Enzyme activities at different stages of plant biomass decomposition in three species of fungus-growing termites

Rafael R. da Costa¹#, Haofu Hu¹, Bo Pilgaard², Sabine M.E. Vreeburg³, Julia Schückel¹,
Kristine S.K. Pedersen¹, Stjepan K. Kračun⁴, Peter K. Busk², Jesper Harholt⁵, Panagiotis
Sapountzis¹, Lene Lange³, Duur K. Aanen³ and Michael Poulsen¹

Short title: Plant biomass breakdown in farming termites

¹Centre for Social Evolution, Section for Ecology and Evolution, Department of Biology,
University of Copenhagen, Universitetsparken 15, 2100 Copenhagen, Denmark.
²Department of Chemical and Biochemical Engineering, Technical University of
Denmark, Søltofts Plads, 2800 Kgs. Lyngby, Denmark.
³Laboratory of Genetics, Wageningen University, Droevendaalsesteeg 1, 6708 PB,
Wageningen, The Netherlands.
⁴Department of Plant and Environmental Sciences, Section for Plant Glycobiology,
University of Copenhagen, Thorvaldsensvej 40, 1871 Frederiksberg C, Denmark.
⁵Carlsberg Research Laboratory, J. C. Jacobsens Gade 4, 1799 Copenhagen V, Denmark.

#Correspondence
Rafael R. da Costa, Centre for Social Evolution, Section for Ecology and Evolution,
Department of Biology, University of Copenhagen, Universitetsparken 15, Building 3,
2100 Copenhagen East, Denmark, email: Rafael.dacosta@bio.ku.dk, phone:
+4535337284.
Abstract

Fungus-growing termites rely on the mutualistic fungus *Termitomyces* and gut microbes for plant biomass degradation. Due to a certain degree of symbiont complementarity, this tripartite symbiosis has evolved as a complex bioreactor, enabling decomposition of nearly any plant polymer, likely contributing to the success of the termites as the main plant decomposers in the Old World. Here we evaluate which plant polymers are decomposed and which enzymes are active during the decomposition process in two major genera of fungus-growing termites. We find a diversity of active enzymes at different stages of decomposition and a consistent decrease in plant components during the decomposition process. Furthermore, our findings are consistent with the hypothesis that termites transport enzymes from the older mature parts of the fungus comb through young worker guts to freshly inoculated plant substrate. However, preliminary fungal RNAseq analyses suggest that this likely transport is supplemented with enzymes produced *in situ*. Our findings support that the maintenance of an external fungus comb, inoculated with an optimal mix of plant material, fungal spores, and enzymes, is likely the key to the extraordinarily efficient plant decomposition in fungus-growing termites.
Importance

Fungus-growing termites have a substantial ecological footprint in the old world (sub)tropics due to their ability to decompose dead plant material. Through the establishment of an elaborate plant biomass inoculation strategy, and fungal and bacterial enzyme contributions, this farming symbiosis has become an efficient and versatile aerobic bioreactor for plant substrate conversion. Since little is known about what enzymes are expressed, and where they are active at different stages of the decomposition process, we used enzyme assays, transcriptomics and plant content measurements to shed light on how this decomposition of plant substrate is effectively accomplished.
Introduction

Plant biomass is one of the most nutritious and abundant carbon sources utilized by a range of different organisms (1). Primarily consisting of cell walls, plant substrates present a complex structure of polysaccharides, proteins, and lignin, differing between plant species in their monomeric composition and linkages (2). The complex arrangements of different plant cell wall polymers make them resistant to degradation, yet many microorganisms can effectively degrade these polysaccharides through the secretion of enzymes that cleave complex saccharides to release oligo-, di- and monosaccharides (1). Most animals do not have the necessary enzymes to break down recalcitrant plant-derived components for nutrition (3), and thus co-opt microbial symbionts for plant biomass decomposition (1, 4).

Fungus cultivation in termites evolved ca. 30 million years ago in sub-Saharan Africa, when the subfamily Macrotermitinae engaged in a mutualistic association with a fungus of the genus Termitomyces (Agaricales, Lyophyllaceae) (5, 6). The termites have become major biomass decomposers in the Old World (7, 8), where they play an important role in turnover of dead plant material (9-11). They decompose up to 90% of the available dead plant material in African savannahs (12), with a consequently major impact on carbon cycling (13, 14). All 11 fungus-growing termite genera occur in Africa and four in Asia, and approximately 330 species have been described (15, 16). Approximately 40 species of Termitomyces have been described, all of which engage in mutualistic associations with the Macrotermitinae (17). Termitomyces serves as the primary plant, decomposer and as the main food source for the termites (18, 19). In addition to Termitomyces, fungus-growing termites harbor a complex and co-diversified gut microbiota (20-22) that complements the plant decomposition properties of Termitomyces spp. (23, 24).
The two major fungus-growing termite genera *Macrotermes* and *Odontotermes* process plant biomass in a similar way (25-27), involving two gut passages and external decomposition in fungal gardens (combs) (28, 29). Old workers collect plant substrate and transport this to the mound (28, 30). Within the mound young workers ingest the plant material along with asexual *Termitomyces* spores produced in fungal nodules in the mature parts of the fungus comb (31). This mixture passes through young termite guts (first gut passage), which possibly contributes to lignin cleavage (32), and the resulting excrements are used to build the fungus comb (hereafter referred to as “fresh comb”) (28). *Termitomyces* produces plant biomass-degrading enzymes (24, 33-35) and possibly also cleaves lignin as it grows during comb maturation (19, 35-38). After maturation of the fungus comb, hereafter referred to as “old comb”, it is consumed by old workers in a second gut passage, after which essentially all organic material is utilized (28). Gut microbial enzymes are believed to facilitate final plant decomposition during this second gut passage and to contribute to fungal cell wall degradation (23, 24).

The first gut passage serves as an effective means with which the termites assure that the plant substrate is densely inoculated with *Termitomyces* spores; however, this gut passage has also been proposed to contribute transport of carbohydrate-active enzymes from nodules to fresh comb to boost initial plant decomposition (29). This transport of enzymes could be complemented with enzymes produced by *Termitomyces* mycelium within the comb. Here we use enzyme assays to investigate what enzymes are active and what plant components are broken down at different stages of the decomposition process, and supplement this with fungal RNAseq data to shed light on the locations of fungal carbohydrate-enzyme expression.
Materials and methods

Termite collections

Samples were collected in 2015 and 2016 in South Africa at four geographical sites (Table 1). In 2015, old major workers, *Termitomyces* nodules, fresh and old comb were obtained from three colonies from each of *Odontotermes* sp., *Odontotermes cf. badius*, and *Macrotermes natalensis* (Table 1). In 2016, both old and young, minor and major workers, *Termitomyces* nodules, fresh and old comb were collected from one colony of *Odontotermes* sp. and three colonies of *M. natalensis* (Table 1). From each nest, approximately 100 mg of fungus comb and nodules was weighed, put into 1.5 mL Eppendorf tube, and frozen at -80°C. Termites from the same nests were collected and whole guts, including gut content, were dissected until 100 mg was obtained (typically 15-20 guts) and frozen at -80°C. To characterize plant polymer content in the forage harvested by fungus-growing termites, we collected and froze 50-200 g forage at -20°C, and sampled foraging termites for taxonomical identification from 18 foraging sites in 2016 (Table 2).

