Integrated ′Omics Reveals the Details of Metabolic Adaptation of *Clostridium thermocellum* ATCC-27405 Grown on Switchgrass

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Project Goals: The BioEnergy Science Center (BESC) is focused on the fundamental understanding and elimination of biomass recalcitrance. BESC's approach to improve accessibility to the sugars within biomass involves (1) designing plant cell walls for rapid deconstruction and (2) developing multi-talented microbes or converting plant biomass into biofuels in a single step [consolidated bioprocessing (CBP)]. BESC research in biomass deconstruction and conversion targets CBP by studying thermophilic anaerobes to understand novel strategies and enzyme complexes for biomass deconstruction and manipulating these microorganisms for improved conversion, yields, and biofuel titer. BESC researchers provide enabling technologies in biomass characterization, ′omics, modeling and data management in order to (1) understand chemical and structural changes within biomass and (2) to provide insights into biomass formation and conversion mechanisms.

Switchgrass is a perennial C4 grass and a dominant grass in North America. Due to favorable growth characteristics, it is a model herbaceous bioenergy crop of significant interest to the U.S. Department of Energy. As such, switchgrass is a promising feedstock, capable of augmenting and potentially replacing corn for the production of bioethanol. The plant cell wall polysaccharides (cellulose, hemicelluloses, and lignin) are a source of carbon and energy for cellulolytic microorganisms that are capable of degrading these somewhat complex biopolymers. However, the biosolubilization of these recalcitrant biopolymers requires a sophisticated microbial enzymatic system with diverse catalytic activities.

Clostridium thermocellum is a thermophillic, anaerobic Gram-positive bacterium that produces large extracellular complexes, termed cellulosomes, that are quite efficient at solubilizing and deconstructing cellulose for eventual production into biofuel materials. This cellulolytic microbe is a candidate for converting lignocellulosic biomass directly into ethanol; however, the microbial capacity to overcome the recalcitrant nature of the lignocellulosic biomass necessary for biofuel production remains challenging to fully capture at an industrial scale. *C. thermocellum* can degrade cellulose from complex lignocellulosic feedstocks, such as switchgrass and *Populus*, to form cellobiose and other small, soluble cellodextrins as the main products. Cellobiose is ultimately utilized by the organism to generate end products of ethanol, acetic acid, lactic acid, hydrogen, and carbon dioxide. The generation of lactate, formate and acetate can divert the metabolic flux away from the desired ethanol product. In order to maximize the ethanol yield, *C. thermocellum* can be genetically modified to eliminate the competing pathways. Despite studies investigating *Clostridium thermocellum* on various cellulose substrates, relatively little work has been done to systematically characterize the comprehensive range of linked proteins/metabolites across

a detailed time-dependent growth of *Clostridium thermocellum* on switchgrass. In this study, we investigated *C. thermocellum* grown on switchgrass in batch fermentation by integrating a multi-′omics approach (proteomics, transcriptomics, metabolomics) to better understand the detailed molecular machinery and regulation of cellulolytic microbial growth on this complex lignocellulose substrate.

Acetic acid and ethanol were observed as the major fermentation products for *C. thermocellum* grown on dilute acid pretreated switchgrass. Ethanol stopped accumulating after 120 hours, whereas acetic acid slowly kept accumulating in the fermentation broth. The ethanol concentration profiles show that the cultures were most metabolically active between 19 and 45 h post-inoculation, which correspond to exponential growth phase in this study. Metabolomics provided key information about the range of end products from microbial growth on this complex biomass, and revealed major hemicellulose catabolic metabolites, including xylobiose, xylose, xylitol, arabinose, and arabitol. A large accumulation (almost 9-fold) of xylitol was measured as the culture reached stationary phase. Other 5-carbon sugar alcohols, such as arabitol, ribitol, and phenolic acids of the lignin pathway, including caffeic acid, ferulic acid and p-coumaric acid were also noted to increase dramatically in abundance; some of these compounds have been found to be inhibitory to microbial growth, and thus warrant further consideration. The accumulation of long chain saturated fatty acids, and unique branched fatty acids was quite notable in *C. thermocellum* during the stationary phase, indicating cell membrane modification to tolerate increasing concentration of fermentation end-products. To better understand this process, we used proteomic and transcriptomic analyses to characterize the range of enzymatic molecular machinery that is used by this microbe during complex cellulose solubilization.

Proteomic analysis of this system (*C. thermocellum* growing on switchgrass) revealed 1551 non-redundant proteins, representing ~50% of predicted proteins, with about a third of the proteins (566) having significant changes in abundance during transition from early exponential phase to late stationary phase. Most of the metabolically related enzymes (e.g., amino acid and protein synthesis, glycolysis) decreased in abundance with growth time; in contrast, cellulosomal proteins, S-layer, and bacterial secretion systems increased in abundance, even at late stationary phase. Clustering analyses revealed significant protein groups that varied substantially with time; these were mainly populated with ABC transporters, catabolic enzymes (cellulosomal proteins), and transport related proteins. Enzymes responsible for atypical glycolysis were highly abundant and remarkably time dependent, suggesting a malate shunt pathway. In response to damage of cellular membrane integrity caused by ethanol accumulation, *C. thermocellum* diverts activity into the pentose phosphate pathway and biosynthesis of branched fatty acids. In addition, we also found that valine, leucine and isoleucine producing enzymes changed significantly, suggesting a possible diversion of PEP/pyruvate towards amino acid biosynthesis. These results indicate that the growth of *C. thermocellum* on switchgrass is a complicated process in which plant substrate is used for microbial growth, but inevitable inhibitory end-products retard microbial metabolism and thus have to be considered in the potential application of this type of celluloytic organism for effective biofuel production.

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