**Developing anaerobic fungal tools for efficient upgrading of lignocellulsic feedstocks**

Casey Hooker[1,3*] (hookerc@udel.edu), Radwa Hanafy[3], Ethan Hillman[1,2†], Javier Muñoz Briones[1,2], and **Kevin Solomon[1,2,3]**

[1]Agricultural & Biological Engineering, Purdue University, West Lafayette, IN; [2]Purdue University Interdisciplinary Life Sciences (PULSe) Program, Purdue University, West Lafayette, IN; [3]Department of Chemical and Biomolecular Engineering, University of Delaware, Newark, DE;

[†]Present Address: Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, MI

http://solomonlab.weebly.com

**Project Goals: This project develops genetic and epigenetic tools for emerging model anaerobic fungi to identify the genomic determinants of their powerful biomass-degrading capabilities, facilitate their study, and enable direct fungal conversion of untreated lignocellulose to bioproducts.**

Deconstruction of plant cell wall biomass is a significant bottleneck to the production of affordable biofuels and bioproducts. Anaerobic fungi (*Neocallimastigomycota*) from the digestive tracts of large herbivores, however, have evolved unique abilities to degrade untreated fiber-rich plant biomass by combining hydrolytic strategies from the bacterial and fungal kingdoms[1]. Anaerobic fungi secrete the largest known diversity of lignocellulolytic carbohydrate active enzymes (CAZymes) in the fungal kingdom (>300 CAZymes), which unaided can degrade up to 60% of the ingested plant material within the animal digestive tract[2,3]. Unlike many other fungal systems, these CAZymes are tightly regulated and assembled in fungal cellulosomes to synergistically degrade plant material, including untreated agricultural residues, bioenergy crops, and woody biomass, with comparable efficiency regardless of composition[1,4–6]. Our efforts to characterize gut fungal CAZymes reveal industrially relevant properties such as remarkable stability and activity towards untreated plant biomass[6-7]. Gut fungal CAZymes liberate sugars from cellulosic substrates for timeframes beyond 192 hours after inoculation. These accumulated sugars can be fed to model bioproduction hosts (e.g. *K. marxianus*) to create coculture systems capable of upgrading sugars from plant biomass to high value products (e.g. ethyl-acetate and 2-phenylethanol)[7]. Similarly, anaerobic fungal biosynthetic enzymes possess unique cofactor substrate preferences that support higher catalytic efficiencies, which are easily overlooked via heterologous expression due to the extremely high AT content (~83%) of gut fungal genomes and biased codon preferences[7]. Thus there is an unmet need to build genetic tools and methods to study these enzymes natively in anaerobic fungi.

As a first step towards genetic tool development, we sequenced the genomes of three novel specimens of anaerobic fungi representing two genera of Neocallimastigocota. Previous anaerobic fungal genomes were highly fragmented into as many as 30,000 scaffolds thus limiting efforts to mine for basic genetic parts (e.g. promoters and terminators). By leveraging high quality genome isolations, long-read sequencing, and Hi-C (chromosomal conformation capture) sequencing, we

have improved genome assembly by an order of magnitude to generate the first genomes for this phylum of organisms with chromosomal resolution. Our assemblies incorporate more than 99% of the genome into 12-25 chromosomes with N50 < 10. These high quality reference genomes show extensive genome duplication and horizontal gene transfer events that partially contribute to the ability of gut fungi to robustly degrade crude plant biomass. We are investigating the epigenetic regulation of these genes by monitoring expression through transcriptomic and proteomic approaches supported through the JGI-FICUS program. These studies show epigenetic control, induced in part by microbial competition, mediates enzyme expression and substrate preference, and thus which parts of plant biomass are preferentially degraded. Thus, these reference genomes and datasets reveal a wealth of regulatory sequences and CAZymes for study. We have synthesized more than a dozen promoters and terminators as well as nearly 20 codon optimized reporter proteins through the JGI-BERSS program for evaluation along with reporter proteins and other putative parts for gene expression. By combining these regulatory sequences with selectable markers we have verified functionality of 2 fluorescent reporters via flow cytometry. Current efforts are aimed at improving transformation efficiencies and expression of selectable markers by mining for centromere binding sequences, Kozak sequences, and autonomously replicating sequences to further enhance plasmid stability and maintenance. Together, our rich high quality reference genomes and datasets are expediting efforts to mine for key regulatory sequences and parts needed to build a stable episomal plasmid.

In summary, the ongoing work has harnessed anaerobic fungal isolates and genes for bioproduction, begun to identify parts for a genetic toolbox and provides an atlas of anaerobic fungal genomes complete with targets for study and genomic regions to avoid. Once validated, these parts will form foundational tools to generate a deeper systems-level understanding of anaerobic fungal physiology while establishing fundamental knowledge about regulation of gut fungal CAZymes. Ultimately, we enable predictive biology in anaerobic fungi and derive insight into microbial plant deconstruction to advance the development of economical biofuels and bioproducts.

## References


**1** Haitjema, C. H. *et al. Nature Microbiology* 2, 17087 (2017)
**2** Seppälä, S. *et al. Metabolic Engineering* 44, 45–59 (2017)
**3** Youssef, N. H. *et al. Appl. Environ. Microbiol.* 79, 4620–4634 (2013)
**4** Solomon, K. V. *et al. Science* 351, 1192–1195 (2016)
**5** Solomon, K. V. *et al. Fungal Genetics and Biology* 121, 1–9 (2018)
**6** Hooker, C. A. *et al. Biotechnology for Biofuels* 11, 293 (2018)
**7** Hillman, E. T. *et al. Biotechnology Progress* 37, e3172 (2021)
**8** Hillman, E. T. *et al. Microorganisms* 9, 1986 (2021)



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