Systems biology towards a continuous platform for biofuels production: Heterologous gene expression and isobutanol synthesis in *B. megaterium* SR7 and biofuel extraction 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 goals (1) and (3).

Conventional microbial biofuel production systems are subject to several long-standing challenges associated with bioreactor culturing and compound harvesting, including contamination, end product toxicity, and energy-intensive downstream bioproduct purification. However, utilizing the sustainable solvent supercritical carbon dioxide ($scCO_2$) for *in situ* extraction of hydrophobic bioproducts would uniquely enable relief of end-product toxicity while creating a lethal environment for non-target organisms. In addition, depressurization of $scCO_2$ enables collection of purified, dehydrated biofuel products, eliminating the need for further downstream distillation. While previous studies have successfully demonstrated a broad diversity of *in vitro* biocatalytic reactions utilizing $scCO_2$ as a solvent and/or substrate, $scCO_2$ has previously been considered inaccessible to *in vivo* product biosynthesis due to its sterilizing effects on most microbes. Therefore, a bioprospecting approach was utilized in an attempt to isolate $scCO_2$-resistant microbial strains through enrichment culture and serial passaging of deep subsurface fluids from the natural McElmo Dome $scCO_2$ reservoir. This approach enabled isolation of multiple strains, of which strain *Bacillus megaterium* SR7 demonstrated superior growth upon inoculation as endospores under $scCO_2$ conditions. During the first project year we showed that SR7 growth under $scCO_2$ was significantly improved by chemical induction of spore germination by media amendment with amino acid L-alanine, developed a genetic system for heterologous gene expression in SR7 during growth under $scCO_2$, and engineered strain SR7xKY to produce isobutanol by introducing a two-enzyme (2-ketoisovalerate decarboxylase (KivD) and alcohol dehydrogenase (Adh)) pathway and feeding substrate 2-ketoisovalerate (α-KIV).

Over the previous project year (Year 2) we have validated heterologous gene expression in strain SR7 under $scCO_2$ using the LacZ reporter system, demonstrated isobutanol expression during growth under $scCO_2$ in 10 mL batch bioreactors, scaled up SR7 growth to larger reactor volumes with improved design for real-time monitoring or subsampling, and characterized butanol extraction efficiency over a range of reactor operating conditions. Average LacZ specific activity (μmol/min*mg) values in transformed strain SR7xL cultures grown under $scCO_2$ demonstrate

that heterologous enzyme production increased 13.1-fold by induction with xylose ($p = 0.026$), indicating potential for multi-gene heterologous pathway expression under $scCO_2$. This hypothesis was verified upon incubating transformed biofuel strain SR7xKY under $scCO_2$, which resulted in generation of up to 93.5 mg/L isobutanol and 29.7 mg/L isopentanol from 580.6 mg/L (5 mM) of substrate α-KIV, indicating a yield of 21.2%. Direct extraction of 5.2% of the total isobutanol product by the $scCO_2$ represents the first demonstration of heterologous multi-enzyme expression, biofuel generation, and product harvesting in a single $scCO_2$-exposed bioreactor.

After demonstrating biofuel production and extraction in bench scale (10 mL) biphasic batch reactors, efforts were initiated to achieve consistent growth for robust biofuel production at increased scales (25 mL and 0.3 L) in biphasic reactors with improved design to allow for continuous visual assessment of culture turbidity (25 mL reactor with sapphire view window) or subsampling (custom-built 300 mL capacity bioreactor with a Rushton impeller stirring at 300 rpm). At the 0.3 L scale, cultures of SR7 in optimized semi-defined minimal media under $scCO_2$ headspace demonstrated cell growth after 4 days, reaching concentrations above 10^8 cells/mL. However, profiles of cell density, metabolite production, and glucose consumption in fluids removed from the reactor indicated that growth was localized to a recessed, unmixed region at the base of the reactor that remained subject to full pressurization. Subsequent investigation of unmixed SR7 cultures under $scCO_2$ in the 25 mL capacity batch bioreactor also revealed turbidity after 4 days. Observations using epifluorescence microscopy revealed vegetative cells dispersed in the bulk media as well as cell aggregates encased in suspected biofilm extrapolymeric substances (EPS). We are currently examining the role of increased surface area to volume ratios, oxygen amendments, and modified stirring regimes on spore germination and outgrowth in the bulk media at the 25 mL and 0.3 L scales.

Upon the establishment of SR7 growth at the 0.3 L scale, we will introduce SR7 transformed with a biofuel pathway into the 0.3 L capacity reactor for compound generation and extraction by semi-continuous $scCO_2$ flow-through. In advance of anticipated short-to-medium chain alcohol bioproduction, we investigated the extraction of butanol, pentanol, and hexanol within the 0.3 L reactor under abiotic conditions. The objective was to examine the extraction efficiency in the 0.3 L reactor, which has not previously been established. We found that extraction rates and efficiency improved with alcohol chain length (i.e., hexanol > pentanol > butanol), broadly consistent with their equilibrium behavior. We modeled the extraction using the 2-film approximation to estimate mass transport coefficients; these were in reasonable agreement with literature values obtained for ethanol extraction. Correlations developed for liquid-liquid extraction agreed within approximately 25% with the estimated values of mass transfer coefficient. In addition, we modeled the energy efficiency of extraction for different CO_2/alcohol ratios. We found that extraction rates in the fermenter would be sufficient for industrial purposes; however, extraction efficiency (which was on the order of 100 moles CO_2 required per mole of alcohol extracted) were not industrially practical. Instead, a multi-stage extractor design will be needed to achieve industrially relevant extraction efficiencies. Overall, progress associated with biotic culturing and abiotic biofuel extraction provides a clear basis for continuing development of this sustainable, integrated biofuel production and extraction technology.

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