Title:

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 working towards the following goals (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.

Abstract:

Supercritical carbon dioxide ($scCO_2$) is an attractive substitute for conventional organic solvents due to its unique transport and thermodynamic properties, its renewability and labile nature, and its high solubility for compounds such as alcohols, ketones and aldehydes. However, biological systems that use $scCO_2$ are currently limited to *in vitro* processes due to its strong inhibition of cell growth. Using the broad microbial lethality and solvent chemistry of $scCO_2$ to our advantage, we hypothesize that a dual-phase reactor of growth media and $scCO_2$ will simultaneously provide a sterile growth environment and the capacity to continuously strip off strain-produced biofuels, thus alleviating long standing bioprocess challenges of culture contamination and end-product toxicity. Towards this goal, environmental strain isolation provides an opportunity to discover organisms capable of growth in harsh environments, such as under $scCO_2$. Using a targeted bioprospecting approach by sampling fluid from a natural, deep-subsurface $scCO_2$ well, several species of gram-positive, endospore forming Bacilli were isolated that demonstrate consistent, robust growth in the presence of $scCO_2$. The species that showed the highest frequency and magnitude growth was identified as a strain of *Bacillus megaterium*, from here on referred to as SR7. The genome and plasmids of SR7 have been sequenced and annotated to determine the metabolic potential of this organism and compare it to related strains of *B. megaterium.* We have established optimal growth conditions and media by studying the single carbon preference of SR7. Spore germination was found to be crucial for achieving successful growth of SR7 under pressure and the addition of germination enhancers, such as L-alanine, significantly improved the growth frequency and magnitude of SR7 under $scCO_2$. High-level growth under $scCO_2$ enabled the measurement of the fermentation products lactate and acetate, representing the first biological products observed under $scCO_2$ and confirmation of active metabolism of SR7 cultured under high pressure.

Next we sought to develop SR7 as a biotechnologically relevant organism by implementing bioproduction pathways to generate products that would preferentially partition into $scCO_2$. Transformation of SR7 is possible using a protoplast-based method, which has permitted the identification of promoters (including two that have not been previously used in *B. megaterium*) capable of inducible heterologous protein expression in both aerobic and anaerobic conditions. Furthermore, the xylose-inducible promoter was evaluated under $scCO_2$ and found to have similar expression compared to anaerobic cultures. We engineered SR7 to produce isobutanol by introducing a two-enzyme (2-ketoisovalerate decarboxylase (KivD) and alcohol dehydrogenase (Adh)) pathway. A library of Adh proteins was screened to identify enzymes that rapidly convert the isobutyraldehyde intermediate in the pathway since this compound is expected to highly partition into the $scCO_2$ phase. Combining our recombinant biofuel strain with $scCO_2$ culturing, isobutanol production was observed, representing the first recombinant bioproduct generated from bacteria grown under $scCO_2$. For cultures that showed high metabolic activity under $scCO_2$, we found almost 50% conversion of the α-ketoacid substrate to biofuel product.

Extraction of alcohols into $scCO_2$ was measured using a custom-built, two-phase reactor/fermenter. We discovered that the efficiency of extraction as well as extraction rate is greater for longer chain length alcohols (*n*-hexanol > *n*-pentanol > *n*-butanol), which is the opposite of what is found for gas stripping of these molecules. Isobutanol extraction was compared to that of *n*-butanol, and was observed to be moderately faster and more efficient. We found that there is a strong dependence on efficiency of extraction with product concentration, necessitating the improvement of biological production for an economically viable $scCO_2$-based extraction strategy. Using the empirically collected data on extraction of alcohols as well as production titers observed in similar microbes, we have developed a process model for $scCO_2$ culturing and have found conditions that are comparable if not better than existing *in situ* extraction techniques such as gas stripping. Furthermore, we observed after collecting the alcohol-$scCO_2$-water mixture, upon intermediate depressurization, an alcohol-rich phase occurs, resulting in high purity product.

Currently we are working on building a genome scale model of our strain of *Bacillus*, inputting data from a transcriptomics study of SR7 grown in various environments. We are also using our transcriptomic data set to design a set of promoters to be used for future engineering of SR7, to understand the natural metabolic capacity of SR7 especially towards bioproducts of interest such as isopentanol, and to aid in on going work to elucidate the $scCO_2$ tolerance mechanism. Additionally, we are designing and optimizing metabolic pathways to make fuels directly from common carbon substrates using the known metabolism of SR7, and developing genomic integration/knockout protocols to enhance the metabolic engineering of this unique host. We have begun implementing a portion of the biofuel pathway to produce 4-methyl-pentanol, to evaluate the functionality of the carboxcylic acid reductase in SR7. Lastly, we are working to characterize this unique phase behavior of the alcohol-$scCO_2$-water mixture to better understand the process variables that maybe changed to optimize the $scCO_2$-based extraction strategy.

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