microelectrode tip could not be quantize unified. The oxygen concentration profiles were asymmetric for both cases showing penetration of the tip of the microelectrode to agar/gut/agar, which might be because of the different thicknesses and compositions of the agar above and below to lead to different degrees of oxygen penetration to the gut. All the measured profiles clearly demonstrated that the microhabitats were aerobic, which is in accordance with the earlier result of Brune *et al.* (1995a); but because we placed a thin layer of agar above the gut to mimic termite body instead of their microchamber for normal oxygen penetration, this likely resulted in the average oxygen concentration being higher than the result previously published (Brune *et al.*, 1995a). Compared to Brune *et al.* (1995a), our microsensor was apparently more advanced in tip sensitivity to be able to give precise oxygen distribution along the whole gut, including foregut, to better reveal the lignin oxidation situation in each segment. The gut segments also showed different oxygen gradients

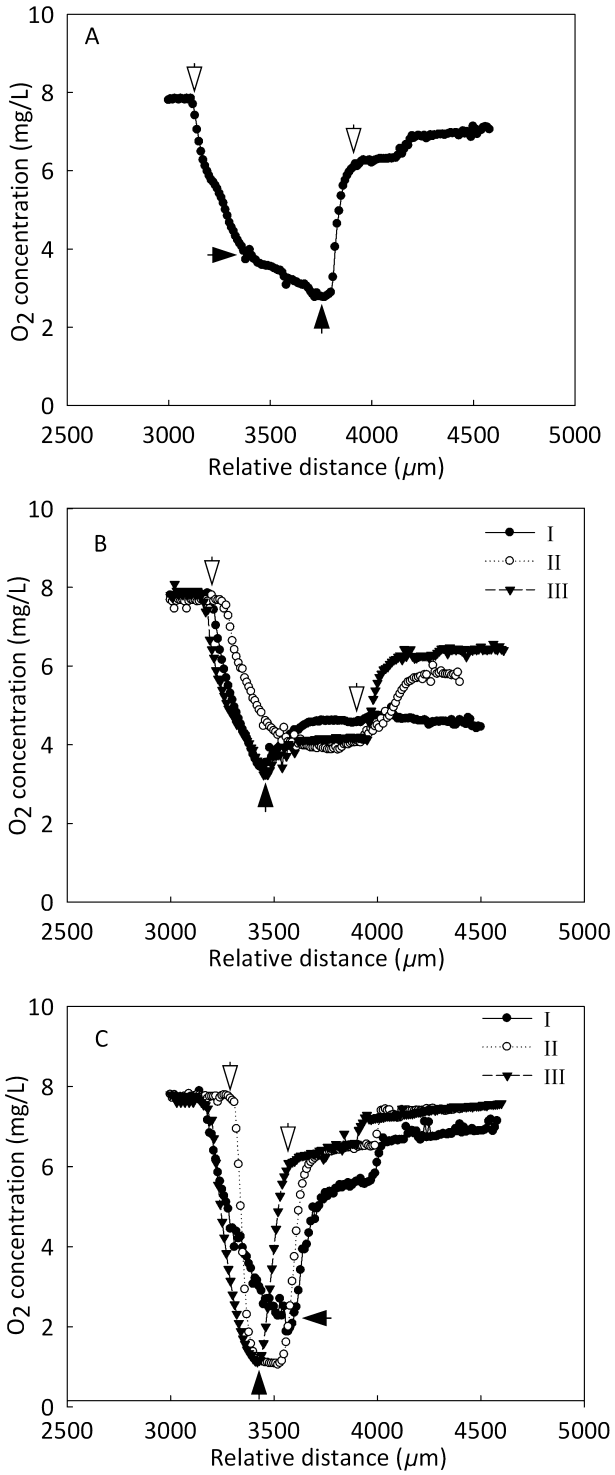


Fig. 2 Radial oxygen profiles along the gut of *C. formosanus.* The relative distance refers the position relative to initial starting location. Initially the measurements were started from an arbitrary selected location near the surface. The data are based on a single replicate since the distance of the microelectrode tip could not be quantize unified. The hollow arrowheads indicate the points at which the tip entered the gut wall or emerged from the opposite side; the solid arrowheads indicate the points of inflection of oxygen profiles. (A) Profiles obtained from a typical foregut. (B) Profiles obtained in the midgut, illustrating the difference among the median midgut (II), posterior (I), and anterior (III) regions. (C) Profiles for the hindgut, illustrating the differences among the colon (III), posterior paunch (I) and anterior paunch (II) regions. Note the much sharper decrease of oxygen profiles than those in foregut and midgut.

indicating different oxygen penetration or consumption rates in these gut segments. Figure 2A shows a slow decline of the radial oxygen concentration in the foregut of *C. formosanus*; similar decreasing gradients were obtained for *R. flavipes* (Fig. 3A). In both foreguts, there were zones of abrupt oxygen transition (marked by solid arrowheads).