Termite identification

Termites in the genus *Odontotermes* require molecular identification due to uncertainty in morphological identification (39). In contrast, *M. natalensis* is the only *Macrotermes* species reported in the sampling area (40), and molecular identification is thus not necessary. DNA extractions of three *Odontotermes* worker heads per colony were performed using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). Cytochrome oxidase II gene (COII) was amplified for comparison to available sequences in GenBank. PCR was prepared using A-tLeu forward primer and B-tLys reverse (41).
The PCR reaction tube contained 8.5 µL sterile distilled water, 1 µL of each primer, 2 µL template and 12.5 µL REDTaq Ready Mix (Sigma-Aldrich, St. Louis, USA). The conditions for PCR were as previously described (42). PCR products were visualized on agarose gels and purified using MSB SPIN PCRapace (STRATEC Molecular, Berlin, Germany). Purified PCR products were submitted to sequencing at Eurofins MWF Operon (Ebersberg, Germany). Sequences were aligned in Geneious PRO 4.8.5 using MUSCLE (43) and compared to Odontotermes COII sequences available from GenBank. A Neighbor-Joining tree was generated in MEGA 6.06 (44) with Kimura two-parameter estimates. The tree was built using termites sampled from the four Odontotermes nests involved in the enzyme and polymer content analyses (Table 1), eight samples from foraging sites (Table 2), sequences included in Otani et al. (12) and from GenBank.

**Enzyme assays**

Azurine-Crosslinked (AZCL) enzyme assays

We conducted AZCL enzyme assays to determine enzyme activities at different stages of the decomposition process: old major worker guts, fungal nodules, fresh comb, and old comb from the 2015 collection, in addition to young minor and major workers from 2016 were examined (Table 1). Seventeen AZCL polysaccharide media were prepared using 0.1 g/L of substrate in agarose medium (1% agarose w/v, 23 mM phosphoric acid, 23 mM acetic acid, 23 mM boric acid). For fungus comb measurements, the pH was adjusted separately for each substrate according to the manufacturer’s description (Megazyme, Bray, Ireland and de Fine Licht et al. (45), and pH 6.0 was used for gut samples to mimic natural gut conditions (46). One hundred mg sample was crushed with a pestle in a 1.5 ml-Eppendorf-tube containing 1 ml distilled water, followed by vortexing and centrifugation (15,000 g). Fifteen µL supernatant was applied in triplicates in ca. 0.1 cm²
wells within the AZCL assay plates, which were photographed after 24 hrs of incubation at 25°C. Enzyme activity was inferred from measuring the halos around the wells using ImageJ v. 1.6.0. (U. S. National Institutes of Health, Bethesda, Maryland, USA).

Principal Coordinates Analyses (PCoA) were performed in RStudio (47), and clustering analyses using Euclidean distance with bootstrap support after 10,000 permutations were performed in PAST v. 2.17c (48).

Enzyme screening using chromogenic polysaccharide hydrogel (CPH) substrates

To validate the AZCL enzyme activity results, we used a new generation of versatile chromogenic substrates for high-throughput analysis of biomass-degrading enzymes provided by GlycoSpot™ (Frederiksberg C, Denmark, see Table S1 in the supplemental material) on samples from M. natalensis (Table 1). Briefly, 200 µL activation solution was added per well and plates were incubated for 15 min to activate the CPH substrates. The remaining activation solution was removed using a vacuum manifold at full pressure, and washed twice with water to remove the stabilizer that keeps CPH substrates solid before they are washed. Samples were ground in 1 mL of 100 mM reaction buffer (50 mM sodium acetate, pH 5.0 for fungal samples and pH 6.0 for gut samples). Five µL enzyme solution (Table S1) with final enzyme concentration of 0.1 U/ml in the well for the positive control and 25 µL sample volumes were added as in Kračun et al (49). The plates were sealed using adhesive PCR seals (Thermo Scientific, VWR, Herlev, Denmark) and incubated for 23 hrs at 25°C at 150 rpm. After incubation, the liquid phase retained in the gel (reaction products) was separated by vacuum manifold and the absorbance was measured in a plate reader at 595 and 517 nm for blue and red substrates, respectively (49).
Enzyme activity relative to fungal content in fresh and old combs

We tested whether the differences in enzyme activities in fresh vs. old comb were due to differences in the amount of fungal material present, i.e., a question of concentration, or if fresh comb activities were more similar to their presumed origins (nODULES via young worker guts). To do so, we estimated the relative amount of fungal biomass in fresh and old comb. We did this by determining the amount of N-acetylglucosamine, which is deacetylated to glucosamine (GlcN) during hydrolysis, allowing for quantification by comparison to a standard curve of commercially-available N-acetylglucosamine (Sigma-Aldrich, St. Louis, USA) (50). We subsequently normalized the fresh comb AZCL activities relative to fungus content and repeated the PCoA to evaluate the effect of this normalization on their relative positioning in PCoA space.

RNA extraction, sequencing and analyses

RNA extraction and sequencing

Approximately 20 mg of nodules, fresh comb, or old comb (Table 1) were placed in 1.5 mL-Eppendorf tubes and frozen in liquid nitrogen within a few hours after sampling. Samples were ground with pestle to a fine powder. RNA was isolated using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer protocol. After RNA purity and quality were determined in NanoDrop spectrophotometer (Thermo Scientific, Wilmington, USA) and RNA yield and integrity analyses in Experion (Bio-Rad Laboratories, Hercules, USA), only samples from *M. natalensis* Mn156 and *Odontotermes* sp. Od127 and Od128 were of sufficiently high quality for RNAseq. mRNA was enriched by oligo dT beads to construct cDNA libraries, which were subsequently sequenced with 125bp paired-end reads on the Illumina Hiseq2500 platform. Sequence data was deposited to the SRA database with accession...
numbers SRR5944781-SRR5944786 and SRR5944350-SRR5944352 (see Table S2 in the supplemental material).

Transcript assembly and quantification

Raw sequencing reads were first quality controlled, and reads were excluded if they contained more than 10% Ns or if their quality value was below 5 for more than 50% of the bases (Table S2). High-quality reads were de novo assembled using Trinity (v2.3.2) (51) with default parameters. High-quality reads were then mapped to assembled transcripts using Bowtie2 (v2.2.9) (52). To quantify the abundance of transcripts in each sample, reads mapped to transcripts were subsequently counted by RSEM (v 1.3.0) (53) to obtain Transcripts Per Million (TPM) values of individual transcripts. Transcript sequences were deposited to the TSA database (Table S2).

