

Carbon Partitioning in *Rubrivivax gelatinosus* CBS for Increased Isoprene Production

Maxwell Keirn, MS, Chemical Engineering, Summer 2017

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To the University of Wyoming:

The members of the Committee approve the thesis of Maxwell Keirn presented on 7/17/2017.

Dr. Karen Wawrousek, Chairperson

Dr. Mark Gomelsky, Outside Member

Dr. Joseph Holles

APPROVED:

Dr. Vladimir Alvarado, Department, Division, or Program Chair, Chemical Engineering.

Dr. Michael Pishko, College Dean/Provost

Abstract

Metabolic engineering can be used to alter or enhance various metabolic pathways in microorganisms for the purpose of producing fuels, chemicals, and pharmaceutical products. In this work, we aim to discover if certain genetic modifications can redirect carbon flux to increase product yields of isoprene, which is used to synthesize rubber. Here, *Rubrivivax gelatinosus* CBS is used as a bacterial platform for investigating the carbon flux from CO through terpenoid pathways. Methods for increasing feedstock consumption, introduction of foreign genes, and deletion of native genes are all utilized in an attempt to increase isoprene titers.

Pathways targeted for deletion are responsible for the production of polyhydroxyalkanoates, which are energy storage molecules. Portions of the mevalonate pathway are added along with an isoprene synthase gene to enable increased production of isoprene. Previous works have shown successful expression of the mevalonate pathway in cyanobacteria, as well as production of isoprene. But no work has been done to investigate redirecting carbon flux from energy storage pathways towards isoprene production. Also, previous works investigating the conversion of CO to isoprene have not used a photosynthetic microorganism to alleviate the energy constraints of growth on CO. We also investigate the transcriptional regulators tied to growth on carbon monoxide, which have not been fully elucidated in CBS.

Background

Metabolic Engineering

An aim of the field of metabolic engineering is to develop microbial systems that can produce fuels and chemicals to supply global demands. Currently, the majority of our fuels and chemicals are derived from fossil fuels. (Foust 2009) This has two main problems. Petrochemical production methods have negative impacts on the environment, and petroleum is a finite resource. Because of this, methods for extracting goods from this resource may have to be substituted with more environmentally friendly procedures. One alternative is to use microorganisms as biocatalysts to produce fuels and chemicals.

The field of metabolic engineering came into existence in the early 1990's as applied molecular biology and genetic engineering technologies were rapidly progressing. There were a few seminal papers at the beginning of this decade that initiated interest in the field leading to a Journal (Metabolic Engineering) and the first book in the field. (Stephanopoulos 2012). The field is defined as the "*directed modulation of metabolic pathways using methods of recombinant technology for the purpose of overproducing fuels and chemical and pharmaceutical products*". (Bailey 1991) Although genetic engineering of bacteria dates back to the 1970's, metabolic engineering did not differentiate itself from this form of applied biology until researchers began to also optimize the pathways being expressed. (Stephanopoulos 2012)

Currently, metabolic engineering is seen as one solution to producing biofuels and commodity chemicals sustainably. Because microorganisms can break down and metabolize many different carbon based feedstocks, fuels and chemicals can be produced by these microorganisms from lower value feedstock materials. Currently, small scale operations can choose feedstocks of their choice that are easy to obtain and have a cheap price. However, larger scale production systems that will satisfy global demands need to choose feedstocks based on greater criteria.

Methods for producing biofuels have changed over time, resulting in 1st, 2nd, 3rd, and 4th generation biofuels. Currently, the majority of bioethanol is produced as a first generation biofuel where biofuel is made through fermentation of simple carbohydrates like sugars or starch. Although first generation biofuel production methods are the only economically viable routes currently for supplying global demands, there are problems that must be addressed. (Kennes 2016) One problem is that using a food source that requires land to be grown on, is not the most efficient use of land and food. Second generation biofuels utilize lignocellulose, which is not a food source and is considered a renewable resource. However, the lignocellulose must undergo saccharization via processing and pretreatments before it can be used as a feedstock for 2nd generation biofuel production. Third generation biofuels focus on extracting hydrocarbons from microalgae, and 4th generation biofuel produce biofuels from gasified biomass (syngas) as a feedstock. Other methods for biofuel production, not discussed here, are also grouped with 4th generation biofuels however.

