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Isolation and Characterization of Lignocellulolytic Bacteria from Fertile Soil for Enzyme Production

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Abstract

Lignocellulolytic bacteria have promised to be a fruitful source of new enzymes for next-generation lignocellulosic biofuel production. Puerto Rican tropical forest soils were targeted because the resident microbes decompose biomass quickly and to near-completion. Isolates were initially screened based on growth on cellulose or lignin in minimal media. 75 Isolates were further tested for the following lignocellulolytic enzyme activities: phenol oxidase, peroxidase, β-D-glucosidase, cellobiohydrolase, β-xylopyranosidase, chitinase, CMCase, and xylanase. Cellulose-derived isolates possessed elevated β-D-glucosidase, CMCase, and cellobiohydrolase activity but depressed phenol oxidase and peroxidase activity, while the contrary was true of lignin isolates, suggesting that these bacteria are specialized to subsist on cellulose or lignin. Cellobiohydrolase and phenol oxidase activity rates could classify lignin and cellulose isolates with 61% accuracy, which demonstrates the utility of model degradation assays. Based on 16S rRNA gene sequencing, all isolates belonged to phyla dominant in the Puerto Rican soils, Proteobacteria, Firmicutes, and Actinobacteria, suggesting that many dominant taxa are capable of the rapid lignocellulose degradation

characteristic of these soils. The isolated genera *Aquitalea*, Bacillus, Burkholderia, Cupriavidus, Gordonia and Paenibacillus represent rarely or never before studied lignolytic or cellulolytic species and were undetected by metagenomic analysis of the soils. The study revealed a relationship between phylogeny and lignocellulose-degrading potential, supported by Kruskal–Walli's statistics which showed that enzyme activities of cultivated phyla and genera were different enough to be considered representatives of distinct populations. This can better inform future experiments and enzyme discovery efforts.

Keywords: Soil lignocellulolytic bacteria, Cellulose, Lignin, Tropical Forest, Biomass degradation, Biofuels

Introduction

Bioenergy from biomass is the leading form of renewable energy production in the United States. However, the main hurdle in production is the plant cell wall's recalcitrance to saccharification due to its tightly interwoven structural components – cellulose, hemicelluloses, and lignin. Therefore, these structures and bonds must be more efficiently broken to liberate more fermentable sugars that are inexpensive enough for biofuel production.

The isolation and characterization of environmental strains are relatively simple strategies in the age of metagenomics, but they are still crucial for understanding the broad range of natural microbial functions. For example, the isolation of bacteria subsisting on antibiotics by Dantas et al. was a major discovery that uprooted paradigms about microbial metabolism and antibiotic resistance. In our own lab, physiological study of a tropical soil isolate *Enterobacter lignolyticus* SCF1 was shown to use lignin as an assimilatory and dissimilatory carbon source, which would have been impossible to discern based on sequence analysis alone . Even when metagenomes of an environment are available, the characterization of pure strains can provide useful information for physiological inferences since metagenomes are only an "incomplete list of parts". Though laboratory cultivations have only isolated a small fraction of bacterial organisms , the range of cultivable soil bacteria is expanding with simple improvements to cultivation strategies.

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The diversity and functionality of lignocellulolytic Puerto Rican tropical forest soil bacteria have not been as thoroughly investigated as termite hindgut and compost, despite that tropical forests have the fastest rates of terrestrial litter decomposition and a vast unknown microbial diversity. Fast decomposition combined with frequent low and fluctuating redox potential suggests the presence of potentially novel and efficient lignocellulolytic bacteria. Small subunit ribosomal RNA (rRNA) amplicon pyrosequencing indicates the presence of many uncultivated phyla in these soils. Furthermore, the metagenomic data contains numerous uncharacterized glycoside hydrolases and glycosyl transferases that cannot be assigned to specific families, supporting the possibility of a vast, as-yet undiscovered functional diversity.

The goal of this broad survey was to collect novel lignocellulolytic strains and determine any associations between phylogeny and function that will better inform larger scale cultivation or sequencing efforts.