Peptide pattern recognition (PPR) and Homology to Peptide Pattern (Hotpep)

Hotpep (54) with PPR generated peptide patterns for all enzyme families in the Carbohydrate-Active enZymes (CAZy) database (55) was used to identify CAZymes in the translated transcripts as previously described by Busk et al. (56). Shortly, Hotpep uses PPR generated short, conserved peptides (57) to assign protein sequences to specific groups within enzyme families. When functional information is available as is the case for many of the carbohydrate-active enzymes, Hotpep uses this information to predict the function of the annotated polypeptide sequences (55, 56).

Plant polymer degradation

Extraction and fractionation of plant cell wall material
To determine plant polymer content, triplicate measurements were performed for forage substrate, fresh comb, old comb, and termite guts. Ca. 100 mg material was freeze-dried and transferred to screw cap plastic vials with stainless steel beads and ground to a fine powder. To extract the plant cell wall material (Alcohol insoluble residues: AIR), a wash step in 70% v/v ethanol followed by a wash in methanol-chloroform (1:1, v/v) and an acetone extraction step, were carried out to remove pigments, proteins, alkaloids, tannins, soluble sugars, and other low molecular weight metabolites. Samples were subsequently air dried overnight before further processing (58, 59).

Comprehensive microarray polymer profiling (CoMPP)

To look at structural polymer composition with high resolution, we performed CoMPP analyses using approximately 10 mg AIR (58). Samples were treated with an aqueous solution of cyclo-hexane-diamine-tetra-acetic acid (CDTA) to solubilize water-soluble cell wall components and Ca$^{2+}$-chelated pectins followed by a NaOH extraction to solubilize hemicellulose and cellulose. Four technical replicates, each in four different serial dilutions of the extracts/supernatants, were printed at room temperature and 55% humidity onto nitrocellulose membranes (Amersham Protran 0.45 µm NC) using a microarray printer (Sprint, Arrayjet, Roslin, UK). Arrays were incubated overnight in 5% milk powder (w/v) in Phosphate-Buffered Saline (PBS) at 4°C for blocking to prevent antibodies from binding to the background (60). After blocking, arrays were incubated in primary monoclonal antibodies (mAbs) for two hours (61). After washing, arrays were probed with secondary antibodies (see Table S3 in the supplemental material) conjugated to alkaline phosphatase for two hours before washing and developing in a BCIP/NBT (5-bromo-4-chloro-3-indolyphosphate/nitro-blue tetrazolium chloride) substrate (58). The developed microarrays were scanned at 2400 dpi and the signals were quantified using...
Array-Pro Analyzer 6.3 (Media Cybernetics, Rockville, USA). An average signal intensity for three technical replicates and four dilutions was calculated, the maximum value set to 100, and all other values normalized accordingly. A heatmap was generated and a cut-off of 5 was imposed to avoid false positives due to background signal (59, 62).

**Lignin**

Twenty mg of AIR per sample were hydrolyzed with 25% acetyl bromide (v/v in glacial acetic acid) at 70°C for 30 minutes (63). After incubation for complete digestion, the samples were cooled in an ice bath, mixed with 0.9 mL 2M NaOH and 0.1 mL 5M hydroxylamine-HCl, and finally six mL glacial acetic acid was added to complete lignin solubilisation. Samples were centrifuged at 1,400 g for five minutes, supernatants were collected, and absorbance measured at 280 nm (64). A standard curve was built with alkali lignin (Sigma-Aldrich, St. Louis, USA) and the absorptivity value (ε) was 22.9 g⁻¹ L cm⁻¹.

**Cellulose and non-cellulosic monomers**

Five mg of AIR were hydrolyzed for one hour at 120°C using 300 µL 2 M trifluoroacetic acid (TFA), after which samples were spun down at maximum speed and ethanol was added until a concentration of 70% (v/v) was obtained. The tubes were centrifuged at 10,000 g, the ethanol was removed, and the samples were air dried for one hour at room temperature. 300 µL 72% (v/v) sulfuric acid was added and samples were incubated for one hour at room temperature, after which 55µL sample was added to 945 µL water to reach a 4% (v/v) sulfuric acid concentration. Samples were incubated at 120°C for one hour, after which the tubes were centrifuged at 10,000 g, the supernatant was retrieved and diluted 50 times before quantification with High Performance Anion Exchange...
Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) using a Dionex ICS 5000+ DC system equipped with a 4 μm SA-10 column with 2x250 mm dimensions and a pre column. Run conditions were 0.3 ml/min, column temperature 40°C, 1 mM NaOH eluent for 0-8 min. followed by 100 mM NaOH for up to 20 min. with 10 min. subsequent equilibration in 1.0 mM NaOH. To measure non-cellulosic monomers, a 3-5 mg AIR sample was hydrolyzed with 800 μL 2M TFA for 1 hour at 120°C (65). After incubation, samples were cooled in an ice bath, centrifuged at 10,000 g, and the TFA removed by evaporation under vacuum overnight. The hydrolysis products were re-suspended in 300 μL of deionized water (65). Samples were further diluted 20 times in MilliQ water before quantification of monosaccharide constituents by HPAEC-PAD as described above.

Statistical analyses

Table S4 in the supplemental material provides an overview of the statistical analyses performed and their results. Assays were evaluated separately, analyses were performed in R v. 3.3.2 (47), and p-values were Bonferroni corrected for multiple comparisons. The hist function in R was used to test if response variables had equal variance; if so, we used linear mixed models (LMM) with colony component (nodules, guts, old comb, and fresh comb), species (M. natalensis, O. sp., and O. cf. badius), and year (2015 and 2016) as fixed variables and colony origin as a random variable. We tested if different plant substrates differed in lignin and cellulose content, but since colony information was not available for these tests, we used linear models (LM) with foraging substrate and termite species as fixed factors. For each model, one-way nested ANOVAs were used to determine the effects of the fixed factors. Pilot experiments (data not shown) indicated a linear association between concentration of enzyme extracts and halo areas. Thus, for
AZCL and CPH substrate variables with unequal variance (Table S4), we conducted non-parametric Kruskal-Wallis rank sum tests. For the remaining AZCL and CPH substrates, we conducted one-way nested ANOVAs fitted on linear mixed models. To evaluate the diversity of the AZCL enzymes, we determined Shannon indices, which were transformed exponentially to assure equal variances, and subsequently fitted a linear model using the Shannon values as the response variable and colony component (guts, fresh comb, nodules, and old comb) and termite species as fixed factors.

For non-cellulosic monomers, cellulose, and lignin content measurements with equal variances, we used linear mixed models (LMM) to test for differences between colony components, while we used Kruskal-Wallis rank sum tests for variables with unequal variance. Similarly, we employed linear models (LM) to test for differences between forage types for contents with equal variance and Kruskal-Wallis rank sum tests for variables with unequal variance (Table S4). For the CoMPP data, we employed one-way ANOVAs followed by unpaired Student’s t-tests on absorbance as the response variable and substrate, old worker guts, fresh comb, and old comb as main factors.