Due to concerns related to food supply, 2nd, 3rd, and 4th generation biofuels have more promise to alleviate global energy demands. (Verma 2016) Synthesis gas, or syngas, is produced through the gasification of biomass. In gasification, nearly *all* of the biomass can be converted to syngas, whereas lignocellulose processing for 2nd generation biofuels does not utilize the entirety of the biomass.

An advantage to pursuing syngas fermentation over lignocellulolytic fermentation is that syngas can also be produced from man-made waste materials. Here, we have the possibility to re-use our waste instead of creating landfills and polluting the oceans. Another advantage comes from the composition of syngas, in that it contains the toxic gas carbon monoxide. A problem

that bio-refineries are often affected by its contamination. Because carbon monoxide is toxic to many organisms, using syngas as a feedstock decreases the potential for contamination.

Unfortunately, studies on syngas fermentation are in their infancy. Most studies have limited their scope to acetogenic bacteria because they are the most widely known organisms that can process CO. However, energetic constraints involved with growth on syngas by acetogens, initially gave a dreary outlook for syngas fermentation. (Bertsch 2015) Studies showed that when acetogens process CO, there is not enough energy left over for simultaneous production of a product. (Bertsch 2015) Fortunately, acetogens are not the only species capable of carbon monoxide metabolism. Surprisingly, this fact is overlooked in many papers that talk about syngas metabolism. Other bacteria, such as PNSB have been found to metabolize CO, and do so in a manner that is different and energetically favorable to CO metabolism in acetogens. (Drzyzga 2015) For this reason, metabolic engineering of these species may improve syngas fermentation strategies.

Biofuels and Biomolecules for a Sustainable Future

Petroleum and Sustainability

Growing concerns about the finiteness of fossil energy sources and climate change are the two largest driving forces behind the development of sustainable biofuels and bio-chemicals. Currently, the fuel and chemical commodity industries are almost completely based on fossil fuels. (Johannes 2015) The need for alternative, sustainable forms of fuel and chemical production is now well acknowledged. As energy requirements increase, global petroleum reserves will continue to decrease. Biofuel additives and bio-produced chemicals are one solution to the presumed fear of coming fossil fuel shortages. With research being put into biofuel production by microorganism, and legislation by various governments, it is clear the world is headed towards using more and more biofuel additives in fuel. One example of legislation in the U.S. is the new Renewable Fuel Standard (RFS2) mandate, established by the EPA, asserting that 36 billion gallons of biofuels have to be blended annually by 2022. (Monge 2014) Further amendments to this mandate have increased the amount of biofuels that must be blended by this date to 60 billion gallons. (Poulomi 2010) By blending these biofuels into transportation fuels we can prolong our oil dependency in an effort to develop alternative energy and transport systems.

Using biofuel additives in transport fuels also lowers the carbon impact from combustion. Production and combustion of 4th generation biofuels is envisaged to have less of a carbon impact than traditional fuel production and use for a few reasons. Biofuels can be considered carbon neutral because they come from plant biomass, which is a product of CO₂ assimilation. Also, the carbon released in manufacturing biofuels is less than traditional fuel sources. (Abideen 2014) Lastly, if carbon produced by manufacturing can be converted by microorganism into fuels/chemicals, instead of going directly to the atmosphere, this would further reduce greenhouse gas emissions.

Evolution of Biofuel Generations

Some criticisms of first generation biofuels stem from the choice of feedstock used, which are human food sources. Examples of this are evidenced by ecological imbalances that are observed when large tracts of forest are replaced with oil palms used for palm oil production. (Ahmad 2011) As these first generation biofuels could lead to continued deforestation, second generation biofuels were an apparent solution. Also, second generation biofuel conversion

processes from non-edible food sources have been shown to be more efficient and environmentally friendly than first generation biofuel production. (Ahmad 2011)

Third generation biofuels arose to produce biodiesel more effectively than second generation biofuel production schemes. Micro-algae are seen as the most promising organism for production of bio-diesel because of their high photosynthetic efficiency and higher amounts of lipid production. (Minowa 1995)

However, using micro-algae for bio-diesel production cannot work in all climates, and does not address biological production of other hydrocarbons. This has led to the emergence of fourth generation biofuels that utilize syngas.