Materials and Methods

Isolate Cultivation

Isolates were cultivated from soils collected from the Luquillo Experimental Forest, part of the NSF-sponsored Long-Term Ecological Research Program in Puerto Rico. The samples were collected and transported under USDA permit number P526P-08-00634. Soil collected from two sites, the Bisley watershed ridge rain forest site and Short Could Forest site, were used as inoculum. The rain forest site is in a lower montane wet tropical forest at approximately 270 m above sea level (18°18′ N, 65°50′ W), and receives approximately 3.5 m of rainfall annually. The cloud forest site is located in a upper montane tropical cloud forest at approximately 1050 m above sea level (18°18′ N, 65°50′ W) and experiences approximately 4–5 m rainfall annually, and a high frequency of low redox conditions. Soil cores were transported to the lab in plastic bags at ambient temperature, diluted, and used as inoculum for growth within one week of collection.

About one gram of soil was added to 10 mL of one of the two minimal salts bases with 0.1% sodium pyrophosphate and 0.03% Tween 80. The minimal salts bases were modified VL55 or basal salts minimal medium (BMM); the details of the media composition are described

below. The mixture was homogenized by 2 rounds of vortexing for 1 min and sonicating for another minute. A serial dilution of the slurry was created in the minimal salts base. Aliquots of 100 µL of dilutions from 10^{-1} to 10^{-10} were spread onto isolation media agar plates using a sterile glass spreader, parafilmed to maintain moisture, and incubated at room temperature, 30 °C, 37 °C, and 55 °C.

Isolation media agar consisted of one of the two different defined media (VL55 or BMM) and one of the three carbon sources (Sigma–Aldrich microgranular cellulose, carboxymethyl cellulose, or alkali lignin). Modified VL55 defined medium contained 0.10 mM $MgSO_4$, 0.30 mM $CaCl_2$, and 0.20 mM $(NH_4)_2HPO_4$ and 2.50 mL L^{-1} trace minerals pH 6.0 (1.50 g L^{-1} of Nitrilotriacetic acid disodium salt, 3.0 g L¯1 $MgSO_4 \cdot 7H_2O$, 0.50 g L^{-1} $MnSO_4 \cdot H_2O$, 1.0 g L^{-1} NaCl, 0.10 g L^{-1} $FeSO_4 \cdot 7H_2O$, 0.10 g L^{-1} $CaCl_2 \cdot 2H_2O$, 0.10 g L^{-1} $CoCl_2 \cdot 6H_2O$, 0.13 g L^{-1} ZnCl, 0.01 g L^{-1} $CuSO_4 \cdot 5H_2O$, 0.01 g L^{-1} $AlK(SO_4)_2 \cdot 12H_2O$, 0.01 g L^{-1} Boric Acid). The VL55 was buffered at pH 5.5 using 3.90 g L^{-1} of the buffer 2-[*N*-morpholino] ethanesulfonic acid (MES). Carbon sources were added at 0.1% or 0.05% final concentration. Immediately before pouring into petri dishes, 10 mL of vitamins solution (2 mg L^{-1} D-Biotin, 2 mg L^{-1} Folic acid, 10 mg L^{-1} Pyridoxine HCl, 5 mg L^{-1} Riboflavin, 5 mg L^{-1} Thiamine, 5 mg L^{-1} Nicotinic acid, 5 mg L^{-1} Pantothenic acid, 0.1 mg L^{-1} of Vitamin B12, 5 mg L^{-1} of P-amino benzoic acid, and 5 mg L^{-1} of D,L-6,8-thiotic acid) was added. Some plates were amended with 1 mL of 1% antibiotic streptomycin or 1% antifungal cycloheximide per liter in attempt to prevent the overgrowth of bacteria or fungi. A second defined media, BMM defined medium, was also used. BMM salts media contained 0.80 g L^{-1} NaCl, 1.0 g L^{-1} NH_4Cl, 0.10 g L^{-1} KCl, 0.10 g L^{-1} KH_2PO_4, 0.80 g L^{-1} $MgCl_2 \cdot 6H_2O$, and 4.0 g L^{-1} $CaCl_2 \cdot 2H_2O$. BMM media was also buffered with 10 g L^{-1} MES at pH 6, in addition to the trace minerals, vitamins and one cellulose or lignin substrate at the previously listed concentrations. Plates were poured with media and 15 g L^{-1} agar. Isolates were re-streaked to isolation before further testing. Isolated colonies were subcultured onto 10% Tryptic Soy Broth (TSB) agar to maximize biomass growth in preparation for enzymatic assays. Frozen stocks were prepared without any serial transfers of cells.