Results

Odontotermes species identification

In addition to two nests (Od127 and Od128) already identified as Odontotermes sp. by Otani et al (12), we successfully amplified COII from termites derived from four Odontotermes nests sampled in 2015, of which one nest was Odontotermes sp. (Od159) and three were Odontotermes cf. badius (Od145, Od150, and Od151) determined by their phylogenetic placement (see Fig. S1 in the supplemental material) (sequences available from GenBank with accession numbers: MF092801-MF092804). Of the termites
collected at the 18 foraging sites (Table 2), five were *Odontotermes* sp. and three were *Odontotermes cf. badius* (accession numbers: MF092793-MF092800) (Fig. S1).

**RNA sequencing and PPR-based - Hotpep analysis**

Since we were able to sequence mRNA from only three termite nests, one colony of *Macrotermes natalensis* and two of *Odontotermes* sp., the results of the RNAseq remains preliminary, but we include it to supplement the findings from the enzyme and content analyses. Nodules, fresh and old comb of the fungal symbiont of both *M. natalensis* and *Odontotermes* sp. showed expression of a wide spectrum of biomass-degrading enzymes, including targeting lignin, cellulose, hemicellulose, and pectin (Tables 3, 4; Tables S5, S6 in the supplemental material). Other key CAZymes in the modification of plant cell wall during the decomposition process include Auxiliary Activities (AAs), such as laccases, versatile peroxidase, alcohol oxidase and lytic polysaccharide monooxygenases (LPMOs), Polysaccharide Lyases (PLs) (mainly pectase lyase and poly-β-D-mannuronate lyase targeting pectin, and Carbohydrate Esterases (CEs) (Tables S5, S6). Transcripts of a full set of enzyme functions, targeting all plant components, were observed for all three nests (Tables 3, 4).

Some patterns emerged from the expression analyses: enzymes targeting cellulose, particularly Cellulose-1,4-β-cellobiosidase (reducing end) (EC: 3.2.1.176), were by far the most highly expressed, with highest expression in old comb, followed by a laccase (EC: 1.10.3.2) with highest expression in fresh comb in the two *Odontotermes* nests, but highest in old *M. natalensis* comb. Following these were enzymes targeting hemicellulose, which also in two out of three nests were most expressed in the old comb (Tables 3, 4).
Enzyme capacities across different stages in the decomposition process

Highest enzyme activities were identified against xylan, arabinoxylan, barley β-glucan and HE-cellulose, and these were highest in fungal nodules, followed by guts, fresh combs, and old combs (Fig. 1, see Table S7 in the supplemental material). Amylose ($\chi^2_{(2)} = 3.899$, $p = 1.000$), arabinoxylan ($F(2) = 2.494$, $p = 1.000$), barley β-glucan ($\chi^2_{(2)} = 2.710$, $p = 1.000$), casein ($F(2) = 9.279$, $p = 0.7670$), debranched arabinan ($\chi^2_{(2)} = 8.956$, $p = 0.1476$) and xylan ($F(2) = 3.124$, $p = 1.000$) degrading activities were not significantly different across termite species (Fig. 1; Table S4). In contrast, HE-cellulose ($F(2) = 10.95$, $p < 0.0001$), collagen ($\chi^2_{(2)} = 33.08$, $p < 0.0001$), curdlan ($\chi^2_{(2)} = 47.50$, $p < 0.0001$), galactomannan ($\chi^2_{(2)} = 60.51$, $p < 0.0001$), galactan ($\chi^2_{(2)} = 13.28$, $p < 0.0001$), xyloglucan ($\chi^2_{(2)} = 75.32$, $p < 0.0001$) and rhamnogalacturonan ($\chi^2_{(2)} = 13.28$, $p = 0.0169$) activities were significantly different between termite species. Worker guts, nodules, fresh and old comb were significantly different in enzyme activity across all AZCL substrates (Table S4) except for debranched arabinan ($\chi^2_{(6)} = 15.05$, $p = 0.2579$).

Euclidian PCoA and Shannon indices analyses ($F(6) = 52.86$, $p < 0.0001$) showed distinct enzyme capacities between nodules, old, young, minor and major worker guts, and fresh and old fungus combs samples, associated with different stages in the decomposition process (Figs. 1, 2a,b). Nodules, guts, fresh comb, and old comb were significantly different for almost all substrates (Table S4). Fresh and old comb were similar in enzyme diversity and activity; however, differences (e.g., in enzyme activities to cleave debranched arabinan and galactomannan exclusively found in the fresh comb) led to separate clusters in the PCoA (Fig. 2b). Nodules and guts from all termite species were similar in enzyme activities and diversity and they clustered together (One-way...
PERMANOVA: $F(7) = 3.957$, $p = 1.000$; (Figs. 1, 2b). Worker guts clustered relatively close to each other and to nodules; however, there was a tendency that old major worker guts were separating from other worker guts (Figs. 1, 2b). After correction of fresh comb AZCL activities based on the ratio of glucosamine in old and fresh combs (up to almost three times higher in old comb; see Table S9 in the supplemental material), comb samples remained as two distinct clusters, with old combs being more similar to old major worker guts and fresh comb being more similar to nodules and old and young minor worker guts (Fig. 2c).

CPH assays corroborated our AZCL enzyme assays; however, galactomannan, xyloglucan and rhamnogalacturonan-degrading activities were not statistically different across colony components ($F(6) = 3.227$, $p = 0.870$ for galactomannan, and $\chi^2(6) = 9.353$, $p = 1.000$ for xyloglucan and $\chi^2(6) = 14.24$, $p = 1.000$). As we only had sufficient material to carry out CPH on M. natalensis (see Table S8 in the supplemental material), our main analyses and discussion focuses on the AZCL results. The comparisons of the results of AZCL and CPH (see Fig. S2a in the supplemental material) indicated that (i) although AZCL and CPH use different measurement scales (cm² and absorbance), comparable patterns were obtained (correlation analyses; $R^2 = 56.5$%), and (ii) some activities were detected with CPH that were absent in the AZCL assay; e.g., amylase was only detected with AZCL in guts, while CPH detected activities across all colony components (Table S7, S8). The clustering analysis of CPH and AZCL compositions (Fig. S2b) showed that the enzyme activity estimates were largely comparable.

Which plant polymers are decomposed in the fungus-growing termite symbiosis?
We were able to characterize 40-64% of the plant-derived components content in foraged substrates (Fig. 3a, see Table S10 in the supplemental material), with the remaining content most likely being microbial biomass, soluble sugars, and ash. Cellulose, lignin, xylose (derived from xylan), and glucose were the most abundant compounds in all forage substrate types (averages 27.9±2.2%, 12.2±1.0%, 8.2±1.1%, and 3.0±0.3%) (Fig. 3a), but lignin was the only one of these major component that was not significantly different between forage types (Table S10). The minor components arabinose, mannose, galactose, fucose, glucuronic, and galacturonic acid accounted for on average only 4.0±0.1% of substrate content, but all but glucuronic acid were significantly different between substrates. These differences appeared to be largely driven by the smaller amounts of most polymers in cow dung Fig. 3ain combination with the small sample sizes (Table S10).

