Title: Novel Systems Approach for Rational Engineering of Robust Microbial Metabolic Pathways

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Project Goals:

The goal of this project is to develop and implement a process for improving bioproduction under conditions that are appealing for industrial processes, such as high temperature and low pH. Our approach addresses the failure of metabolic reactions due to inhibition, denaturation, mis-folding or disorder of individual enzymes. We will develop and implement a framework that identifies these enzymes and then identifies their robust replacement enzymes from sets of extremophiles. The engineering strategy of replacing enzymes to improve bio-production is well-established, but rarely applied to system-wide stressors. We apply a systems genomics approach to improve bio-production, with *E. coli* as the model organism. Butanol production at high temperature and succinate production at low pH are the model systems. This approach is complementary to improvement of microbial robustness by engineering the cell membrane and has advantages relative to evolutionary-based organism improvement by prioritizing bio-production rather than growth.

Abstract Text:

Aim 1: Metabolic Systems Modeling. Flux balance analysis methods will be used to model changes in system temperature and intracellular pH through enzyme inhibition and changes to reaction thermodynamics. Models will be tuned with experimental data from Aim 3 and validated against existing literature data. An iterative process using these models, existing data and work from Aim 2 will be used to identify rate-limiting enzymes and evaluate the effects of their replacements.

Aim 2: Computational Enzyme Assessment. Enzymes from thermophilic organisms known to be robust under high temperature and low pH conditions will be queried as replacements for the rate-limiting enzymes identified in Aim 1. This approach utilizes the huge and rapidly growing body of knowledge regarding protein sequence, structure and evolution. Sequence matching will be used to assess protein vulnerability and to identify candidate replacement enzymes. Structures of sequence variants will also incorporate entropy evaluations to assess protein stabilities.

Aim 3: Organism Characterization and Engineering. Building on previously developed engineering strategies, the enzymes and replacements identified above will be recursively assessed for their impact on organism performance. Experimental data from Aim 3 will feed back to Aims

1 and 2. The final temperature-tolerant butanol producer and acid-tolerant succinate producer will both be characterized at the 0.5L-scale. Success in organism engineering will be judged in terms of the sensitivity of product titer, rate and yield to temperature or acidification.

The tasks span three length scales: enzyme sequence (Aim 2), the performance of metabolic pathways as enzyme networks (Aim 1), and the functional output of metabolic pathways in the form of organism activity (Aim 3). Our approach strikes out on a new path to build on existing knowledge, by complementing existing organism with enzyme replacement strategies, and at the same time is generic in terms of production organism and product identity. The ability to adjust metabolic models for changes in temperature and intracellular pH is also relevant to systems analysis of food spoilage organisms and pathogenesis. This framework could also be applied to other stressors that impact enzyme activity, such as salinity and concentration of alcohols or solvents. The proposed approaches for enzyme robustness and stability could likewise be applied for the selection of enzymes for pan-organism *in vitro* systems, such as in the production of bioprivileged molecules that can serve as precursors for drop-in petroleum replacement and novel molecules.

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