

Modifying Carbon, Nitrogen, and Electron Metabolism in *Clostridium thermocellum* to Enhance Cellulosic Biofuel Yield and Titer

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Project Goals: The BioEnergy Science Center (BESC) focuses on fundamental understanding and elimination of biomass recalcitrance. BESC's approach to improve accessibility to the sugars within biomass involves (1) improved plant cell walls for rapid deconstruction and (2) multi-talented microbes for converting plant biomass into biofuels in a single step [consolidated bioprocessing (CBP)]. Biomass research works with two potential bioenergy crops (switchgrass and *Populus*) to develop improved varieties and to understand cell wall biosynthesis pathways. We test large numbers of natural variants and generate specific modified plants samples. BESC's research in deconstruction and conversion targets CBP manipulating thermophilic anaerobes and their cellulolytic enzymes for improved conversion, yields, and titer. Enabling technologies in biomass characterization, 'omics, and modeling are used to understand chemical and structural changes within biomass and to provide insights into mechanisms.

A sustainable future will require the development of renewable alternatives to petroleum-derived fuels and chemicals, and one potential solution for the replacement of gasoline involves the conversion of plant biomass into liquid fuels. Due to its native ability to rapidly consume cellulose and its existing ethanol production pathway, *Clostridium thermocellum* is a leading candidate organism for implementing a consolidated bioprocessing strategy for biofuel production, wherein biomass deconstruction and fermentation occur in a single vessel without added enzymes. In addition to producing ethanol, *C. thermocellum* converts cellulose and soluble cellodextrins such as cellobiose to lactate, formate, acetate, H₂, amino acids, and other products. Therefore, metabolic engineering is required to optimize flux to a single product.

A mutant strain of *C. thermocellum* was constructed to remove major side product formation, resulting in *C. thermocellum* $\Delta hydG \Delta ldh \Delta pfl \Delta pta-ack$. This strain no longer produces formate, acetate and lactate; hydrogen production is decreased four-fold; and the ethanol yield is doubled compared with the wild type on cellobiose, crystalline cellulose Avicel, and pretreated biomass. Laboratory strain evolution has allowed for further improvement of growth rate, yield, and titer, resulting in a strain capable of converting crystalline cellulose to ethanol at a titer of 22.5 g/L at

75% of the maximum theoretical yield. Genome resequencing revealed mutations that occurred during strain evolution and suggests mechanisms responsible for the improved phenotypes.

We also introduced four genes from a high ethanol yielding strain of engineered *T. saccharolyticum* that are likely important for ethanol production – *adhA*, *nfnAB*, and a mutant *adhE* – into wild-type *C. thermocellum*. We observed significant improvements to ethanol yield and titer in the resulting strain. Further engineering of this *C. thermocellum* strain by eliminating hydrogen production did not improve ethanol yield, but instead decreased ethanol titer on higher initial substrate concentrations. We hypothesize that we will need to further engineer *C. thermocellum* metabolism to more closely resemble that of *T. saccharolyticum* via an approach that includes both gene deletions and heterologous expression.

While these mutants exhibit higher ethanol yield, amino acids are still produced as end products. Therefore, genetic analysis of nitrogen metabolism is being investigated to understand the mechanism of ammonium assimilation and to devise strategies to prevent production of amino acids as fermentation products. Progress in understanding and altering nitrogen metabolism in *C. thermocellum* and the impact on product formation also will be discussed.

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