Fourth generation biofuels, produced via syngas fermentation have been identified as a sustainable alternative for increasing energy demands and have several advantages over previous generation biofuels; including low feedstock costs, higher availability of biomass for feedstocks, and no competition with food sources for supplying feedstock. (Verma 2016) Although there are still major problems that need to be solved to implement large scale syngas fermentations, the high potential of microbial catalysts for converting syngas to biofuels and valuable bio-chemicals is evidenced by various successful scientific studies and patent applications. (Drzyzga 2015) INEOS Bio, Coskata, and Lanzatech are a few companies that currently apply this technology. (Verma 2016)

Lignocellulose and Syngas

Lignocellulosic biomass is the most abundantly available raw material on earth. (Alonso 2010) For this reason, it has received considerable attention as a sustainable feedstock for the replacement of fossil fuels. Generally, lignocellulosic biomass has three main components: hemicellulose (25-35%), cellulose (40-50%), and lignin (15-20%). (Alonso 2010) However, lignocellulosic biomass must be processed before it can undergo biological conversion. This is mainly due to β -O-4 linkages in the lignin matrix which are difficult for microorganisms to degrade. (Liu 2015)

There are two main ways to process this biomass before it can undergo biological conversion, by thermochemical or hydrolysis pretreatment. For the hydrolytic route, the lignocellulose is processed into more easily convertible sugars via a series of pretreatment steps. These include: crushing of the feedstock, an acid pretreatment, and hydrolysis. (Kennes 2016) These extra steps are necessary to yield soluble sugars that can then be fermented by microorganisms. Alternatively, the thermochemical route involves gasification of this biomass to produce synthesis gas. Syngas, or synthesis gas, is primarily composed of CO, CO₂, and H₂, though it can also contain CH₄ and C₃ tars along with other volatiles. (Drzyzga 2015) Some bacteria are capable of growing on syngas mixtures of H₂, CO₂, and CO. Thus, syngas fermentation is a viable option for producing carbon compounds that can be used as feedstock for microorganisms.

Although syngas fermentation has been touted as part of a possible solution to our energy crisis, there are many challenges that must be addressed before peak efficiency can be achieved. First and foremost is the low mass transfer rate of H₂, CO₂ and CO into liquid medium. (Drzyzga 2015) With limited substrate available to the microorganism, growth rates and product yields are limited. To improve the mass transfer limitations, advancements in reactor design and development of aiding technologies will be necessary. (Mohammadi 2011) So far, only a limited number of chemicals have been produced by syngas fermentation, mainly ethanol and acetate. (Mohammadi 2011) This is partly because the organisms currently used are not efficient producers of other chemicals and are not easily genetically manipulated. (Mohammadi 2011)

This is further reason to pursue syngas fermentation in non-acetogenic bacteria, which are capable of a multitude of syntheses and are easier to manipulate genetically. (Lindberg 2010, Bentley 2014)

Bacteria for Syngas Fermentation

Metabolizing syngas involves the ability to process H₂, CO, and CO₂. Two important types of CO oxidizing bacteria are acetogens and certain phototrophs. Some phototrophs, such as purple-non-sulfur bacteria, are capable of syngas fermentation in light and dark, however CO oxidation is more energetically favorable in the presence of light. In light, anaerobic PNSB (purple non sulfur bacteria) use a CO dehydrogenase to convert CO and H₂O to H₂ and CO₂ via a gas-shift reaction: $H_2O + CO \rightarrow H_2 + CO_2$. (Drzyzga 2015, Wawrousek 2014) CO₂ is speculated to be incorporated into cellular components via the CBB (Calvin Benson Bassham) cycle (Wawrousek 2014) and H₂ is oxidized by a hydrogenase to form a proton gradient used for energy production, in light. (Maness 2004)

Acetogens process H₂, CO₂ and CO very differently from phototrophs. Through use of the WL (Wood–Ljungdahl) pathway, these anaerobes incorporate syngas components into acetyl-CoA. (Drzyzga 2015) However, syngas fermentation in acetogens has been limited by an incomplete understanding of their energy metabolism and difficulties with transformation. (Mohammadi 2011, Verma 2016)