Statistical analyses of the polymer content of forage substrate, fungus comb and guts were not done, because the samples were not comparable, but we were able to perform comparison between fresh and old comb. These indicated that there was a total reduction in plant polymers from 38-46% of the total biomass in fresh combs to 30-44% in old combs (Fig. 3a). Again, cellulose, lignin, xylose, and glucose were the most abundant compounds that were reduced from 13.7±0.5%, 12.9±0.5%, 6.7±1.1%, and 4.0±0.5% in fresh combs to 12.5±1.6%, 11.6±0.7%, 5.5±0.6%, and 4.0±0.3% in old combs (Fig. 3a).

Old workers eat the old comb, and polymer contents in guts was thus unsurprisingly further reduced to only 2.4±1.1%, 4.0±0.3%, 0.6±0.1%, and 4.9±0.3%, for cellulose, lignin, xylose, and glucose, respectively. Thus, while xylan and cellulose appeared to be most decomposed plant components within the comb, lignin was not greatly reduced ($F_{(1)}$...
before reaching old major worker guts. Glucose was the only monomer that increased in old major worker guts (Fig. 3a, Table S8).

The CoMPP analyses confirmed the overall pattern obtained by acid hydrolyses, but provided a more detailed overview of changes in plant components (Fig. 3b, see Table S11 in the supplemental material). A one-way ANOVA revealed a significant difference in polymer composition across termite species ($F_{(7)} = 3.934, p = 0.0004$); however, the relative amount of polysaccharides was not statistically significantly different between forage material and fresh combs ($F_{(10)} = 0.76, p = 0.666$). On the other hand, the diversity of polysaccharides was higher in fungus combs than forage material ($t_{(25)} = -2.917, p = 0.007$). Hemicelluloses were abundant and these polysaccharides were consumed during the decomposition process (Fig. 3b). While most plant components decreased from forage material to fungus comb and subsequently worker guts (Fig. 3b), some (e.g., pectins) were higher in fresh comb than forage material, probably because other substrates were harvested by these colonies than those included in our content analyses. As expected, pectins were either absent or present in very low amounts in termite guts ($F_{(7)} = 3.934, p = 0.0004$, Fig. 3b).

**Discussion**

*Plant biomass decomposition in the fungus-growing termite symbiosis*

The presence of a diverse assembly of plant-degrading enzymes, involving key GHs, AAs, CEs, and PLs, coded for by *Termitomyces*, underlines previous suggestions that the termite fungus can produce the enzymes necessary to decompose complex substrates (24). Their differential presence at different stages of the decomposition process...
highlights how the integrated combination of gut passage and an external fungal symbiont serve as an effective means to obtain very efficient plant biomass degradation.

It was first proposed in 1981 that the ingestion of nodules by termite workers was a strategy to increase the fungal load in the top part of the fungus comb (10). Later, this was confirmed to be important for substrate inoculation and to assure monoculture farming through frequency-dependent selection of a single *Termitomyces* strain within a colony (66). It was also hypothesized that the first gut passage could facilitate enzyme transport to new substrates (‘the ruminant hypothesis’) (29). Accordingly, *Termitomyces* could use the first gut passage to efficiently move ligno-cellulosic enzymes from the old to the fresh comb. In our comparisons, fresh and old comb were distinct from each other and separated from guts and nodules (Fig. 2a), but normalization of enzyme activities indicated that this was not driven by differences in enzyme concentrations due to fungal content, because fresh comb became more similar to nodules and young worker guts than old comb in PCoA space (Fig. 2c). Although our enzyme assays cannot discriminate between fungal, termite, and bacterial enzymes expressed within guts, the most parsimonious explanation for the similarities in composition and activity of guts and nodules is that most of the enzymes that are active in guts are of fungal origin and originating from the exclusively fungal nodules (28, 29).

Our preliminary fungal RNAseq indicated the expression of a wide range of *Termitomyces* enzymes in nodules, fresh and old comb. This implies that even if our assertion that guts serve to transport enzymes is true, this transport is complemented by the expression of enzymes *in situ*. This combination of enzyme transport and *in situ* expression may be key to efficient boosting of plant biomass degradation after substrate
inoculation; possibly a key innovation contributing to these insects becoming major decomposers in the Old World (7, 8).

Which plant polymers are decomposed in the fungus-growing termite symbiosis?

Forage materials contained high concentrations of lignin, cellulose, and hemicellulose, of which most was depleted during the decomposition process. The high enzyme activities in guts could support previous suggestions that decomposition is initiated here (32, 67). However, the considerable amount of plant content in fresh combs suggests that guts are unlikely to be the prime location for decomposition. Recent work has suggested that gut passage in young Odontotermes formosanus workers could initiate lignin degradation (32). In contrast, we found high lignin content in the fresh comb, but instead that the expression of AAs targeting lignin was high in the fresh comb. Although we cannot rule out that this contrast is due to different methods applied, it could suggest that termite-Termitomyces species pairings differ in lignin processing. After old workers digest the old comb in a second gut passage, plant biomass is essentially completely degraded (28), which is consistent with our findings of very low polymer content in old worker guts, which did contain some plant polymers as we sampled during the process of digestion and not from the excreted final feces, where all polymers are expected to have been utilized.

There was a highly significant decrease in the amount of cellulose content from forage material to the old worker termite guts, suggesting that Termitomyces uses cellulose as one of its main nutrient sources, and the transcriptome analysis showing the expression of a host of cellulose- and hemicellulose-degrading enzymes. The low expression of enzymes targeting pectin (PLs) likely reflects that the substrate collected by the termites...
is mostly dead plant material with low pectin content. One of the most distinct changes in plant polymer amounts was in old termite guts, for which both plant polymer assays showed a substantial reduction in plant-cell wall polymers, with only glucose increasing in concentration. *Termitomyces* is able to grow on many carbon sources, but among the mono-, di- and oligosaccharides, it grows best on cellobiose (24), and this was corroborated by the high expression of cellbiohydrolases in our transcriptomes. Gut microbes encode genes to break down fungus-cell wall components and simple sugars, and since *Termitomyces* does not grow well on glucose alone (24), the termites may obtain glucose when digesting old comb (cf. 68).