The midguts of both WFTs contained significant amounts of oxygen, despite their small diameters (Figs. 2B and 3B), and they exhibited a more gradual loss of oxygen than the hindguts did. For *C. formosanus*, the anterior and posterior portions of the midgut revealed steeper oxygen gradients than the middle region; while the difference in *R. flavipes* seemed not so obvious.

In the three hindgut regions of both *C. formosanus* and *R. flavipes* (Figs. 2C and 3C), there were linear decreases and then increases in oxygen concentrations from the ambient concentrations at the agarose surface toward the gut. Only the central region of the paunch was relatively anoxic, with oxygen concentration of approximately 1 mg/L. This was most obvious in the second region of the paunch. The oxygen gradient in the hindgut of *R. flavipes* was less sharp, and exhibited more oxygen than that of *C. formosanus*. The slopes of the oxygen gradient were quite reproducible for all regions.

Axial distribution of oxygen in the gut of termites from foregut to hindgut The anterior-to-posterior oxygen profiles of termite guts were determined by measuring the concentration in the center of each gut region with three times replication in three different worker termite guts for each species (Fig. 4). The error bar and averages were derived from single measurements from multiple termites. Because of the artificial restriction of oxygen flux toward the gut by the surrounding agarose, the measured oxygen concentrations might be slightly different from that *in vivo* (Ebert & Brune, 1997; Brune *et al.*, 1995a). Thus, the axial oxygen profiles shown in Fig. 4 represent conservative estimates of the oxygen profiles in the guts