Isoprene and Terpenoid Pathways

Isoprene, or 2-methyl-1,3-butadiene (C₅H₈), is a colorless liquid that is volatile at room temperature. (Morais 2015) It is an essential commodity chemical used to manufacture a range of consumer items, but also present in small amounts in the metabolisms of various species. From rubber bands to shoe soles, many elastomers are made from isoprene. The most notable use of isoprene however, is in tire manufacturing. (Morais 2015) A small percentage (5%) of isoprene is also used to produce chemicals which can be used for the synthesis of pharmaceuticals, nutraceuticals, and many other commodity chemicals. (Morais 2015) Isoprene can also be converted to multiple different biofuels with varying properties depending on the use. (Harvey 2014)

Although plants around the globe emit 600 teragrams of isoprene annually (Lantz-Guenther 2006), which is enough to satisfy the world's tire production needs for the next 50 years, harvesting this isoprene for commercial purposes is economically unfeasible. (Morais 2015) Alternatively, most commercial isoprene is produced from fossil resources. (Morais 2015) Isoprene is produced differently around the world, but both ways rely on fossil resources. In Russia, isoprene is produced either from isobutylene and formaldehyde, or from dehydrogenation of isopentane. (Morais 2015) In the United States, isoprene is isolated directly from C₅ streams in petroleum refineries. (Morais 2015)

Recently, in light of unsteady oil prices and environmental concerns, interest in microbial production of isoprene has gained significant momentum. Due to its volatile nature, with a boiling point of 34° Celsius, isoprene produced biologically can be easily separated from culture medium. This distinguishes isoprene generation and separation from other biomolecules that may require additional separation techniques. Furthermore, pathways for isoprene generation are already known and certain strategies for increasing titer have been developed.

Isoprene is produced biologically from dimethylallyl diphosphate (DMAPP) via the enzyme Isoprene Synthase (IspS), which must be heterologously expressed in bacteria, as bacterial isoprene synthases have yet to be discovered. In the last decade, work on increasing

isoprene titers has mainly centered around increasing DMAPP production. Two biosynthetic terpenoid pathways, the MVA (mevalonate) and MEP (methyl-erythritol phosphate) pathways can produce DMAPP. The MVA pathway is found in all eukaryotes, archaea, fungi, and some eubacteria, while the MEP pathway is present in prokarya, plant chloroplasts, and algae (Zhao 2010).

Because the terpenoid pathways are involved in the bio-production of many chemicals of interest, developing strategies for increasing flux through these pathways benefits the production of other isoprenoid chemicals.

Introduction to Project

Novelties of Project

This project aims to address problems related to multiple areas of metabolic engineering. One important aspect is the pursuance of the feasibility of syngas fermentation and its improvement. It is clear that syngas from lignocellulosic biomass has the potential to avert future energy crises. Therefore, it is imperative to study syngas metabolism and develop strategies for improving its efficiency.

Strategies for increasing growth on syngas are relatively unexplored, but there are two general ways for increasing growth and product production from syngas. One way is to identify unnecessary byproduct formation and redirect metabolic flux toward product formation. Alternatively, we aim to identify the metabolic processes that consume and utilize syngas components. If we can discover which metabolic and signal transduction pathways are responsible for this metabolism, we can begin to optimize various parts of the system. In *R. gelatinosus* CBS CO metabolism is thought to be controlled by the transcriptional regulator RcoM. It is hypothesized that overexpressing *rcoM* may also improve growth rates on CO.

Next is the goal of increasing flux through the terpenoid pathways. Previous works have shown that heterologous expression of the MVA pathway results in superior DMAPP and isoprene production when compared to efforts to improve the native MEP pathway. (Bentley 2014, Lindberg 2010) The MVA pathway is fed by the precursor acetyl-CoA. (Figure 1) Therefore, we will attempt to redirect acetyl-CoA from other processes in the cell towards the MVA pathway. One strategy is to focus on pathways that produce polyhydroxyalkanoates (PHAs), which are energy storage molecules. PHB (polyhydroxybutyrate) is a type of PHA produced from acetyl-CoA, so it follows that removing proteins responsible for PHB production may lead to a buildup and redirection of acetyl-CoA. Any strategies that are verified will be relevant to bio-production of chemicals beyond isoprene. Because DMAPP and isoprene are the basic building block to many terpenoids, a finding like this would immediately impact any metabolic engineering strategies utilizing the MVA pathway and perhaps other projects involving the redirection of acetyl-CoA.