Our findings support that the maintenance of an external fungus comb, inoculated with an optimal mix of plant material, fungal spores, and enzymes, is likely the key to the extraordinarily efficient plant decomposition in fungus-growing termites. The transcript analysis identified enzymes targeting not only all complex polysaccharides, but also oxygen-dependent enzymes (e.g. LPMOs), supporting that the comb serves as a versatile aerobic plant biomass conversion bioreactor. The efficiency of this bioreactor is likely comparable to decomposition in ruminants, but naturally with major differences between the two: The enzymes (functions and families) involved are markedly different between, because biomass conversion is aerobic in the fungus comb. In the anaerobic rumen, biomass is converted to oligo-, di- and monosaccharides that can be fermented to short-chain fatty acids and alcohols, which serve as nutrients for the host animal, while degraded plant components are primarily consumed by *Termitomyces*, with fungal biomass only later providing nutrition for the farming termites.

**Conflict of interest**
The authors declare no conflict of interest.

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Author Contributions

RRDC designed the experiments and analyses, collected in the field, carried out AZCL and CPH enzymes assays, barcoded the termites, extracted RNA, performed plant polymer measurements, and drafted the first version of figures, tables, and the manuscript; KSKP helped perform plant polymer measurements; HH assembled and annotated RNA sequences; BP, PKB, and LL carried out Hotpep analysis and contributed with data interpretation; JS and SKK helped design CPH enzyme screening and provided suggestions on substrates; JS facilitated access to CoMPP; SV helped design the study, helped in the field, and extracted RNA; JH helped with plant content analyses; DKA helped design the experiments and with interpretations, PS helped in experimental design and statistics; and MP supervised RRDC, designed the study, contributed with comments.
on analyses and the first versions of Figures, tables, and text. All authors contributed to writing the manuscript.

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Fig. 1. Carbohydrate active enzyme activities through the plant decomposition process. (a) Heatmap of enzyme diversity and activity in nodules and guts from different fungus-growing termite species, and similarity analyses based on Euclidean clustering with bootstrap support after 10,000 permutations. AZCL substrate and the enzymes targeting them, respectively.

Fig. 2. Enzyme activities across different stages of the decomposition process. (a) Schematic illustration of the decomposition process within a fungus-growing termite mound: (i) Old major workers collect plant substrate from surroundings of the mound. (ii) These forage plant substrate are processed by young and old minor workers. These minor workers ingest the plant substrate along with *Termitomyces* asexual spores found in the nodules. (iii) This mixture passes through the termite gut (first gut passage) and is deposited as fresh comb. (iv) Once this mixture is inoculated in the fresh comb, *Termitomyces* breaks down complex plant cell wall components, and, as the comb matures, new nodules are produced. (v) When the plant substrate is utilized by *Termitomyces*, old and young major (dark green) workers feed on the old part of the fungus comb (blue) and after a second gut passage, all the organic matter is essentially decomposed. (b) Principal Coordinate Analyses (PCoA) of AZCL enzyme activities in colony components and worker castes and ages. (c) PCoA of enzyme activities in colony components and worker castes and ages after normalization of fungus comb enzyme activity based on the relative abundance of fungus biomass. Color code: old major workers: purple, young major worker: dark green, young minor worker: red, old minor workers: petrol blue, nodules: orange, fresh comb: light green.
Fig. 3. Plant biomass degradation in fungus-growing termites. (a) Content of

polysaccharides expressed in % per gram of AIR sample. Cellulose content was measured
by using 4% sulfuric acid hydrolysis, lignin was measured using Acetyl-bromide and
non-cellulosic polymers were measured using trifluoroacetic acid. Although these
analyses were not performed on nodules, we include an image of them to show their
presence within comb. (b) CoMPP heatmap illustrating the distribution and relative
amount of plant polymers in forage material, fungus comb and termite guts based on
NaOH extraction. The spot signal values (Table S5) are correlated to color intensity.

Polysaccharide epitopes and their monoclonal antibodies are shown above. The values

correspond to averages of nests (M. natalensis: n=6, O. sp.: n=4, and O. cf. badius: n=3)

and forage substrate based on its type (dry wood n=4, n=4, n=2; cow dung n= 2, n=1,
n=1; for M. natalensis, O. sp and O. cf. badius, respectively, and decaying wood and bark
n=1 for M. natalensis) (Table 1).
Tables legends

Table 1: Termite species, year of collection, geographical location, GenBank accession numbers for *Odontotermes* samples that needed identification and biological replicates used the different experiments. An “x” indicates that samples from those nests were used in the given experiment. *O.* sp: *Odontotermes* sp., *O.* cf. *badius*: *Odontotermes* cf. *badius*, *M. natalensis*: *Macrotermes natalensis*, EF: Experimental farm, MO: Mookgophong, ARC: Rietondale, F.C.: Fresh comb, O.C.: Old comb, Nod: Fungal nodules, *O.* mj. w.: Old major worker guts, *O.* mi. w.: Old minor worker guts, *Y.* mj. w.: Young major worker guts, *Y.* mi. w.: Young minor worker guts. * samples that were RNA sequenced successfully.

Table 2: Forage substrate sampling information, type of substrate, geographical location, termite species, GenBank accession numbers for *Odontotermes* foragers identified with Cytochrome Oxidase II gene, and date of substrate collection.

Table 3: Expression level and distribution of transcripts on target substrates. *Termitomyces* fungal samples from *Macrotermes natalensis* and *Odontotermes* sp. The expression levels in Transcripts per Million are given at the left, while the diversity of enzymes within different CAZy families are given on the right. The transcripts were classified according to EC number functions (top) and to predicted substrate specificities (bottom). For the full results, see Tables S5 and S6. Nod.: Fungal nodules, F.C.: Fresh comb, O.C.: Old comb.
Table 4: Expression levels (Transcript per Million – TPM) of CAZymes across different sites of the decomposition process in one colony of Macrotermes natalensis and two Odontotermes sp. colonies. Functions were predicted using Hotpep (PPR), sorted by substrate targets and listed according to predicted functions based on EC number. White shading represents the lowest number of transcripts, yellow intermediate and red highest number. For the full results, see Tables S6 and S7. Nod.: Fungal nodules, F.C.: Fresh comb, O.C.: Old comb.
Figure 2.

(a) Fresh comb

(iv) Nodules

(iii)

(ii)

(v)

Old comb

(b) Odontotermes sp.