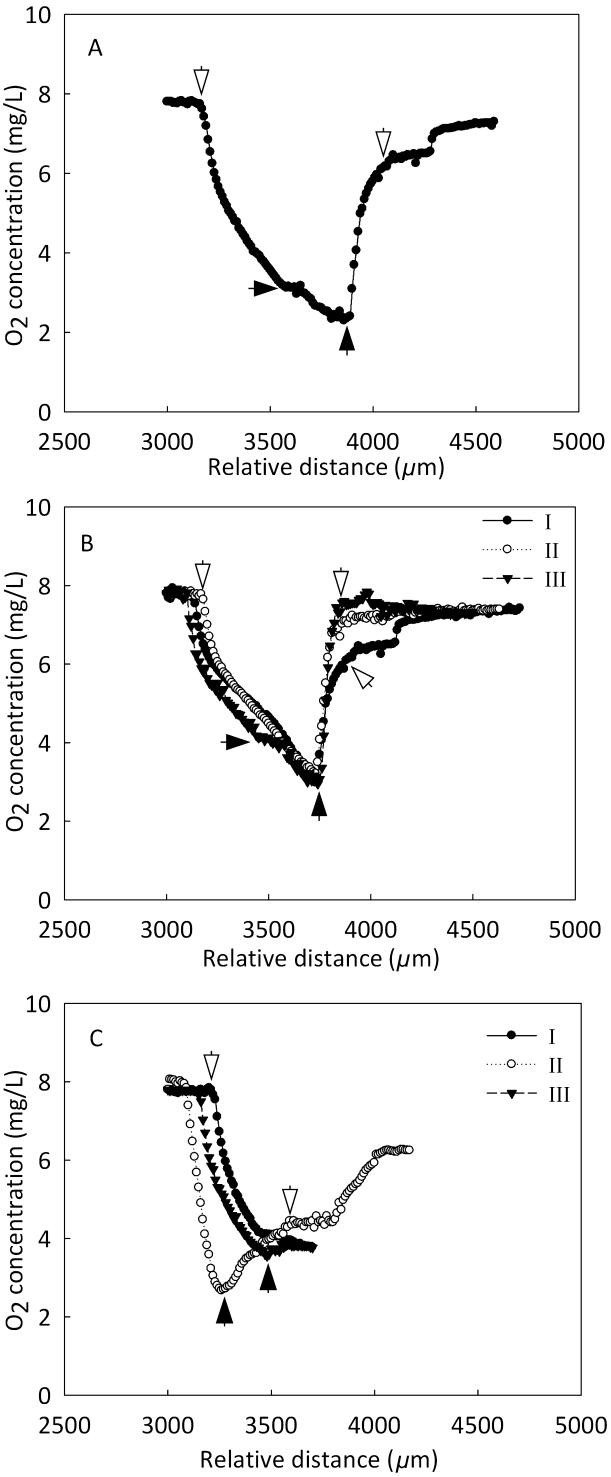


Fig. 3 Radial oxygen profiles along the gut of *R. flavipes.* The relative distance refers to the position relative to initial starting location. Initially the measurements were started from an arbitrary selected location near the surface. The data are based on a single replicate since the distance of the microelectrode tip could not be quantize unified. The hollow arrowheads indicate the points at which the tip entered the gut wall or emerged from the opposite side; the solid arrowheads indicate the points of inflection of oxygen profiles. (A) Profiles obtained from a typical foregut. (B) Profiles obtained in the midgut, illustrating the differences among the median midgut (II), posterior (I) and anterior (III) regions. (C) Profiles for the hindgut, illustrating the differences among the colon (III), posterior paunch (I) and anterior paunch (II) regions. Note the much sharper decrease of oxygen profiles than those in foregut and midgut.

of *C. formosanus* and *R. flavipes*. However, they do illustrate the relative oxygen status in various gut regions. For both termites, even the center of the hindgut, which had the least oxygen, was not definitely anoxic. *C. formosanus* exhibits a more rapid decrease in oxygen along the axis of the gut than *R. flavipes*.

Lignin modification in different gut segments

Differences in gut segment sizes and food intake affected the amount of wood particles in gut segments of the two termite species tested. To estimate lignin modification of each sample, we calculated the proportions of pyrolysed lignin compounds in the total aromatic compounds. Twenty-three percent of all aromatic compounds were contained in the pyrolysis products from the undigested wood sample: 6%, 12%, 12%, and 41% in the *C. formosanus* sample of foregut, midgut, hindgut and feces, respectively; and 20%, 6%, 31%, 37% for *R. flavipes*, respectively.

Figures 5 and 6 show pyrograms obtained from gut samples of *C. formosanus* and *R. flavipes*, and the undigested wood control, after being pyrolyzed at 500°C for 1 h. Meanwhile, cellulose-fed termite guts proved to be lignin-free (Figs. 7 and 8). Lignin-derived compounds were concentrated after passage through the termite gut in comparison to undigested samples, mainly because of the degradation of cellulose and hemicellulose. The aromatic compounds concentrated in the feces of both termites were similar, indicating similar mechanisms were in process in the two species. These compounds were guaiacol, vinyl guaiacol, isoengenol, 4-acetyl-2-methoxyphenol, vanillyl methyl ketone and coniferyl alcohol. The two compounds with aldehyde groups, 4-acetyl-2-methoxyphenol and vanillyl methyl ketone, were not observed until the hindgut, and seemed to reflect byproducts from the lignin degradation process. This was consistent with the suggestion that oxidation occurs at hydroxyl groups on

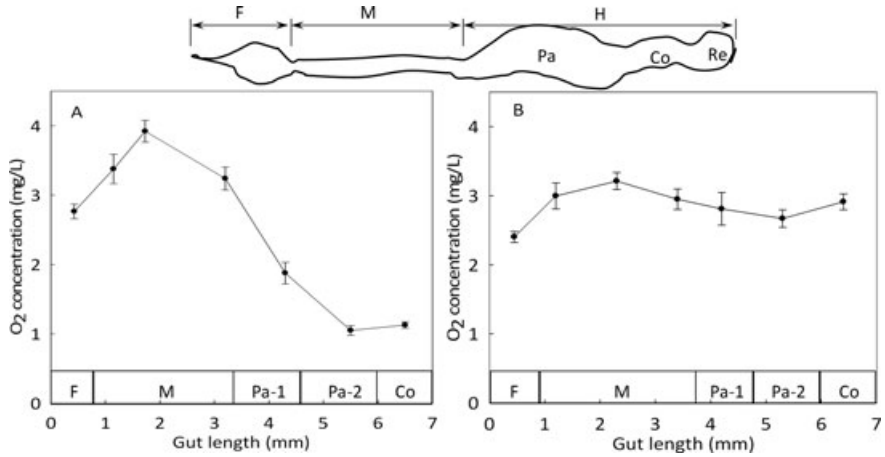


Fig. 4 Axial oxygen distribution in guts. The guts of both termites, *C. formosanus* (A) and *R. flavipes* (B), included the foregut (F), midgut (M) and hindgut (H). The hindgut consisted of paunch (Pa), colon (Co) and rectum (Re). Since the rectum is not thought to be an important digestive part for lignin modification, it has not been included. The reported oxygen concentrations were determined at the center of each gut region with three times replication in three different worker termite guts for each species.

