Systems biology towards a continuous platform for biofuels production: Engineering an environmentally-isolated *Bacillus* strain for biofuel production and recovery under supercritical CO_2.

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Project Goals: We are developing *Bacillus megaterium* as a host for continuous biofuel production coupled with *in situ* product extraction by supercritical CO_2 ($scCO_2$) stripping. We employ a cross-disciplinary collaborative approach to achieve the following (1) Develop a supercritical CO_2 tolerant strain of *B. megaterium* into a bioproduction host for biofuels (2) Engineer de novo pathways for biosynthesis of longer chain fuels in *B. megaterium* and (3) Develop and model a two-phase stripping chemostat for continuous biosynthesis and in situ extraction of biofuels using $scCO_2$ as a sustainable extractive solvent. The abstract below reports on progress towards goal (2).

Medium-chain, branched alcohols have advantages as fuels over molecules such as ethanol due to their higher energy density and lower research octane number, enabling their use as drop-in fuels with properties similar to conventional gasoline. End-product toxicity is one limitation to their production biologically, resulting in low titers that require energy intensive separations. *In situ* product extraction offers a solution to product toxicity by removing compounds from the culture medium as they are generated. We intend to utilize the unique thermodynamic and transport properties of supercritical CO_2 ($scCO_2$) in a bi-phasic bioreactor to strip off the alcohol products made by an $scCO_2$-tolerant strain of *Bacillus megaterium* (SR7) that has been engineered to produce biofuels. Additionally, $scCO_2$ is a sustainable solvent, which when depressurized leaves the alcohol product at high concentrations, eliminating costly downstream distillation. Furthermore, $scCO_2$ has broad microbial lethality, providing a contamination-free reactor for the SR7 to grow in. In this work we have begun developing the genetic tools to engineer SR7 for isobutanol production. Additionally, we are modifying a biofuel pathway to increase the bioproduction 4-methyl-pentanol, which will be later implemented in the $scCO_2$-tolerant *Bacillus*.

Developing tools to modify SR7 began through characterizing the physiology of the organism. Through a screen of 71 carbon sources, we identified ones that enabled high growth. One of these compounds, L-malic acid, when used in combination with a previously optimized protoplast transformation method, resulted in at least a 10-fold increase in transformation efficiency. After successful plasmid transformation, we have developed two inducible and one constitutive promoter for heterologous protein expression in SR7. Two of these promoters have not previously been demonstrated in *Bacillus megaterium*. Further, we have found xylose-inducible expression of protein

under $scCO_2$, providing evidence that the promoter is functional under these harsh growth conditions and suggesting heterologous production of biofuels under $scCO_2$ is possible. We are currently looking to expand the genetic toolbox for SR7 by genomic incorporation of heterologous genes and developing strategies to knockout or knockdown endogenous enzymes.

We began to engineer SR7 to produce isobutanol by introducing a two-enzyme (2-ketoisovalerate decarboxylase (KivD) and alcohol dehydrogenase (Adh)) pathway. The two-step conversion occurs at approximately 70-80% from 2-ketoisovalerate when grown aerobically; however, the intermediate aldehyde was found to accumulate at short culture times. Due to the high partition coefficient for the aldehyde to the $scCO_2$ phase, five alternative homologous alcohol dehydrogenases were tested to eliminate its buildup. A variant was identified that lowered the build-up of isobutyraldehyde and resulted in conversion of 2-ketoisovalerate to isobutanol above 85% in aerobic cultures. The optimized, two-step conversion of 2-ketoisovalerate to isobutanol was tested in SR7 grown under $scCO_2$ with and without xylose induction. For two out of five of the induced cultures, over 20% of the 2-ketoisovalerate was converted to isobutanol, while no isobutanol was observed in the non-induced cultures. Furthermore, we have developed a five-enzyme pathway that converts pyruvate to isobutanol, and have found production of 400 mg/L isobutanol from glucose in SR7 when grown aerobically. However, this pathway has shown genetic instability and lower titers than expected, which we are modifying by genomic incorporation and using the previously developed promoters.

In addition to production of isobutanol in SR7, we have engineered a 4-methyl pentanol (4MP) production pathway in *E. coli*. Due to the limited genetic tools in SR7, its low transformation efficiency, and complexity of the 4MP pathway, we decided to engineer it for increased production in *E. coli* first. We identified the build up of isobutyrate as a potential bottleneck in the pathway. We tested three homologous propionate-CoA transferases and identified one that showed at least a 1.5-fold increase in 4MP titers. We have begun to create a more redox-balanced pathway by identifying homologous enzymes that have preference for NADH over NADPH. Lastly, we are analyzing a set of 40 keto-aryl-CoA reductases for their specificity for branched chain substrates as well as NADH cofactor preference. We intend to use the findings from engineering the 4MP pathway in *E. coli* to inform the incorporation of the pathway in SR7 to increase its likelihood of success.

This work was supported by the Office of Biological and Environmental Research in the DOE Office of Science (DE-SC0012555).