Enzyme Assays

Enzyme activities were determined by measuring the degradation of four different 4-methylumbelliferone (MUB) linked carbohydrate substrates and two phenolic L-3,4-dihydroxyphenylalanine (L-DOPA) solutions: MUB-β-D-glucopyranoside, MUB-β-D-cellobioside, MUB-β-D-xyloside, MUB-*N*-acetyl-β-D-glucosaminide dihydrate, L-3,4-dihydroxyphenylalanine (L-DOPA), L-DOPA with 0.3% H_2O_2. To prepare the cells for the enzymatic assays, cell cultures of isolates were grown overnight in 10% TSB at 30 °C, shaking at 200 rpm. The cultures were then diluted using 1× Phosphate Buffered Saline (PBS) to a normalized concentration of 0.2 Optical Density (OD) at 600 nm; cells were sometimes used at 0.10 OD if there was insufficient biomass to achieve 0.20 OD.

Cells were mixed at 1:1 volumetric ratio with each of the 0.10 mM MUB-substrates or 10 mM L-DOPA in technical replicates of three or more. Negative controls consisted of the substrate alone and cells alone. Cells were allowed to digest substrates for 2–9.5 h during which fluorescence or absorbance readings were taken. The quantity of released MUB was calculated using a standard curve of 0–50 μM MUB in 50 mM sodium acetate buffer (pH 5.5). Rates of MUB-substrate degradation are expressed as μmoles of MUB-substrate degraded per $OD_{600\ nm}$ cells loaded per hour. Rates of L-DOPA degradation are expressed as L-DOPA absorbance at 460 nm per $OD_{600\ nm}$ cells loaded per hour.

CMCase and xylanase activity were tested qualitatively by a Congo red based plate assay. Aliquots of 5 μL from 48-h-old liquid culture were spotted onto 10% TSB agar with either 0.5% CMC or xylan. Cells were incubated at 30 °C for 7–10 days before staining with 0.1% Congo Red solution as described in Teather et al. but extended for an hour and followed by 2 washes in 1 M NaCl for an hour each. Lastly, plates were stained with 2% HCl for 5 min to develop better contrast. Any indication of clearing was considered a positive result.

Isolate Identification and Characterization

Isolates were genotyped using the 16S rRNA gene sequence. The 16S rRNA gene was PCR amplified using 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) primers. Each 50 μL PCR reaction contained 1× Takara ExTaq Buffer with $MgCl_2$, 300 pM of both 27F and 1492R primer, 1 mg mL^{-1} of Bovine

Serum Albumin, 200uM of each dNTP, and 2.5 U of Takara ExTaq. The PCR amplification protocol was a 95 °C denaturation step for 3 min, then 25 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min, followed by a 72 °C for 8 min to allow final extension.

A small amount of cell biomass from a single colony growing on 10% TSB agar plates was added to 50 µL of the PCR reaction as template ("colony PCR"). When colony PCR failed, Qiagen DNeasy Blood and Tissue kit was used to extract DNA from isolates, then PCR was performed using 10–50 ng of DNA template. Several isolates required 5 rounds of snap freezing and thawing followed by extracting using a CTAB extraction method. The CTAB/NaCl buffer is 41 g NaCl and 100 g CTAB per liter of solution. Cell biomass from agar plates was resuspended in 740 µL of TE and the concentration adjusted such that the $OD_{600\ nm}$ equaled 1. At this point 20 µL of lysozyme (100 mg mL^{-1}) was added and incubated for 5 min at room temperature, then 40 µL 10% SDS and 8 µL proteinase K (10 mg mL^{-1}) were added and incubated for an additional 1 h at 37 °C. Then, 100 µL 5 M NaCl and 100 µL CTAB/NaCl (pre-heated to 65 °C) were added, then incubated at 65 °C for 10 min. After that, 0.5 mL of chloroform: isoamyl alcohol (24:1) was added mixed, and centrifuged for 10 min. The aqueous phase was transferred and extracted again with 0.5 mL of phenol: chlorofom: isoamyl alcohol (25:24:1), mixed and centrifuged, and the organic phase discarded. DNA in the aqueous phase was precipitated by isopropanol precipitation with one wash step, then resuspended in TE plus RNAse, and incubated at 37 °C for 20 min.

