Bio-production of synthetic rubber using engineered *Escherichia coli*

Introduction: In a society with both a growing dependence on energy and a depleting reservoir of fossil fuels, it has become increasingly important to design chemical syntheses that are sustainable, renewable and cost-effective. One synthesis of particular concern is that of isoprene, a precursor to synthetic rubber, since the current production relies on finite petrochemical sources.[1] Recent advances in synthetic biology and metabolic engineering have made it possible to biosynthesize isoprene using glucose extracted from plant biomass, a renewable feedstock. Despite these advances, previously studied synthesis pathways report low product yield due to poor catalytic activity, making them economically unfeasible for large scale production.[2,3]

In order to overcome this roadblock, I propose to use a keto acid-mediated pathway to biosynthesize isoprene. Keto acids can be used as a selection in directed evolution (DE), a vital tool in the enhancement of enzyme activity.[4] In order to employ DE however, enzyme specificity must be high enough for the desired conversion. Computational techniques, such as docking and funnel metadynamics, can be utilized to elucidate key amino acids involved in binding and catalytic active sites. These amino acids can then be mutated to enhance enzyme specificity. In this work, I focus on the conversion of citramalate to butanoic acid, a key step in the synthesis of isoprene, by expressing carnitine-CoA ligase (*CaiC*) and carnitine-coA transferase (*mvaE)* in *E. coli*. **I hypothesize that engineering specificity and activity in heterologous *CaiC* and *mvaE* enzymes using a combined theory-experiment approach will increase butanoic acid yield**, making the biosynthesis of isoprene more feasible for scale-up.

Research Aims: The primary objectives of this project are to (1) manipulate specificity and (2) increase activity of *CaiC* and *mvaE* to optimize conversion of citramalate to butanoic acid, which can then be converted to isoprene through a series of organic reactions (Fig. 1).

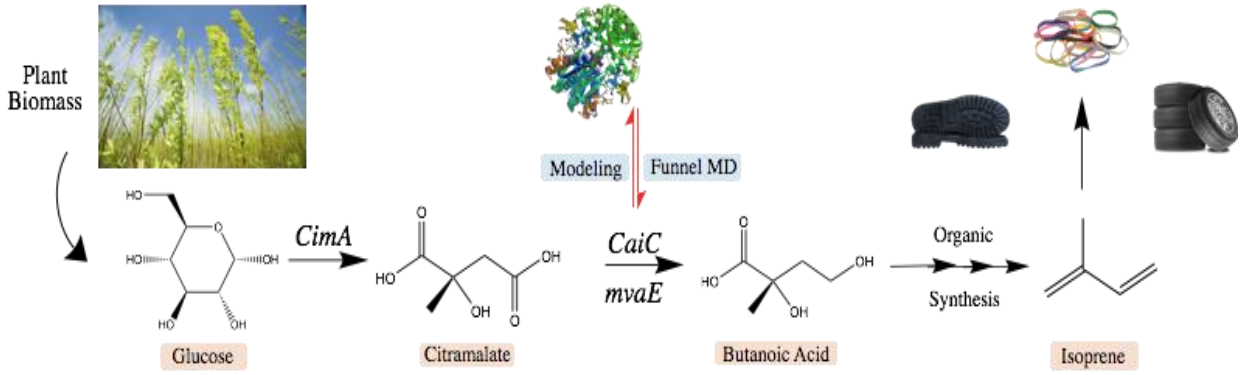

Figure 1: Workflow to sustainably produce isoprene from plant biomass by designing enzymes, supplementing experiment with modeling to engineer protein design, and using results from computation to inform experiment.

Preliminary results: During the summer of 2018, I obtained preliminary data by working on the upstream synthesis of isoprene using engineered *E. coli* in Dr. Kechun Zhang's lab at the University of Minnesota through the NSF-funded Center for Sustainable Polymers. Specifically, I was able to produce citramalate from glucose via citramalate synthase (*CimA*) using a keto acid-mediated pathway (Fig. 1). The next phase of the project is to convert citramalate to butanoic acid, the next intermediate in the biosynthesis of isoprene.

Methods: (Aim 1) *CaiC* and *mvaE* enzymes are known to perform the desired reduction on carnitine, a molecule of similar structure and functional group composition to that of citramalate.[5,6] I will use computational modeling to design more specific enzyme active sites for citramalate. The crystal structure for *mvaE* will be obtained from the Protein Data Bank. I will

then build a homology model for *CaiC* based on the crystal structure of L-carnitine CoA-transferase (*CaiB*) and generate force fields describing carnitine and citramalate from quantum chemical calculations. I will run molecular docking of *CaiC* and *mvaE* enzymes with citramalate followed by molecular dynamics (MD) simulations to equilibrate systems. I will then use funnel metadynamics to calculate protein-substrate binding free energy to provide an estimate of the binding affinity between both enzymes and substrates.[7] Insights gained from these simulations will inform key amino acid mutations to improve enzyme specificity.

(Aim 2) Next, I will use directed evolution to improve enzyme activity using a keto acid-pathway and growth-based selection.[4,8] I will use error-prone polymerase chain reactions (PCR) to create mutant libraries of *CaiC* and *mvaE* enzymes. PCR products will be cloned into a DNA backbone, electroporated into *E. coli*, and plated on Luria broth (LB) plates containing 100 μg/mL Ampicillin. I will measure the total product formed from enzyme conversion using a 4-aminoantipyrene assay.[8] Enzymes with readings 50% greater than parental standards will be selected for rescreening. Once both enzymes are optimized for activity and specificity, their DNA sequences will be ordered, replicated using PCR, ligated into a DNA backbone, and transformed into *E. coli*. I will then run fermentation for 48 hours in a 37 °C thermoshaker running HPLC and OD_{600} every 12 hours to monitor butanoic acid production.[9]

Resources and support: In order to address these aims, I will work in collaboration with the Zhang and Truhlar groups at the University of Minnesota (UMN) to develop the experimental and theoretical components of isoprene synthesis, respectively. Access to supercomputing time through NSF's XSEDE will allow for the proposed computational simulations.

Intellectual Merit: The combined theory-experiment workflow outlined in this proposal is used to overcome low yield by improving enzyme activity and specificity in heterologous *CaiC* and *mvaE* enzymes. This methodology can be applied in any biosynthetic reaction to synthesize novel non-natural metabolites at higher yields over varying conditions.[7] Even if highly accurate free energies are challenging to obtain from simulation, mechanistic information obtained from MD can be used to inform the next stage of experiment. Future directions of this project include converting butanoic acid to isoprene and investigating gene knockouts to further increase yield.

Broader Impacts: The synthesis of isoprene, a commodity used in countless goods, such as adhesives, tires, and shoe soles, is currently unsustainable due to reliance on limited petroleum resources. Biosynthesizing isoprene using fermentation offers a renewable and cost-effective alternative. Increasing yield will make it feasible to utilize this technology in large scale production to move away from harmful, depleting syntheses and towards a more sustainable future. I plan to regularly present results from this work at conferences and make publications available to the public via open-access publication methods. Lastly, because parts of the above project, such as using recombinant DNA technology and running fermentation, lend themselves to undergraduate research, I will mentor and engage undergraduates in order to provide them with access to authentic research experiences early in their careers.

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