Title: Dynamic Kinetic Models Capture Cell-Free Metabolism for Improved Metabolic Engineering

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Project Goals: We aim to develop a predictive model of metabolism in bacterial cell-free systems for the purpose of rapidly prototyping heterologous metabolic pathways. This model may be used to both optimize the production of metabolites in cell-free systems, as well as to understand how results in these systems should inform design in related cell-free metabolic pathways or even metabolism in living organisms.

Abstract: The optimization of biosynthetic production remains a challenge in metabolic engineering. This is particularly true for products made via longer heterologous pathways, which may require manual tuning of all component reactions. While cell-free systems rapidly increase the experimental throughput of testing pathway combinations, they remain complex systems and produce large amounts of difficult-to-interpret data. Toward this goal, we are developing a dynamic kinetic model to better understand this complex system and enable rapid data analysis for pathway optimization.

We are currently using this model to study butanol production via acetyl-CoA in *E. coli* cell-free systems. Because these experiments have exhibited complex dynamics, wherein the transient behavior of the heterologous butanol pathway interacts with core metabolism and vice versa, our model is both mechanistic and dynamic to robustly capture these experimental phenomena and predict optimal engineering solutions. However, compared to steady-state models, dynamic models have additional degrees of freedom that demand non-stationary flux measurements and different parameterization methods than those typically used for models of living cellular steady-state metabolism. To this end, we have developed a dynamic modeling framework which utilizes a variety of literature kinetic values, thermodynamic calculations, and Monte Carlo methods for parameter sampling. An ensemble of models is first pruned according to their fit to timecourse metabolomics, and the resulting top-performing models are fine-tuned by local parameter optimization algorithms. Each model also simulates several phenomena unique to cell-free systems, including gas-liquid equilibrium and transient pH measurements. By using this framework, we have successfully captured complex dynamic behavior, such as shifts in core metabolism that were experimentally observed when butanol production varied. Because these models are mechanistic in nature, detailed analysis was able to be performed to understand the metabolic causes of many of these dynamic behaviors. Lastly, the final ensemble of models was

used to provide experimental recommendations for metabolite and enzyme level changes to improve butanol production and has additionally identified a key bottleneck in the butanol pathway.

In future work, we plan to refine this model by retraining on data in which butanol pathway enzymes were individually adjusted, instead of simply knocked out. By preserving the parameters associated with core metabolism learned in the previous work and adjusting only the parameters of enzymes within the butanol pathway, we aim to learn detailed kinetics of the butanol pathway while minimizing model refitting or experimental measurements. Ultimately, we aim to translate these trained mechanistic parameters into models of *in vivo* production strains, which will accelerate model-building and product optimization workflows.

Funding Statement: This poster is based upon work supported by the U.S. Department of Energy, Office of Science, Office of Biological and Environmental Research under Award Number DE-SC0018249.