For this project, *Rubrivivax gelatinosus* CBS has been chosen as a strain for a few main reasons. CBS has been shown to metabolize CO, (Maness 2004) its genome has been sequenced, (Hu 2012, Wawrousek 2014) and it has been shown to accumulate large amounts of PHAs (Maness 1994). Also important is that genetic modification of this strain has been performed successfully. CBS also has an impressive metabolic diversity, which is an important characteristic of strains used for metabolic engineering. For example, this strain can perform chemotrophic and phototrophic growth, has multiple methods of sugar metabolism, performs fermentation, nitrogen fixation, and H₂ gas production. (Hu 2012) Metabolic versatility, high amounts of PHA, and the ability to consume CO make CBS an ideal strain for this project.

Plan and Hypotheses

There are three phases to this project. First, a pathway for isoprene production is added to the genome of CBS. Next, we attempt to shunt carbon flux toward isoprene by knocking out competing pathways. Finally, we attempt to increase the speed at which the microbe can process CO. This is also tied to CO₂ and H₂ processing as described above.

To begin, we add pathways for isoprene production. Isoprene is produced biologically from dimethylallyl diphosphate (DMAPP) via the enzyme Isoprene Synthase. The first manipulation made to CBS was to introduce *ispS*, which encodes the isoprene synthase enzyme. Next, we attempt to increase carbon flux in the cell towards DMAPP. Two biosynthetic pathways, the MVA (mevalonate) and MEP (methyl-erythritol phosphate) pathways can produce DMAPP. (Zhao 2011) The unmodified CBS genome naturally contains the MEP pathway, but not the MVA pathway. (Figure 1) Previous works show increased isoprene and DMAPP titers from addition of MVA pathway enzymes. (Bentley 2014, Yoon 2008) Five genes from the MVA pathway (Hmg-CoA reductase (HmgR), mevalonate kinase (MK), phosphomevalonate kinase (PMK), mevalonate-5-pyrophosphate decarboxylase (PMD), and IPP isomerase (FNI)) are being introduced in tandem with targeted gene knockouts.

At the same time, we are exploring the effects of deleting pathways involved in energy storage and pathways involved in fermentation. It has been shown that eliminating pathways that produce fermentation end products can divert carbon flux towards a product of interest. (Li 2012) This same logic should apply to energy storage pathways. Energy storage molecules, such as polyhydroxybutyrate, are synthesized by bacteria for use in times of nutrient deficiency. However, bacteria used for chemical production should not require energy storage molecules like this because feedstock will be supplied at all times. Past studies have shown that knockout of these PHA genes leads to viable cells. (Martino 2014)

Next, we will identify the fermentation end products that are produced during growth on CO. HPLC analysis of CBS grown on CO reveals several such products. Identification of these unknowns will provide insight into the carbon flux from syngas metabolism in CBS. This will be done with the help of another lab on campus, using an LC-MS. Once we have identified the molecules our bacteria loses carbon flux towards we can explore the effects of deleting these pathways—with the hope of increasing flux to terpenoid pathways and increasing isoprene titers.

Lastly, we aim to increase the consumption of syngas. One method to increase CO metabolism is to target the transcriptional regulators tied to the CO dehydrogenase. We know of two such regulators, CooA and RCoM (regulator of CO metabolism). (Wawrousek 2014) It has been observed that overexpressing RCoM in CBS allows cells to transition to growth using CO more rapidly. Prior works have shown that overexpression of *cooA* can also increase the rate of CO consumption. (Ainala 2014).

Materials and Methods

Media

M1 media was prepared with 120 mg/L MgSO₄ · 7H₂O, 75 mg/L CaCl₂ · 6H₂O, 11.8 mg/L FeSO₄ · 7H₂O, 20 mg/L EDTA, 2.8 mg/L H₃BO₃, 1.6 mg/L MnSO₄ · H₂O, .75 mg/L Na₂MoO₄ · 2H₂O, .24 mg/L ZnSO₄ · 7H₂O, .04 mg/L Cu(NO₃)₂ · 3H₂O, .8 mg/L CoCl₂ · 6H₂O, .8 mg/L NiCl₂ · 6H₂O, .6 mg/L KH₂PO₄, .9 mg/L K₂HPO₄, 1 mg/L Thiamine HCl, 15 µg/L Biotin, 1 mg/L Nicotinic acid, 10 µg/L B-12, 100 µg/L p-aminobenzoic acid, and 1.5 g/L 10% NH₄Cl. BG media was prepared by combining M1 media with 0.5 g/L yeast extract and 5 g/L DL-malic acid. M1 + YE denotes M1 with 0.5 g/L yeast extract. Components of media were

autoclaved or sterile filtered before a final mixing. After mixing, the pH of this solution was adjusted to 6.8-6.9 and media was sterile-filtered into sterile glass media jars using a .22 µm bottle top vacuum filter.