Odontotermes cf. badius

Macrotermes natalensis

Outlined symbols: from 2015

Not outlined symbols: from 2016

PCO2 - 12.5%

PCO1 - 66.4%

Axis 2

Axis 1

PCO2 - 6.2%

PCO1 - 86.5%
Table 1.

| Termite species and colony code | Year of collection | Location | GenBank accession numbers | GPS | Enzyme assays | Plant polymer content | RNA extraction | Fungal biomass measurement |
|--------------------------------|-------------------|----------|---------------------------|-----|---------------|-----------------------|---------------|---------------------------|
| O. sp. Od127                  | 2015/2016         | EF       | EF KJ4590690 (Otani et al. 2014) | S25 44.562 E28 15.391 | x x x x & s x x | x x x x s x x x x x |  & x x x x & x x x x | x x x x & s x x x x |
| O. sp. Od128                  | 2015              | EF       | EF KJ4590691 (Otani et al. 2014) | S25 44.544 E28 15.397 | x x x x & s x x | x x x x s x x x x |  & x x x x & x x x x | x x x x & s x x x x |
| O. cf. badius Od145           | 2015              | EF       | MF092801                  | S25 45.118 E28 15.525 | x x x x & s x x | x x x x s x x x x |  & x x x x & x x x x | x x x x & s x x x x |
| O. cf. badius Od150           | 2015              | ARC      | MF092802                  | S25 43.666 E28 14.112 | x x x x & s x x | x x x x s x x x x |  & x x x x & x x x x | x x x x & s x x x x |
| O. cf. badius Od151           | 2015              | ARC      | MF092803                  | S25 43.650 E28 14.128 | x x x x & s x x | x x x x s x x x x |  & x x x x & x x x x | x x x x & s x x x x |
| O. sp. Od159                  | 2015              | EF       | MF092804                  | S25 44.826 E28 15.337 | x x x x & s x x | x x x x s x x x x |  & x x x x & x x x x | x x x x & s x x x x |
| M. natalensis Mn156           | 2015              | EF       | NA                      | S25 44.623 E28 15.391 | x x x x & s x x | x x x x s x x x x |  & x x x x & x x x x | x x x x & s x x x x |
| M. natalensis Mn160           | 2015              | EF       | NA                      | S25 44.578 E28 15.391 | x x x x & s x x | x x x x s x x x x |  & x x x x & x x x x | x x x x & s x x x x |
| M. natalensis Mn173           | 2016              | MO       | NA                      | S25 44.623 E28 15.391 | x x x x & s x x | x x x x s x x x x |  & x x x x & x x x x | x x x x & s x x x x |
Table 2.

| Type of substrate | GPS          | Location   | Forager species | GenBank accession numbers | Collection date |
|-------------------|--------------|------------|-----------------|----------------------------|-----------------|
| Dry wood          | S25 43.746 E28 14.453 | Rietondale | O. sp.          | MF092793                   | 18-01-2016      |
| Dry wood          | S25 43.712 E28 14.444 | Rietondale | O. cf. badius   | MF092794                   | 18-01-2016      |
| Dry wood          | S25 43.681 E28 14.418 | Rietondale | O. cf. badius   | MF092795                   | 18-01-2016      |
| Dry wood          | S25 43.743 E28 14.523 | Rietondale | O. sp.          | MF092796                   | 18-01-2016      |
| Dry wood          | S25 43.781 E28 14.433 | Rietondale | O. sp.          | MF092797                   | 18-01-2016      |
| Decaying wood     | S26 48.929 E30 42.660 | Iswepe     | M. natalensis   | N/A                        | 24-01-2016      |
| Cow dung          | S26 48.908 E30 42.677 | Iswepe     | M. natalensis   | N/A                        | 24-01-2016      |
| Bark              | S26 48.907 E30 42.678 | Iswepe     | M. natalensis   | N/A                        | 24-01-2016      |
| Bark              | S26 48.911 E30 42.689 | Iswepe     | M. natalensis   | N/A                        | 24-01-2016      |
| Dry wood          | S26 48.836 E30 42.634 | Iswepe     | M. natalensis   | N/A                        | 24-01-2016      |
| Cow dung          | S24 44.499 E28 15.605 | Experimental farm | O. cf. badius | MF092798                   | 01-02-2016      |
| Cow dung          | S24 39.695 E28 47.590 | Mookgophong | M. natalensis   | N/A                        | 03-02-2016      |
| Cow dung          | S24 40.463 E28 48.275 | Mookgophong | O. sp.          | MF092799                   | 03-02-2016      |
| Dry wood          | S24 43.738 E28 14.121 | Rietondale | M. natalensis   | N/A                        | 05-02-2016      |
| Dry wood          | S24 43.784 E28 14.126 | Rietondale | M. natalensis   | N/A                        | 05-02-2016      |
| Decaying wood     | S25 43.893 E28 14.136 | Rietondale | M. natalensis   | N/A                        | 05-02-2016      |
| Dry wood          | S25 43.934 E28 14.140 | Rietondale | M. natalensis   | N/A                        | 05-02-2016      |
| Dry wood          | S25 43.953 E28 14.131 | Rietondale | O. sp.          | MF092800                   | 05-02-2016      |
| Classification | M. natalensis 156 | O. sp. 127 | O. sp. 128 | The number of transcript sequences placed in different CAZyme families |
|----------------|------------------|------------|------------|-------------------------------------------------|
|                | F.C. | O.C. | F.C. | O.C. | F.C. | O.C. | F.C. | O.C. | F.C. | O.C. |
| **Auxiliary Activities** | 1 638 | 2 623 | 4 504 | 140.7 | 7 116 | 3 946 | 2 054 | 6 176 | 2 872 | 194 | 151 |
| **Polysaccharide Lyases** | 738.1 | 9 538 | 13 117 | 407.8 | 1 691 | 2 054 | 6 176 | 2 872 | 194 | 151 |
| **Carbohydrate Esterases** | 6 056 | 3 289 | 5 006 | 738.1 | 13 117 | 4 054 | 6 176 | 2 872 | 194 | 151 |
| **Glycoside Hydrolases** | 5 026 | 7 664 | 11 253 | 437.0 | 13 117 | 4 054 | 6 176 | 2 872 | 194 | 151 |

**Table 3.**

Expression level (Transcript per Million) The number of transcript sequences placed in different CAZyme families.
Table 4.