lignin polymer side-chains to generate aldehyde groups (Gvozdev & Chupka, 2004). Of the other four aromatic compounds pyrolyzed from the feces, only guaiacol obviously increased; vinyl guaiacol (vinyl group) and isoeugenol (prophenyl group) levels were similar to that of the control; coniferyl alcohol decreased in *R. flavipes*. The increase in the methoxylated aromatic compounds (guaiacol) in the feces may indicate loosely packed lignin

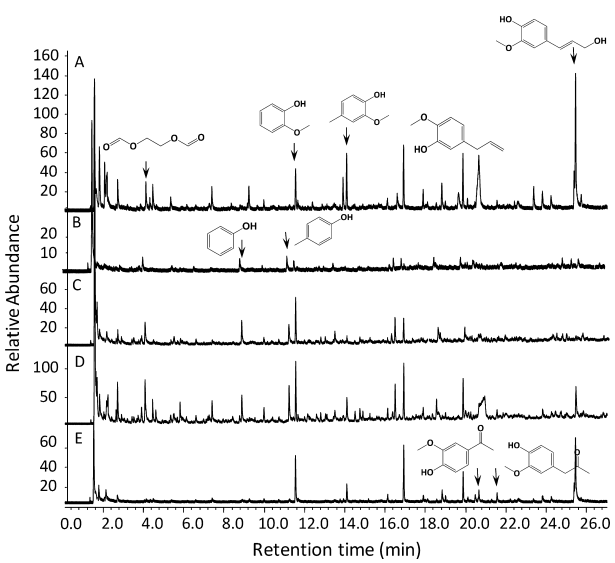


Fig. 5 Lignin modification during passage through gut of *C. formosanus*. (A) Control sample; (B) samples from foregut; (C) samples from midgut; (D) samples from hindgut; (E) feces. Chromatograms show increases in phenol (8.873 min), and *p*-cresol (11.223 min) from the foregut (B), and further increase in the midgut (C); as well as decreases in methyl guaiacol (14.100 min), coniferyl alcohol (25.453 min). The various pyrolysis products from hindgut sample (D) were mainly guaiacol; but only several were left in the feces (E).

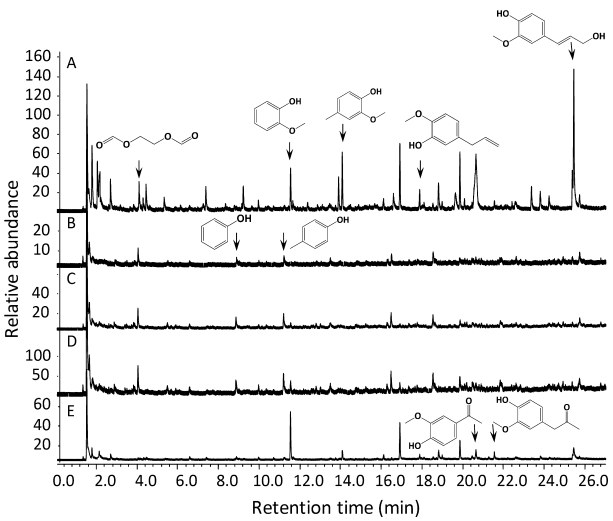


Fig. 6 Lignin modification during passage through gut of *R. flavipes*. (A) Control sample; (B) samples from foregut; (C) samples from midgut; (D) samples from hindgut; (E) feces. Chromatograms show obvious increases in phenol (8.873 min), and *p*-cresol (11.223 min), and decreases in methyl guaiacol (14.100 min), and coniferyl alcohol (25.453 min), in termite-treated wood after passage through foregut (B) and midgut (C). The pyrolysis products of the hindgut sample (D) were mainly guaiacol (11.563 min), vinyl guaiacol (16.923 min) and 5-allyl-2-methoxyphenol (17.897 min). Acetoguaiacon (20.651 min) and vanillyl methyl ketone (21.554 min) increased in the feces (E).

