Title: Application of a Novel ^{2}H Isotope Tracer Approach to Characterizing Metabolic Thermodynamics in *C. thermocellum*

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Project Goals: Thermodynamic analysis can help us understand how energy is transferred and transformed within metabolic networks and has emerged as a powerful tool for pathway design and metabolic engineering. This project integrates thermodynamic analysis with advanced mass spectrometry, computational modeling, and metabolic engineering to develop an approach for *in vivo* determination of Gibbs free energies (ΔG) in metabolic networks. This project also investigates how the thermodynamics of biosynthetic pathways in microbial biofuel producers change dynamically as substrates are depleted or products accumulate.

Abstract: Consolidated bioprocessing (CBP) relies upon efficient cellulolytic microbial activity to break down plant cellulose, making hard-to-access substrates into metabolizable starting material. *Clostridium thermocellum* is an excellent candidate for CBP, as it is an anaerobic bacterium capable of efficient cellulose catabolism. Previous work has begun to characterize the metabolic networks of *C. thermocellum* that facilitate the conversion of cellulose feedstocks into C_2 and C_4 alcohol products. These studies found that the thermodynamics of central metabolism in *C. thermocellum* are vastly different than those of other model fermenters, such as anaerobically-grown *Escherichia coli* and *Thermoanaerobacterium saccharolyticum*, having a lesser overall drop in Gibbs free energy and thus more limited thermodynamic driving force [1,2]. The thermodynamics of *C. thermocellum* metabolism is likely a key limitation to achieving high product titers, and consistent with this hypothesis we observe non-optimal ethanol titers during *C. thermocellum* fermentations with high substrate loading.

Established methods for identifying thermodynamic bottlenecks, which are helpful for identifying engineering targets for increasing product yields, have relied upon feeding microbial cultures isotopically labeled (^{2}H, ^{13}C) substrates. Unfortunately, this option is not viable for high concentrations of cellulose substrates. Here, we present an alternative approach utilizing deuterated water ($^{2}H_2O$, or "heavy water") as the source of isotope label during microbial fermentations. We have successfully used HPLC-MS to measure the incorporation of ^{2}H from heavy water into central metabolism, including intermediates of glycolysis, the TCA cycle, and the pentose phosphate pathway, as well as amino acids. Labeling patterns are consistent with known metabolic signatures in this organism, indicating that our $^{2}H_2O$ isotope tracer approach is a cost-effective method that is not limited by labeled substrate availability. Application of this technique to high substrate fermentation conditions, as well as various *C. thermocellum* mutants, will provide key information about metabolic flux during these fermentations. These data will

contribute to our holistic understanding of feedstock-to-bioalcohol metabolism, allowing us to identify barriers and optimize pathway engineering.

References/Publications

1. Jacobson TB, Korosh TK, Stevenson DM, Foster C, Maranas C, Olson DG, Lynd LR, Amador-Noguez D. 2020. *In vivo* thermodynamic analysis of glycolysis in *Clostridium thermocellum* and *Thermoanaerobacterium saccharolyticum* using ^{13}C and ^{2}H tracers. mSystems 5:e00736-19. doi: 10.1128/mSystems.00736-19
2. Cui J, Stevenson D, Korosh T, Amador-Noguez D, Olson DG, Lynd LR (2020). Developing a cell-free extract reaction (CFER) system in *Clostridium thermocellum* to identify metabolic limitations to ethanol production. Front. Energy Res. 8:72. doi: 10.3389/fenrg.2020.00072 .

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