

## 225. Engineering Anaerobic Gut Fungi for Lignocellulose Breakdown

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**Project Goals:** The goal of this project is to engineer anaerobic gut fungi as novel platform organisms for biofuel production from plant material. To accomplish this goal, a panel of anaerobic fungi will be isolated from different herbivores and screened for their ability to degrade lignocellulose. The basic metabolic networks that govern lignocellulose hydrolysis within anaerobic fungi will also be determined, and models will be generated to describe how important enzyme groups are coordinated during breakdown. Using this information, genetic transformation strategies to manipulate gut fungi will be developed, which would endow them with enhanced functionality against a range of industrially relevant substrates. Collectively, this information will establish the molecular framework for anaerobic fungal hydrolysis, and will guide in the development of lignocellulosic biofuels.

Anaerobic fungi are the primary colonizers of biomass within the digestive tract of large herbivores, where they have evolved unique abilities to break down lignin-rich cellulosic biomass through invasive, filamentous growth and the secretion of powerful lignocellulolytic enzymes and enzyme complexes (fungal cellulosomes). Despite these attractive abilities, considerably less genomic and metabolic data exists for gut fungi compared to well-studied anaerobic bacteria and aerobic fungi that hydrolyze cellulose. This presents a significant knowledge gap in understanding gut fungal function, substrate utilization, and metabolic flux, which has prohibited the genetic and functional modification of gut fungi. Recently, however, advances in sequencing technologies have made it possible to explore the dynamic metabolic networks within gut fungi for the first time. Our approach combines next-generation sequencing with physiological characterization to establish the critical knowledge base to understand lignocellulose breakdown by gut fungi. This project will (1) enable exploration of novel isolates for desirable enzymatic properties, (2) construct metabolic models to describe biomass degradation, and (3) develop new methods to metabolically engineer gut fungi for bioprocessing.

We isolated a panel of novel gut fungi from sheep, goat, giraffe, and elephants at the Santa Barbara Zoo. To date, four unique strains from the *Piromyces*, *Neocallimastix*, *Anaeromyces*, and *Caecomyces* genera have been obtained through roll tube isolation. Proliferation of the fungal isolates was monitored via fermentation gas production, and cellulosomes from each species were isolated through cellulose-precipitation. All of these isolates exhibited high enzymatic reactivity against a range of cellulosic and unpretreated substrates (reed canary grass, switch grass, beechwood xylan), which was repressed in the presence of simple sugars. Within isolated cellulosomes across multiple genera, striking similarities are observed for certain dockerin-fused glycosyl hydrolases, and these proteins are not secreted from fungi when simple sugars are present, supporting the hypothesis that these enzymes are catabolically regulated. We have collaborated with researchers at the DOE-JGI and PNNL EMSL through a 2014 Community Science Program to sequence the genomes/transcriptomes for these isolates, which reveals conserved xylan-degrading machinery and putative cellulosome structure.

Though fungal hydrolytic activity has been shown to be substrate dependent, the underlying regulation mechanisms that coordinate the action of cellulases and cellulosomes from gut fungi remain unknown. We hypothesize that cellulose-degrading machinery is catabolite-repressed to conserve cellular energy,

and our objective is to exploit this regulation mechanism to discover novel enzymes. To address this hypothesis, we have combined next-generation sequencing and proteomic approaches to examine cellulose-degrading enzyme production in a panel of gut fungi isolated from natural ecosystems. Through strand-specific RNAseq and use of the TRINITY assembly platform, we were able to assemble thousands of novel genes de novo from >27,000 transcripts without the need for genomic sequence information. RNA-Seq also elucidated global regulatory patterns in response to catabolite repression of biomass degradation, and in response to growth on cellulosic substrates of increasing complexity. Through these efforts, we have identified hundreds of transcripts encoding novel enzymes for biomass degradation, and the fungal transcriptome is particularly rich in GH6, GH48, and GH43-containing enzyme domains. As hypothesized, most of these transcripts are strongly repressed by the addition of simple sugars and are clustered within distinct ‘regulons’ of coordinated gene expression. Gene set enrichment analysis confirms the upregulation of these regulons across cellulosic substrates, and complementary proteomic analyses reveals that the composition the fungal cellulosomes is tuned by the presence of different substrates. More importantly, the functional enrichment of these regulons suggests a critical role for the divergent and unannotated transcripts that they contain. A dozen co-regulated transcripts from our screen bear no homology to known enzymes, and likely harbor previously undiscovered glycosyl hydrolase domains from nature, which we are currently investigating through protein crystallography in collaboration with Argonne National Laboratory, as well as high-throughput activity screening at NREL. Collectively, this information will establish the molecular framework for anaerobic fungal hydrolysis, ultimately allowing us to refactor this system in anaerobic fungi and beyond.

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