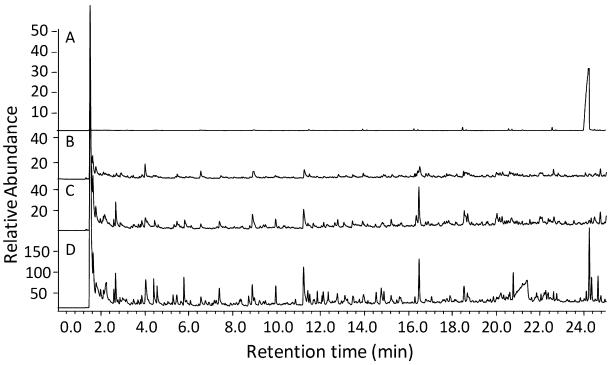


Fig. 7 Filter paper modification during passage through gut of *C. formosanus*. (A) Filter paper; (B) samples from foregut; (C) samples from midgut; (D) samples from hindgut. Few phenol-related compounds showed in each sample.

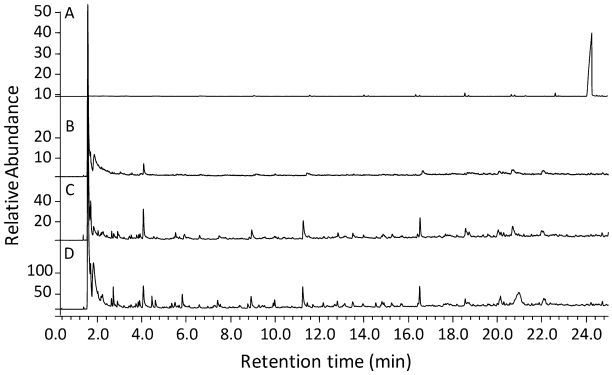


Fig. 8 Filter paper modification during passage through gut of *R. flavipes*. (A) Filter paper; (B) samples from foregut; (C) samples from midgut; (D) samples from hindgut. Few phenol-related compound showed in each sample.

morphology after lignin modification in termite guts which could facilitate the gasification process during pyrolysis, which may indicate carbon-carbon bond or allyl alcohol group cleavage, or some other conversion of the hydroxyl group. The other guaiacol compounds (methyl guaiacol, eugenol, 5-allyl-2-methoxyphenol, homovanillic acid, 4-hydroxy-2-methoxycinnamaldehyde) disappeared in the pyrolysis product from the feces, which suggested that methyl and allyl groups on aromatic rings, as well as carboxyl, aldehyde, hydroxyl groups on side-chains, had been modified or removed. The increase of guaiacol, decrease of guaiacol derivatives, and appearance of new guaiacol derivatives after termite pretreatment suggested that there should be conversions among guaiacol and its derivatives in the gut, presumably as a result of wood-lignin degradation. However, the fact that the concentrations of aromatic compounds in the two WFTs fed on the same diet were not identical indicates that the quantity and efficiency of enzymes or gut symbionts effecting lignin modification differ in the two species.

Changes in lignin content with progression through the termite gut were observed, and provide some insight into the modification processes active in each gut segment. Gut samples from *C. formosanus* (Fig. 5, Table 1) and *R. flavipes* (Fig. 6, Table 1) exhibited similar trends for all compounds. Compared to the control sample, 1,2-diformyloxyethane, dimethyl 3,8-dioxodecanedioate and *p*-cresol were increased in the foregut, and further increased when passing through the midgut. Phenol was observed in the midgut samples. Guaiacol was found to be increased in the midgut, whereas there were decreases of guaiacol derivatives, such as methyl guaiacol, 5-allyl-2-methoxyphenol, homovanillic acid, 4-hydroxy-2-methoxycinnamaldehyde, and coniferyl alcohol there. These findings indicate lignin ring demethylation, decarboxylation and side-chain oxidation (depolymerization) in the foregut and midgut.

When passing through the hindgut, phenol, *p*-cresol, 1,2-diformyloxyethane, and dimethyl 3,8-dioxodecanedioate maintained their concentrations (Fig. 5D, Fig. 6D), but vanished in the feces. This was consistent with the consumption of simpler products from lignin degradation in the hindgut by anaerobic aromatic lignin digesters, as suggested by Breznak and Brune (1994). Other guaiacol derivatives, such as methyl guaiacol, vinyl guaiacol, isoeugenol and coniferyl alcohol, initially decreased in midgut samples, but increased in hindgut samples and maintained their relative contents in feces. Thus, conversions of functional groups on aromatic rings are likely to occur in the hindgut.

