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Enzyme activities of aerobic lignocellulolytic bacteria isolated from wet tropical forest soils

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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–Wallis 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.

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.

Although lignocellulolytic fungi such as *Aspergillus*, *Penicillium*, *Schizophyllum*, *Trichoderma*, *Phanerochaete*, and *Sclerotium* [1] can secrete industrial quantities of extracellular enzymes, bacterial enzyme production can be more cost-efficient. This is because they grow more rapidly, produce multi-enzyme complexes with increased functionality and higher specificity, and can tolerate larger and more diverse environmental stress [2–6]. Lignocellulolytic bacteria could also potentially allow better separation of lignin from cellulose and thereby increase the value of both lignin, which is currently a waste product, and cellulose. The few bacterial species currently known to degrade cellulose and lignin are within *Pseudomonas* (order

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Pseudomonadales), *Cellulomonas* (order *Actinomycetales*), *Streptomyces* (order *Actinomycetales*), and other genera within the order *Actinomycetales* [7,8] and are likely employing extracellular laccases and peroxidases.

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. [9] 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 [10]. 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" [11,12]. Though laboratory cultivations have only isolated a small fraction of bacterial organisms [13], the range of cultivable soil bacteria is expanding with simple improvements to cultivation strategies [14,15].

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 [16–22]. 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 [17]. Furthermore, the metagenomic data contains numerous uncharacterized glycoside hydrolases and glycosyl transferases that cannot be assigned to specific families [17], 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 [15] or basal salts minimal medium (BMM) [23]; 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

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