

## Enhancing Control of Cell-free Metabolism Through pH Modulation

Blake Rasor<sup>1,2,3\*</sup> (b.rasor@u.northwestern.edu), Ashty Karim<sup>1,2,3</sup>, **Michael C. Jewett**<sup>1,2,3,4,5</sup>

<sup>1</sup>Department of Chemical and Biological Engineering, <sup>2</sup>Chemistry of Life Processes Institute, <sup>3</sup>Center for Synthetic Biology, Northwestern University, Evanston, IL;

<sup>4</sup>Robert H. Lurie Comprehensive Cancer Center, <sup>5</sup>Simpson Querrey Institute, Northwestern University, Chicago, IL

<http://jewettlab.northwestern.edu/research/metabolic-engineering>

**Project Goals: We are establishing an interdisciplinary clostridia Foundry for Biosystems Design (cBioFAB) with government and industry partners to accelerate engineering efforts in non-model organisms through *in vitro* metabolic pathway prototyping, computational modeling, and integrated omics analysis. Through these diverse approaches, we seek to utilize genomic information from the David T. Jones clostridia collection to expand our knowledge of clostridia species and realize more efficient strain engineering for the synthesis of biofuels and bioproducts.**

Cell-free systems allow the biosynthesis of enzymes and chemical products without the cell viability and growth constraints that hamper traditional metabolic engineering. Utilizing these systems to rapidly prototype metabolic pathways with many homologs *in vitro* enables the informed design of high-titer combinations of enzymes without constructing hundreds of unique bacterial strains. Such prototyping in *E. coli* extracts has proven useful for butanol biosynthesis in clostridia, a slow-growing, non-model organism with limited genetic tools available. However, prototyping pathways in the cytoplasmic milieu of *E. coli* is inherently different from the cytoplasm of clostridia. Such differences include the acidic environment preference of clostridia. Modifying the *in vitro* platform to better reflect clostridia could enhance its predictive ability, and the open environment of cell-free reactions allows for extensive control over the chemical composition. In this work, we altered the pH of cell-free reactions to provide a chemical environment more similar to extremophiles, including acidophilic clostridia. We found that cell-free reactions containing *E. coli* glycolytic enzymes and clostridial butanol synthesis enzymes consume glucose most rapidly at pH 8 and produce butanol most rapidly at pH 6, which is similar to the cytosolic pH of the respective bacteria. Additionally, this system can serve as a testbed for the pH tolerance of enzymes in a more biologically relevant context than purified protein in buffer. Alcohol dehydrogenases from other extremophiles convert butyraldehyde to butanol less efficiently than the canonical *C. acetobutylicum* homolog at acidic pH but show similar activity at alkaline pH. Overall, this enhances the utility of cell-free metabolic engineering to elucidate optimal chemical environments and sets of enzyme homologs to produce a desired product.

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