The hindgut pyrograms of the two WFTs showed different levels of furfurol (retention time: 5.3–6.0 min) and levoglucosan (20.5–21.0 min), which likely came from hemicellulose and cellulose, respectively. These results may be indicative of different digestion levels of hemicellulose and cellulose in hindguts of the two species; and *R. flavipes* appeared to possess higher efficiency in holocellulose digestion.

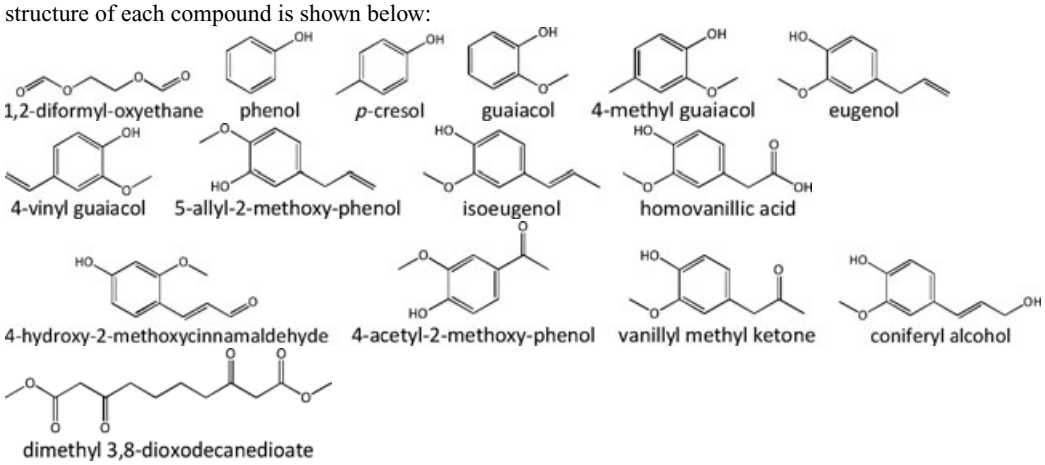
Discussion

This study has provided information on concentrations of oxygen and lignin-derived compounds in various segments of the guts from two wood-feeing termite species. Compared to Brune *et al.* (1995a) and Ebert and Brune (1997)'s work on oxygen profiles in *R. flavipes* gut, we advanced the microelectrode with smaller diameter and increment to be more accurate to reflect oxygen profiles

Table 1 Percentage of pyrolysis products from lignin before and after biological modification by *C. formosanus* and *R. flavipes*

Compounds	Retention time (min)	undigested wood (%)	Foregut (%)	Midgut (%)	Hindgut (%)	Feces (%)
1,2-diformyloxyethane	4.120	1.228	1.414	1.994	1.857	×†
			1.611	1.431	1.597	×
Phenol	8.873	0.089	×	1.228	1.137	×
			3.115	1.115	1.441	0.140
p-cresol	11.223	×	1.561	2.006	1.524	×
			5.326	1.663	2.141	0.126
Guaiacol	11.563	1.739	1.381	3.236	1.851	5.914
			2.778	0.349	6.146	10.295
Dimethyl 3,8-dioxodecanedioate	13.482	0.109	0.218	0.596	0.444	×
			×	0.285	1.356	×
4-methyl guaiacol	14.100	2.297	×	0.338	0.905	2.457
			0.922	×	1.910	2.133
Eugenol	16.130	0.560	0.335	0.329	0.433	1.071
			×	0.285	0.686	1.183
4-vinyl guaiacol	16.923	2.893	0.447	1.772	1.671	7.951
			2.562	×	5.366	7.751
5-allyl-2-methoxyphenol	17.897	0.870	×	0.263	0.454	0.676
			×	0.180	0.688	0.655
Isoeugenol	18.819/19.869	3.446	1.175	1.158	1.450	6.889
			2.412	1.040	4.427	6.565
Homovanillic acid	23.810	0.915	×	×	0.571	0.678
			×	×	×	0.694
4-hydroxy-2-methoxycinnamaldehyde	25.380	1.746	1.105	0.507	0.193	2.145
			1.540	0.484	2.537	0.596
4-acetyl-2-methoxyphenol	20.651	×	×	×	×	1.349
			×	×	1.833	1.592
Vanillyl methyl ketone	21.554	0.327	×	×	0.433	1.117
			×	×	0.952	0.994
Coniferyl alcohol	24.235/25.453	7.683	×	1.136	1.417	11.117
			1.540	0.484	2.537	4.054

