Systems Metabolic Engineering of *Clostridium thermocellum* for Direct Conversion of Cellulosic Biomass to Designer C4-derived Esters

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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.

The C4-derived esters (e.g., isobutyl acetate, butyl acetate, isobutyl isobutyrate) are industrially important chemicals with versatile applications as fuels, flavors, fragrances, and solvents. Biologically, these esters are synthesized by condensation of either (iso)butanol with acyl-CoAs or alcohols with (iso)butyl-CoA using alcohol acyltransferases (AATs). Consolidated bioprocessing using efficient and robust biocatalysts, capable of direct conversion of lignocellulosic biomass to esters at high efficiency, offers a viable solution to sustainable bioeconomy. While the cellulolytic, thermophilic *Clostridium thermocellum* is a promising CBP microorganism due to its robust metabolism to degrade lignocellulosic biomass, it cannot natively produce esters. The critical challenges for optimal ester biosynthesis in *C. thermocellum* are to rewire its metabolism and harness enzymes and pathways that are efficient, robust, and compatible with the host. The aim of our project is to enable systems metabolic engineering of *C. thermocellum* for direct conversion of cellulosic biomass to designer C4-derived esters. Through bioprospecting and model-guided protein engineering, we first repurposed chloramphenicol acetyl transferases (CATs) to function as AATs that are efficient, robust, and compatible with *C. thermocellum* for designer bioester synthesis.[1-2] To eliminate the endogenous ester degradation, we next performed genome mining and enzyme characterization to identify and disrupt two critical carbohydrate esterases (CEs) from the genome of *C. thermocellum* while not affecting its robust capability of cellulose degradation.[3] In combination with gene expression and fermentation optimization, we demonstrated the first-generation of engineered *C. thermocellum* capable of producing 1 g/L and 0.34 g/L of C4-derived esters with and without isobutanol supplemented cellulose fermentation, respectively. To enable biosystem designs of *C. thermocellum*, we have built the genome-scale metabolic model[4-6] and used it to design the modular (chassis) cells that can be assembled with exchangeable production modules for optimal

biosynthesis of designer bioesters with minimal requirement of strain optimization cycles.[7-11] Future studies are to characterize and elucidate the modular design of *C. thermocellum* for the target ester biosynthesis.

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The Center for Bioenergy Innovation is a U.S. Department of Energy Bioenergy Research Center supported by the Office of Biological and Environmental Research in the DOE Office of Science