Final Report to ISEN

METAGENOMIC DISCOVERY OF NOVEL LIGNIN-DEGRADING FUNGI FOR BIOFUEL PRODUCTION

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Total Award: $35,000 Duration: 12 months

BACKGROUND AND OBJECTIVES
The bio-conversion of plant lignocellulose to glucose is an key component of second generation biofuel production, but the resistance of lignin to breakdown is a major obstacle in this process. Filamentous fungi possess the unique ability to decompose the aromatic lignin polymers using enzymes encoded by divergent gene families. White-rot fungi are the primary lignin-degraders, and produce phenol oxidases (laccase) or heme peroxidases (lignin, manganese, versatile peroxidases) to completely mineralize lignin and expose the cellulose in the matrix. Brown rots and soft-rots modify the lignin structure and facilitate the access of enzymes produced by white rots. Nevertheless, few fungi are used as pre-treatments for improved bioconversion. In fact, only three species of wood-rotting fungi, most notably *Trichoderma*, are used to depolymerize plant biomass to simple sugars that, in turn, are converted to chemical intermediates and biofuels. Outside of *Trichoderma*, the process of lignin degradation by other types of fungi is largely unknown, and only partial lignin depolymerization and modification have been documented in bacteria.

The primary objective of this project was to examine the diversity and gene products of a lignin-degrading fungal community to identify keystone species and their functions. The ISEN funding has allowed us to use advances in next-generation sequencing and bioinformatics to discover and describe novel fungi associated with the degradation of lignocellulose in a dry seasonal tropical forest. In these forests, the turnover of plant litter is rapid and the microbial diversity is immense but largely unexplored.

RESULTS
1. Novel fungal assemblages are associated with the degradation of lignin
We selected for lignin decomposing fungi by placing in situ traps filled with lignin alkali medium under different tree species at three locations in a minimally managed forest site in the Yucatan Peninsula, Mexico. Traps remained in the field for five months during the wet season, and were then surveyed for fungal taxonomic and functional genetic diversity using high-throughput ITS and metagenome sequencing (barcoding) on an Illumina platform.

We identified novel assemblages- rather than individual species- of fungi associated with the degradation of lignin substrate. The ITS sequencing identified between 18,000 and 80,000 sequences per lignin trap (Figure 1). The decomposer communities were diverse and comprised 100-600 operational taxonomic units per lignin trap, and spanned approximately 300 genera.
Two of the traps were dominated by members of the Ascomycota (cup fungi; LT2, GT1; Figure 2) and included *Cyphellophora*, which had previously been identified as a decomposer of *Miscanthus* and sugarcane biofuels (Table 1).

Members of the Basidiomycota dominated the remaining four traps (Figure 2). These traps contained well-known lignin degrading fungi such as *Geastrum* and taxa within the Polyporaceae (Table 1). However, these were not always apparently dominant taxa, and we also detected ectomycorrhizal (symbiotic) fungal taxa including *Russula* and *Suillus* species, as well as a large diversity of Ascomycota. These results suggest that the rapid and complete decomposition of lignocellulose requires complex species assemblages rather than the current approach of using one or two fungal species.

**Figure 1.** Observed species richness as a function of the cumulative number of sequences analyzed from duplicate traps at three sampling sites. Abbreviations denote locations as follows: GT, Grande; LMT, Little Mayan; LT, Ladder.

**Figure 2.** The composition of the lignin-degrading fungal community identified in duplicate traps at three sampling sites. Vertical bars of color denote the contribution of different taxa to the community. Note that ‘Ascomyc.’ denotes Ascomycota. Abbreviations denote locations as follows: GT, Grande; LMT, Little Mayan; LT, Ladder.
Table 1. Dominant taxa of Ascomycota and Basidiomycota identified at each sampling site. * Denotes known ligninolytic taxa.

<table>
<thead>
<tr>
<th>Fungal phyla (％ of community)</th>
<th>Sample (Abbreviation)</th>
<th>Dominant Ascomycota</th>
<th>Dominant Basidiomycota</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascomycota (50-63％)</td>
<td>Ladder 2 (LT2)</td>
<td>Berkliasmium</td>
<td>Clitopilus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Xenobotrytis</td>
<td></td>
</tr>
<tr>
<td>Grande 1 (GT1)</td>
<td>Cyphellophora</td>
<td>Exophiala</td>
<td>Fusarium</td>
</tr>
<tr>
<td>Basidiomycota (65-95％)</td>
<td>Ladder 1 (LT1)</td>
<td>Beauveria</td>
<td>Geastrum*</td>
</tr>
<tr>
<td>Little Mayan 2 (LMT2)</td>
<td>Trichoderma</td>
<td>Lepiota</td>
<td></td>
</tr>
<tr>
<td>Little Mayan 1 (LM1)</td>
<td>Fusarium</td>
<td>Ceratobasidium</td>
<td></td>
</tr>
<tr>
<td>Grande 2 (GT2)</td>
<td>Aspergillus</td>
<td>Geastrum*</td>
<td></td>
</tr>
</tbody>
</table>