All PCR products were purified using the Qiagen Qiaquick PCR Purification kit following the manufacturer's instructions. The purified PCR product was sent to either the University of California Berkeley Sequencing Facility (Berkeley, CA) or Quintara Biosciences (Emeryville, CA) for sequencing. Nucleotide sequences produced by the forward and reverse primers were concatenated into a contiguous sequence read, NAST aligned against Greengenes reference strains and checked for PCR chimeras using the Greengenes tool Bellerophon. Strain identification at the genus level is based on the Greengenes batch classification of sequences that provides the best matching taxa from multiple taxonomies. Strains with less than 97% similarity were subsequently identified using the EzTaxon-e server. The 16S rRNA gene sequences can be found on Genbank, with accession numbers JQ917930–JQ918000. Sequences were clustered in 14 subgroups based on a nearest neighbor

algorithm, then one representative isolate from each subgroup was BLAST against Puerto Rican soil metagenomes within JGI IMG s blastn module.

Statistical Analyses

Activity rates from MUB-substrate degradation assays for all enzyme activities were normalized between 0 and 1 for principal component analysis and ordination using the package vegan. The neighbor joining tree was made by inserting Greengenes-aligned and chimera-free 16S rRNA gene FASTA files into program ARB using parsimony insertion. The Greengenes tree was used for genetic distances. Four isolates that had 16S rRNA gene sequences with short reads or poorly aligned (<75%) with Greengenes reference strains were excluded from the tree – cmc19, cmc20, lig16, lig38.

All other statistical analyses were performed in JMP10 Pro. Contingency table analysis was used to compare nominal variables. Enzyme activities from MUB and L-DOPA tests were log transformed to more closely achieve normal distributions of values for logistic regression analysis. Enzyme activities below detectable limit assumed values of 0. Logistic regression's response dichotomous variable set cultivation on cellulose as 1, and lignin growth as 0. Receiver operating characteristics (ROC) analysis was used to evaluate the predictive value of the regression. Untransformed data was used for all nonparametric analyses. Wilcoxon tests (non-parametric) were used to compare 2 groups' distributions. Kruskal–Wallis tests (non-parametric) compared more than 2 populations at once. Steel-Dwass (non-parametric) was used to compare distributions pairwise following Kruskal–Wallis analysis. Statistical results were interpreted using techniques outlined by Peng et al.

Results

Seventy-five bacterial strains were isolated from the Puerto Rican Luquillo experimental forest soils based on their ability to grow on a recalcitrant carbon source (lignin or cellulose) as their sole carbon source. Each isolate was cultivated on carboxymethyl cellulose (cmc), alkali lignin (lig), or microgranular cellulose (mgc) in minimal media agar. Isolates were picked from plates with an inoculum dilution factor ranging from 10^{-1} to 10^{-10} with most colonies appearing on plates streaked from the 10^{-5} dilution (Table S3): 5 colonies of *Gordonia*, 4 of *Bacillus*, 12 of *Burkholderia* were isolated at dilutions of 10^{-5} from at least 3

different plates; 4 colonies of AQUITALEA were isolated at dilutions of 10−6. The high dilutions suggest that the most probable number (MPNs) for GORDONIA, BACILLUS, BURKHOLDERIA and AQUITALEA range from 1 × 105 to 1 × 10^6 cells per milliliter. To ensure isolation, single colonies were picked and re-streaked at least two times, checked for homogeneous morphology, and singular 16S ribosomal RNA sequence was determined by PCR. Isolated strains were tested for phenol oxidase, peroxidases, β-D-glucosidase, cellobiohydrolase, β-xylopyranosidases, chitinase, CMCase, and xylanase activity (Tables 1). Cellulose-derived isolates possess elevated β-D-glucosidase, CMCase, and cellobiohydrolase activity. Isolated strains were identified by assigning nearest neighbor based on BLAST results of 16S rRNA gene sequences. Forty of the 75 strains were from lig minimal media agar, 31 strains on cmc, and 4 on mgc (Table 2). On average, colonies appeared within 2 weeks. To cultivate strains of different temperature optimums, replicates of inoculated agar plates were incubated at temperatures between ambient and 55 °C (Table 2). Only 10 of the 75 strains, all *Gordonia* and *Paenibacillus* species, were successfully cultured from plates incubated at or above 37 °C.