Note: the proportions of pyrolysed lignin compounds were calculated from their percentages in the total aromatic compounds. The data on the top of each unit was from *C. formosanus*; the one at the bottom was from *R. flavipes*. †, no appearance in the pyrograms. The structure of each compound is shown below:



in termites; and further discuss the relationship between wood-lignin modification and oxygen profiles along the whole gut. The entire oxygen profiling pattern of the termite guts for both genera were consistent with the results previously reported by Brune *et al.* (1995a) and Ebert & Brune (1997). However, the detection of oxygen concentration in the center of hindgut illustrates the high sensitivity of the microelectrode used in this study. Consumption of oxygen in some parts of the gut may cause oxygen flux from other sites, possibly leading to maintenance of the anaerobic environment in other sites (Wertz & Breznak, 2007), and further resulting in varied oxygen distributions across the entire gut. This idea supports Yang's conclusion (2005) that interactions of micro-organisms, and differences in microenvironment inside and across habitat boundaries, should influence the structure and diversity of microbial communities within the ecosystem of the termite gut. Lignin degraders might be one component of the microflora that constitutes an oxygen sink in the guts of WFTs. The steep oxygen gradients at the periphery of the hindgut are maintained as a result of oxygen flux to the midgut, or oxygen consumption by the intestinal microbiota (Brune, 1998), which occupy the bulk of the hindgut volume (Breznak, 1984). Earlier reports have indicated that WFTs prefer a diet containing lignin over pure cellulose or fungus-infected wood, suggesting the importance of lignin-modifying bio-factors in termite guts (Kanai *et al.*, 1982). Brune *et al.* (1995b) have studied the metabolism of monoaromatic model compounds by termites and their gut microflora and concluded that gut homogenates of the WFTs *Nasutitermes lujae* (Wasmann) and *R. flavipes* mineralized lignin analogs only if oxygen was present, which was consistent with the oxygen requirement in other lignin-degrading systems. In both termites examined in this study, the middle point of the midgut exhibited higher oxygen concentration than other gut regions, indicating a high possibility of lignin oxidation there.

This work demonstrated both lignin modification and gut microenvironments conducive to lignin modification in WFTs. Early evidence of lignin degradation by termites was obtained only from labeled heterogeneous biomass-lignin or synthetic lignin (Cookson, 1987, 1988; Kyou *et al.*, 1996; Breznak & Brune, 1994; Itakura *et al.*, 1995), leading to ambiguity in the results. Chemical analysis of lignin in WFTs' food and feces have had varied results, ranging from virtually minor changes (Katsumata *et al.*, 2007) to astonishingly high degradation values of 83% (Lee & Wood, 1971). These earlier experiments characterized only the Björkman lignin fraction in insect feces, limiting detection of modification of the entire lignin sample. Uncertainty over whether termites attack lignin was reinforced by metagenome sequencing of the higher termite species *Nasutitermes corniger* hindgut community, in which no genes encoding known lignin-degrading enzymes were found (Warnecke *et al.*, 2007). However, these findings are not in conflict with our hypothesis that oxidation of lignin occurs mainly in the foregut and midgut regions. In support of our hypothesis, tetramethylammonium hydroxide (TMAH) thermochemolysis analysis found significant alteration of the lignin polymer of coniferous wood (softwood) after passage through the gut of a lower WFT (Geib *et al.*, 2008), including propyl side-chain oxidation, ring demethylation and ring hydroxylation.