2. Observable fungal fruiting bodies on decayed wood are a small fraction of the potential lignin-degrading species pool
The first fungi used for lignocellulose degradation were those collected as fruiting bodies on decayed wood. For comparative purposes, we collected 88 morphologically distinct fungi growing on decayed wood, identified them to species level using ITS sequencing, and analyzed their status as facultative decomposers or symbionts using ion ratio mass spectrometry (δ¹³C, δ¹⁵N). Sixty-three species could be assigned decomposer status (δ¹³C -26 ‰, δ¹⁵N< 1 ‰), and some of these species were also detected in the metagenomics analyses, e.g., Cookeina and Trametes. However, this count pales in comparison to the ~600 molecular taxa we estimated from analyses of the lignin traps.

3. Geographic location influences fungal diversity
Summarizing the ITS sequencing using multivariate statistics (Figure 3) showed that lignin decomposer communities within each sampling site were closely aligned. However, comparisons among the three sampling sites showed strong compositional differences in their lignin-degrading communities (Figure 2). While some of these differences may be attributed to shifts in the dominant fungal taxa (Table 1), it appears that fungal community composition may also be driven by the local microclimate conditions. These results suggest a degree of functional redundancy in fungal communities, i.e., irrespective of the geographic location the fungal community houses a sufficiently wide diversity of fungal species with the capacity to utilize lignin.
4. Gene products

The ITS sequencing of lignin trap communities enabled us to identify samples with a range of diversity and different dominant taxa to use for exploration of lignin-degradation related genes. These samples are currently awaiting shotgun metagenome sequencing at Argonne National Laboratories. We expect these results will document the presence of genes encoding laccases and peroxidases, as well as other enzymes active in the degradation of less recalcitrant products of lignin decomposition, particularly in samples with a large diversity of organisms not directly involved in the initial stages of lignin breakdown. Metagenome results will additionally provide a secondary estimate of species diversity based on the sequencing of ribosomal DNA fragments that will provide support to estimates based on ITS sequencing.

Implications for biofuel production

- The lignin degrader community is a dynamic consortia comprising ~300 species, rather than a few select species. Because these species work synergistically to degrade lignin, biofuel research might benefit from examining the effect of combinations of fungal enzymes or isolates on degradation processes.
- These novel fungal consortia may also represent fungal communities associated with different stages of lignin decomposition. Future research will enable us to identify these successional processes and identify keystone species at different stages of the decomposition process that could be implemented to provide a controlled succession for biofuel preparation.
- Fungal communities were also distinct among local microsites. Future research should document microsite temperature and moisture in concert with analyses of fungal composition to identify those environmental conditions that best enhance the degradation processes.
- Our findings also show that species of *Leptota* and *Ceratobasidium* may represent new sources of lignocellulolytic enzymes. Future surveys are likely to discover many other
potential candidates.

**Project participants**
ISEN funding provided summer support for Ph.D. student, Benjamin Morgan, and provided him with the opportunity to develop expertise in next-generation sequencing and bioinformatics, and estimates of the diversity of lignin-degrading fungi that will be a part of his dissertation. This project has also provided learning opportunities for three high school students through the College First program at the Chicago Botanic Garden. Mr Kevin Amses, a participant in the Chicago Botanic Garden- Northwestern University REU program contributed to fieldwork. An additional undergraduate, Ms Aarohi Shah, has started working with our lab group to culture and test fungi for their enzymatic capabilities.

**Presentations resulting from this ISEN project**


**Publications**
The results of this study have provided us with sufficient data for two peer-reviewed publications: one on development of the metagenomics methods, and a second cataloging the diversity of lignin-degrading fungi.

**External proposals submitted as a result of this ISEN project**
Title: Microbial controls over the degradation of fungal necromass: implications for soil organic carbon storage
Sponsoring Agency: NSF Division of Environmental Biology
Budget: ~$472,000   Status: Pending

Title: Characterizing the contribution of fungi to the recalcitrant soil organic C pool
Sponsoring Agency: NSF Low Temperature Geology and Geochemistry Program
Budget: ~$650,000   Status: Pending

Title: Doctoral Dissertation Improvement Grant: Assemblage and diversity of litter-decomposing fungi and consequences for nutrient cycling in restored urban ecosystems
Sponsoring Agency: NSF Division of Environmental Biology
Budget: ~$16,500   Status: Pending

Title: Microbial controls over litter decomposition and soil carbon storage
Sponsoring Agency: NASA/ DOE/ USDA
Budget: $471,779   Status: Declined