Table 1. Enzyme activities summary.

Activity rates	Unit	Mean	Standard deviation
BG	(umoles substrate/(O.D. cells × hour)	2.18	2.09
CBH	(umoles substrate/(O.D. cells × hour)	1.35	0.81
NAG	(umoles substrate/(O.D. cells × hour)	1.48	2.19
XYL	(umoles substrate/(O.D. cells × hour)	0.88	1.40
PO	(O.D. LDOPA/(O.D. cells × hour)	0.16	0.10
HPO	(O.D. LDOPA/(O.D. cells × hour)	0.21	0.26

Empty Cell	Empty Cell	**Frequency (number of isolates)**
CMCase	Positive for activity	36
Xylanase	Positive for activity	33

Table 2. Descriptive statistics of Puerto Rican isolate collection.

Empty Cell	**Isolates *N* = 75**	
Empty Cell	**Frequency**	**Percentage**
Isolation carbon source		
Alkali lignin	40	53
Carboxymethyl cellulose	31	41
Microgranular cellulose	4	5
Genus (Phyla)		
Aquitalea (Proteobacteria)	11	15
Bacillus (Firmicutes)	6	8
Burkholderia (Proteobacteria)	23	31
Cupriavidus (Proteobacteria)	6	8

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Empty Cell	Isolates $N = 75$	
Empty Cell	**Frequency**	**Percentage**
Gordonia (Actinobacteria)	15	20
Paenibacillus (Firmicutes)	3	4
Other	11	15
Isolation temperature		
30°C	12	16
37°C	9	12
55°C	1	1
Ambient	53	71

Discussion

A targeted cultivation strategy was successfully used on Puerto Rican rain forest soil to broaden the known diversity and functionality of bacteria in lignocellulose degradation that would not have been found with metagenomics alone. Puerto Rican soil has not been sufficiently sequenced to determine all the species of bacteria present. Less than 80 16S rRNA gene sequences were annotated in each of the three metagenomes. Pyrotags SSU rRNA of Puerto Rican soil microbial communities trapped on lignin beads could only provide resolution at the phylum and genus level. Without sufficient sequencing to identify dominants, isolation may be the easiest method to discover strain-level diversity.

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The multiple strains of Gordonia, AQUITALEA, Bacillus, and Burkholderia could be dominant taxa in Puerto Rican soil or contributors to rapid plant litter degradation. This agrees with previous SSU rRNA pyrosequencing and Phylochip work where the prevalence of phyla Proteobacteria, Firmicutes, and Actinobacteria most quickly increased in response to lignin enrichments. In addition, the most probable number of the present study's GORDONIA, BACILLUS, BURKHOLDERIA and AQUITALEA strains being at least 105 and 106 organisms per milliliter suggest a large contribution of these to the overall soil population. However, it is still possible that dominant strains eluded cultivation. The most well characterized lignin degrading bacteria like Sphingomonas paucimobilis, Streptomyces VIRIDOSPORUS, and Rhodococcus were not found. Acidobacteria and Chloroflexi constituted a significant amount of some clone libraries or pyrotags from tropical soil but were not cultivated either. Adjusting the media composition and length of incubation may reveal additional diversity of isolates.

One of the few existing culture collections from tropical forest soils also captured similar diversity on lignocellulose from the genera Mycobacterium (genus of ACTINOBACTERIA), BACILLUS and Paenibacillus (genera of FIRMICUTES), BURKHOLDERIA (genus of Betaproteobacteria), Variovorax, Cupriavidus, and PSEUDOMONAS (genera of Gammaproteobacteria). However, no prior reports exist on AQUITALEA, GULBENKIANIA, and PSEUDOGULBENKIANIA as lignocellulose degraders. The latter two strains were potential novel species based on their 16S rRNA sequences but were also functionally different from type strains of the genera, which were either negative for β-D-glucosidase activities or failed to grow on cellobiose in previously published characterizations.

