

## Improving *Clostridium thermocellum* Product Titters by Increasing the Thermodynamic Driving Force of its Glycolytic Pathway

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**Project Goals:** The Center for Bioenergy Innovation (CBI) vision is to accelerate domestication of bioenergy-relevant, non-model plants and microbes to enable high-impact innovations at multiple points in the bioenergy supply chain. CBI addresses strategic barriers to the current bioeconomy in the areas of 1) high-yielding, robust feedstocks, 2) lower capital and processing costs via consolidated bioprocessing (CBP) to specialty biofuels, and 3) methods to create valuable byproducts from the lignin. CBI will identify and utilize key plant genes for growth, composition and sustainability phenotypes as a means of achieving lower feedstock costs, focusing on poplar and switchgrass. We will convert these feedstocks to specialty biofuels (C4 alcohols, C6 esters and hydrocarbons) using CBP at high rates, titers and yield in combination with cotreatment, pretreatment or catalytic upgrading. CBI will maximize product value by *in planta* modifications and biological funneling of lignin to value-added chemicals.

*Clostridium thermocellum* is a cellulolytic, thermophilic anaerobic bacterium and is a candidate organism for the consolidated bioprocessing of lignocellulosic biomass into biofuels. Metabolic engineering of *C. thermocellum* is necessary to improve yields and titers of biofuels products such as ethanol and butanol. With respect to ethanol, *C. thermocellum* has already been engineered to produce ethanol at yields upwards of 80% of theoretical maximum. However, maximum ethanol titers from different engineered strains of *C. thermocellum* tend to be limited to ~30 g/L. Our present challenge therefore is to engineer for higher ethanol titer.

Past metabolic engineering attempts in *C. thermocellum* have most focused on altering the metabolic pathways between pyruvate and ethanol. In this work, we first present recent experimental evidence and thermodynamic analyses of *C. thermocellum* metabolism, which show that *C. thermocellum*'s glycolytic pathway appears to have less thermodynamic driving force than that of other ethanol-producing microorganisms, such as yeast, engineered *Escherichia coli* and *Thermoanaerobacterium saccharolyticum*, and *Zymomonas mobilis*. These findings lead us to the hypothesis that thermodynamic limitations, and not biophysical inhibition, are limiting further improvements to improving ethanol titer. In particular, these analyses have identified that the pyrophosphate-dependent 6-phosphofructokinase (PPi-PFK) and phosphoenolpyruvate to pyruvate reactions in *C. thermocellum* – represented by the pyruvate:phosphate dikinase and malate shunts – as high priority targets to be replaced with more thermodynamically favorable reactions, such as the ATP-dependent 6-phosphofructokinase (ATP-PFK) and pyruvate kinase (PYK) reactions, respectively

Acting upon the aforementioned conclusions, we also present here our metabolic engineering efforts to improve thermodynamic driving force of *C. thermocellum* glycolysis. A major hurdle that had to be overcome was that pyrophosphate (PPi) was both an essential cofactor in glycolysis, and also a potent inhibitor of biosynthetic reactions. Achieving the ATP-PFK and PYK replacements necessitated not only identifying and eliminating the non-biosynthetic source of PPi from *C. thermocellum*, but also required us to completely uncouple PPi turnover from sugar metabolism. We have been successful at removing pyrophosphate's role from *C. thermocellum* glycolysis and have demonstrated that the modifications do in fact lead to a less reversible glycolysis. This engineered strain of *C. thermocellum* is one significant step closer to possessing the metabolism of ethanol producers, while still retaining its cellulolytic capabilities.

## References

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