| Predicted substrate | EC #          | Predicted function | Macrotermes natalensis (Mn156) | Odontotermes sp. |
|---------------------|---------------|--------------------|---------------------------------|------------------|
|                     | Nodules (127) | Fresh comb (127)   | Old comb (127)                  | Nodules (128)    | Fresh comb (128) | Old comb (128) |
| LPMO                |               |                    |                                 |                  |
| AA9 expanded family |               |                    |                                 |                  |
| 1.1.99.18           | 600.8         | 197.5              | 177.3                          | 161.6            | 341.7            | 2 257          |
| Cellulose dehydrogenase (acceptor) | 190.0 | 453.2              | 302.1                          | 166.1            | 84.80            | 96.73          |
| Pyranose dehydrogenase (acceptor) | 13.77 | 36.08              | 8.600                          | 13.88            | 27.29            | 4 790          |
| 3.2.1.14            | 102.5         | 176.3              | 181.9                          | 393.1            | 562.0            | 1 526          |
| β-glucosidase       | 565.3         | 333.9              | 536.1                          | 7 532            | 474.4            | 750.1          |
| 3.2.1.21            | 51.11         | 121.6              | 212.3                          | 230.9            |                  |                |
| Cellulose-1,4-β-cellobiosidase (non-reducing end) | 41.81 | 310.5              | 1 389                          | 226.0            |                  |                |
| Sum                 | 1 571         | 1 629              | 2 816                          | 517              | 1 639            | 30 990         |
| Hemi-cellulose      |               |                    |                                 |                  |
| 3.1.1.6 acetyltransferase | 30.75   | 121.5              | 251.1                          | 253.7            | 41.65            | 30 990         |
| 3.1.1.73 Feruloyl esterase | -     | -                  | -                              |                  |                  |                |
| 3.1.1.72 Acetylxylosidase | 3.756 | 235.1              | 488.1                          | 217.1            | 145.3            | 775.7          |
| 3.2.1.55 α-N-arabinofuranosidase | 33.76 | 44.47              | 352.4                          | 13.44            | 121.5            | 1 594          |
| 3.2.1.8 Endo-1,4-β-xylanase | 110.5 | 760.4              | 984.9                          | 5389             |                  |                |
| 3.2.1.37 Xylan-1,4-β-xylanidase | 717.5 | 413.5              | 795.8                          | 252.9            | 317.9            | 1 976          |
| 3.2.1.131 Xylan-α-1,2-glucuronosidase | 41.80 | 140.5              | 150.8                          | 100.4            |                  |                |
| 3.2.1.177 α-D-xylanide-xylohydrolase | 53.50 | 129.3              | 128.3                          | 6.24             |                  |                |
| 3.2.1.151 xyloglucan-specific endo-β-1,4-glucanase | 36.04 | 112.6              | 104.9                          | 942.6            |                  |                |
| Sum                 | 1 166         | 1 957              | 3 256                          | 1 642            | 1 639            | 10 723         |
| Lignin              |               |                    |                                 |                  |
| 1.10.3.2 Laccase    | 550.4         | 1 662              | 2 816                          | 11 677           | 4 606            | 478.3          |
| 3.1.1* 4-O-methyl-glucuronoyl methyltransferase | 47.97 | 346.6              | 809.8                          | 48.57            | 112.2            | 942.6          |
| 1.11.1.16 Versatile peroxidase | 2.070 | 542.2              | 95.25                          | 1 05.4           |                  |                |
| Sum                 | 600.4         | 2 352              | 2 822                          | 1 686            | 4 722            | 1 422          |
| Pectin              |               |                    |                                 |                  |
| 4.2.2.2 Pectate lyase | 100.7    | 239.8              | 191.9                          | 237.3            | 26.36            | 33.64          |
| 4.2.2.10 Pectin lyase | -        | -                  | -                              | -                | -                | -              |
| Enzyme Class          | Enzyme Name                          | Unit 1 | Unit 2 | Unit 3 | Unit 4 | Unit 5 | Unit 6 | Unit 7 | Unit 8 | Unit 9 |
|-----------------------|--------------------------------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| 3.1.1.*               | Pectin acetyl esterase, rhamnogalacturonan acetyl esterase | 65.90  | 67.51  | 175.4  | 3.530  | 106.8  | 133.8  | 340.5  |
| 3.1.1.11              | Pectinesterase                       | 28.17  | 98.87  | 98.62  | 0.950  | 232.9  | 65.65  | 21.00  | 18.22  | 74.66  |
| 3.2.1.15              | Polygalacturonase                    | 24.97  | 113.7  | 225.9  | 2.010  | 98.16  | 342.7  | 14.81  | 499.1  | 190.3  |
| 3.2.1.67              | Galacturonan-1,4-α-galacturonidase   | 223.9  | 26.45  | 123.7  | -      | 98.29  | 302.8  | 17.38  | 49.92  | 194.4  |
| 4.2.2.*               | Rhamnogalacturonan lyase             | 57.96  | 58.57  | 84.37  | 2.010  | 232.9  | 116.4  | 9.77   | 44.1   | 940.7  |
| 3.2.1.114             | Rhamnogalacturonan rhamnolysase      | 94.06  | 15.68  | 10.02  | -      | 24.36  | 116.4  | 9.77   | 44.1   | 940.7  |
| 3.2.1.40              | α-L-rhamnosidase                     | 30.65  | 147.4  | 170.5  | 2.980  | 77.5   | 142.3  | 92.41  | 40.57  | 221.7  |
| Sum                   |                                      | 626.3  | 766.0  | 1080   | 24.00  | 1127   | 1413   | 542.4  | 834.6  | 2045   |
| 3.2.1.1               | α-amylase                           | 98.25  | 156.3  | 168    | 16.23  | 132.0  | -      | 26.01  | 14.71  | 13.94  |
| 3.2.1.3               | Glucan-1,4-α-glucosidase             | 207.2  | 178.2  | 65.63  | 7.030  | 291.5  | 367.8  | 2.742  | 130.7  | 189.1  |
| 3.2.1.20              | α-glucosidase                       | 101.4  | 128.0  | 146.9  | 199.9  | 75.44  | 349.5  | 95.6   | 78.11  | 155.6  |
| 3.2.1.68              | Isoamylase                          | -      | -      | -      | 0.840  | -      | -      | -      | -      | -      |
| Sum                   |                                      | 406.9  | 462.5  | 380.2  | 224.0  | 499.0  | 717.2  | 2.864  | 223.5  | 358.7  |
| 3.2.1.89              | Arabinogalactan endo-β-1,4-galactanase | 10.88  | 16.30  | 53.68  | -      | 21.02  | 82.69  | 9.720  | 25.61  | 82.49  |
| 3.2.1.22              | α-galactosidase                     | 9.870  | 143.7  | 173.1  | 26.20  | 283.2  | 281.2  | 43.42  | 80.90  | 192.4  |
| 3.2.1.23              | β-galactosidase                     | 383.9  | 101.4  | 348.8  | 3.560  | 649.6  | 135.9  | 74.06  | 229.0  | 206.1  |
| 3.2.1.99              | Arabinan endo-1,5-α-L-arabinanase    | 28.14  | 47.01  | 102.1  | -      | 83.13  | 86.58  | 16.27  | 24.38  | 362.3  |
| 3.2.1.145             | Galactan 1,3β-galactosidase          | 37.09  | 42.69  | 35.22  | 1.830  | 22.96  | 89.03  | 14.68  | 19.14  | 86.73  |
| Sum                   |                                      | 469.9  | 351.1  | 713.0  | 31.59  | 1060   | 675.4  | 158.2  | 379.0  | 930.0  |
| 3.2.1.78              | Mannan endo-1,4-β-mannosidase        | 37.92  | 42.51  | 34.01  | 3.780  | 260.9  | 60.05  | 9.170  | 22.14  | 119.9  |
| 3.2.1.25              | β-mannosidase                       | 147.2  | 103.2  | 151.5  | 25.93  | 133.2  | 261.3  | 10.99  | 36.05  | 100.4  |
| Sum                   |                                      | 185.1  | 145.7  | 185.5  | 31.70  | 394.1  | 321.4  | 20.20  | 58.20  | 220.3  |
| Total Sum             |                                      | 5026   | 7664   | 11253  | 1001   | 33952  | 27963  | 9162   | 46689  |        |