The most active isolates were the cellulose-degrading *Aquitalea* and the lignin-degrading *Gordonia.* The five isolates *Aquitalea* spp. cmc2, cmc3, cmc4, cmc7, and cmc9, (family *Neisseriaceae*) demonstrated the ability to produce all three glycoside hydrolase (GH) activities involved in complete enzymatic hydrolysis of cellulose. These general classes of enzymes are β-1,4-endoglucanase (EC 3.2.1.4) which cleave internal 1-4-glycosidic linkages and are active against CMC; cellobiohydrolase (EC 3.2.1.91) which is an exocellulase that cleaves cellobiose from the non-reducing end and is most active on crystalline cellulose; and

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β-D-glucosidase (EC 3.2.1.21) which finally hydrolyses cellobiose into glucose monomers. Most known cellulolytic strains have rate-limiting steps caused by deficiencies in one or more of these glycoside hydrolases. While some bacteria such as *Bacillus polymyxa* have been found to have all three types of activities, the *Aquitalea* isolates had significantly higher β-D-glucosidase and cellobiohydrolase activity than other isolates of the collection, which include the known cellulose degraders *Paenibacillus, Methylobacterium*, and *Bacillus* . The most active lignin isolates were *Gordonia* species, which supports prior observation of a *Gordonia* strain growing on lignin and its ability to degrade xenobiotics and alkanes including lignin-related alkyl ethers . It is closely related to other lignocellulolytic organisms such as MYCOBACTERIUM, NORCARDIA, and RHODOCOCCUS within the suborder CORYNEBACTERINEAE.

Nutrient contamination is a concern with cultivation studies, but the general association between cellulose isolates and high carbohydrate degradation (β-D-glucosidase, cellobiohydrolase, CMCase) and conversely, lignin isolates and high phenolics degradation (phenol oxidase, peroxidase) makes it likely that the isolates were growing on cellulose or lignin provided as substrates in the isolation media. Current enzyme assays, like Congo Red, are limited in resolution but improvements in sensitivities could strengthen the link between carbon source utilization and enzyme activities so that low CMCase production can be detected in all isolates grown on CMC. However, the general association between isolation substrate and enzyme activity was evident through three different analyses: contingency analysis using CMCase and xylanase rates, principal component analysis of the carbohydrate and phenolics degradation activities, and logistic regression using enzyme activities as a predictor for cellulose and lignin isolates.

This is the first experimental evidence of a possible relationship between lignocellulose degradation activity and phylogeny. Few lignocellulose degrading culture collections have been published within the past decade because of the increasing prevalence of solely culture-independent studies. This has consequently left questions regarding lignocellulolytic function in relationship to phylogeny unaddressed, though at a broad phylogenetic scale there is evidence for phylogenetic coherence based on niche differentiation or the distribution of cellulase genes . A previous isolation study reported no associations between phylogeny and

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function in terms of cellulose utilization {Ulrich, 2008 #112} though there could be niche differentiation between cellulose and lignin based on time of degradation. It has been posited that cellulose degradation emerged via convergent evolution, where traits arise irrespective to lineage. As shown in this study, it is extremely unlikely that the observed activity differences between phylogenetic clades, both at the phyla and genus level, could be attributed to random chance and thereby supports a relationship between phylogeny and function among lignocellulolytic bacteria.

Conclusion

This collection of tropical forest soil isolates are members of a highly lignocellulolytic microbial community and should arguably possess more robust degradation capabilities than bacteria frequently studied only due to literature precedents. Despite the bias associated with cultivation, culture collections can demonstrate the diversity of a functional group of bacteria in a way that molecular analyses alone cannot, and in fact, culture collections overcome cultivation bias if they discover novel or rarely studied species of a selected function as this study has done with lignocellulose degradation. With this approach, we were able to observe that lignocellulolytic function may not be randomly distributed in respect to phylogeny. This investigation of tropical forest soils has provided some of the much-needed insight into the microbial community phylogeny and enzyme capabilities that account for the rapid lignocellulose degradation and whose enzymes could be used for biofuel feedstock deconstruction.

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