Our work examined lignin modification in each segment of the termite gut. The results detail side-chain oxidation/cleavage (depolymerization) and demethylation of ring methoxyl groups of wood-lignin beginning in the foregut, and continuing in the midgut. Ring demethylation makes the substrate more suitable for carbon-carbon bond cleavage; with other reactions of side-chain oxidation, ring opening will thus be facilitated (Goodell *et al.*, 2003), assuming guaiacols favor aromatic ring opening and mineralization. Side-chain oxidation will cause depolymerization of lignin polymers or release of lignin units from lignin–carbohydrate complex. Both of these reactions are believed to require oxygen, as previous experiments have demonstrated (Kedderis & Hollenberg, 1983; Ten Have *et al.*, 2000). All currently known lignin-degrading pathways are aerobic. Aerobic regions of termite guts, housing large populations of aerobic microbes (Tholen, 1997; Brune *et al.*, 1995a; Ohkuma, 2003) support the maintenance of anaerobic conditions in other areas for fermentation of cellulose-derived monosaccharides by termites (Adams & Boopathy, 2005; Johnson & Barbehenn, 2000). The relatively high levels of oxygen observed in the midgut were therefore consistent with these reactions occurring in the midgut. Previous studies have shown that, in fungi, phenol oxidase and peroxidase are responsible for cleavage between the aromatic and aliphatic portions of lignin, and phenolic and non-phenolic oxidation of lignin (Janshekar & Fiechter, 1983; Ten Have & Teunissen, 2001). Our findings support that a similar system of catalysis might exist in the salivary glands/foregut and midgut of WFTs (Tartar *et al.*, 2009). Furthermore, lignin modification should not only happen in the foregut and midgut, but the hindgut might also participate in the process of dealing with simpler products. As lignin depolymerizes, it liberates phenolic groups, breaks away from hemicellulose (Higuchi, 1990), and perhaps releases low molecular weight lignin fragments (Janshekar & Fiechter, 1983; Tartar *et al.*, 2009), it will increase in hydrophilia. It is speculated that when passing through the

hindgut, the easily digested byproducts, such as ester and monomer lignin units, might be taken up through the gut epithelium and oxidized aerobically by termite tissue or gut symbionts; however, it needs further investigations to clarify this. This process may be attributed to the function of esterase and phenol oxidizing enzymes (Kirk *et al.*, 1980).

The presence of aromatic-degrading bacteria in termite guts has been well documented (Watanabe *et al.*, 2003; Adams & Boopathy, 2005), indirectly supporting our observations of degradation of small plant aromatic compounds in the hindguts of WFTs, which may contribute to the carbon and energy requirement of the host. Pasti *et al.* (1990) isolated 11 novel actinomycete strains with specific peroxidase activity from the guts of higher termites (Termitidae). Later, Kuhnigk and König (1997) indicated that the hindgut floras of xylophagous termites were able to produce substrates for their hosts from dimeric lignin model compounds in the presence of oxygen. The presence of bacteria that degrade monomer lignin and phenolic compounds from decomposed wood lignin was reported in the gut of *Nasutitermes takasagoensis, Coptotermes formosanus* (Kinya *et al.*, 1998; Harazono *et al.*, 2003). In addition, as demonstrated by Vu *et al.* (2004), there is redox metabolism of iron in two WFTs; reduction of iron (III) may provide redox potential for oxidation of lignin. Since the epithelial regions of the gut are known to be micro-oxygenic (Figs. 2, 3), iron (II) is likely subject to rapid re-oxidation there (Vu *et al.*, 2004), so that it can again serve as a potent catalyst in the partial oxidation of lignin fractions (Jamil & Hussain, 1992).

Although neither the exact rate of lignin degradation by WFTs nor the specific micro-organisms or enzymes responsible for lignin modification could be determined at this time, their efficiency of wood disruption within 24 h is quite striking (Itakura *et al.*, 1995; Katsumata *et al.*, 2007). In general, the mean retention time of digesta in termite hindgut is estimated to be around 24–26 h (Breznak, 1984). Our results agree with earlier predictions regarding the distribution of phenol oxidase and esterase along the gut of WFTs, as noted by Tartar *et al.* (2009) and Wheeler *et al.* (2007) in the *R. flavipes* gut. Furthermore, genes encoding peroxidases, which are also responsible for wood-lignin disruption, have also been identified from the *R. flavipes* gut (Tartar *et al.*, 2009).

The results acquired in these studies have provided new insights into our understanding of lignin degradation by WFTs. Lignocellulosic degradation occurs throughout the entire gut system, with initiation of lignin modification before the hindgut. It is likely that WFTs have evolved optimal conditions and processes for efficient lignocellulose degradation. Further understanding of the exact reactions and mechanisms that modify lignin in the termite gut will contribute to our understanding of the biochemical roles that these insects play in lignin modification, and promote industrial utilization of these processes for faster and better access of enzymes to polymer carbohydrates.

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