Growth Conditions

CBS strains were grown anaerobically in glass screw top tubes with BG media under light at 32-34° Celsius. Cultures were grown with antibiotic if a selective marker was present. Carbenicillin and kanamycin resistant CBS was grown with 10 and 20 µg/mL antibiotic, respectively. Cultures were started from freezer stocks, and freezer stocks were prepared by mixing culture with sterile filtered 50% glycerol in a 1:1 ratio. For growth and isoprene emissions tests, strains were grown in 27 mL anaerobic culture tubes, under argon, with 10 mL culture under lights and shaking at 215 RPM, without antibiotics. Fluoroelastomer stoppers were used for isoprene emission tests, while all other tests used butyl rubber stoppers. Light intensity for all growth experiments was 80 micro-einsteins and temperature was 32-34°. Optical density (OD) values were obtained using a Unico 1100-RS spectrophotometer and absorbance measurements were taken at 660 nm.

DNA Preparation and Cell Handling

Plasmid Construction

Primers and plasmids were designed using Snapgene software. Primers were designed based on recommendations by NEBuilder HiFi DNA Assembly Cloning Kit. Archetype, was used for searching CBS genomic DNA. Archetype was used for codon optimization of genes introduced into the chromosome of CBS, not including antibiotic resistance genes. Antibiotic resistant genes were left unmodified. PCR was performed using a Bio-Rad T100 Thermal cycler. A PCR mixture was prepared following NEB guidelines by combining NEB Q5 Reaction buffer, Q5 GC enhancer, and dNTP solution. This mixture was added to template DNA, primers, and NEB Q5 Polymerase. The following method was used for PCR reactions: 98°C for 3 min, 30 cycles of 98°C for 10 seconds, anneal temp for 30 seconds, 72°C extension for 1-2 minutes, followed by a 72°C final extension for 2 minutes, hold at 4°C.

Backbone plasmid regions were prepared by digesting a plasmid made by a previous graduate student, which tested an *rcoM* deletion. An important component of the backbone plasmid region is the *ori* (pMB1), or origin of replication, which directs the host cell to initiate replication of the plasmid.

After PCR and restriction digest reactions produce DNA fragments, plasmids are constructed by isolating the DNA fragments and performing a Gibson reaction. Gel electrophoresis with TAE agarose (2%) gels were used with .2 µL/mL SYBR-safe DNA dye to isolate DNA fragments. Gels were run in 1x TAE buffer at 60 V and 400 mA for 2 hours. Gels were imaged using a Protein Simple FluorChem imager using the SYBR-Green setting. DNA was extracted from gels using a Zymoclean Gel DNA Recovery Kit. DNA was quantified after every extraction using a NanoDrop 2000 by Thermo Scientific. Plasmids were then assembled using NEBuilder HiFi DNA Assembly Cloning Kit. Assembled plasmids were transformed into chemically competent Mix and Go *E. coli* (Zymo Research).

Transformation of *Rubrivivax gelatinosus* CBS

Preparation of electrocompetent cells and transformation protocols were adapted from Ouchane et al, 1996. Electrocompetent CBS was prepared by triple rinsing cells, with decreasing volumes of ice cold DI water (10ml, 6ml, 4ml), at 4,700 RPM (4816 G) and addition of 10% ice

cold glycerol to the final cell pellet in a 1:1 ratio by volume. A 280 μL aliquot of electrocompetent CBS was transformed with .1-1 μg DNA using an ECM 630 Electro Cell Manipulator with 2 mm electrode gap cuvettes. Electroporation parameters used were 2 kV, 200 Ω , and 25 μF , which produced time constants close to 5 ms. Electroporated cells were then diluted in 900 μL of BG media and incubated in darkness at 32° Celsius with gentle shaking for 6 hours. Various concentrations of 100 μL amounts of this mixture were plated onto BG plates containing antibiotic, and streaked for colonies. Streaking plates for colonies is necessary for later isolation of single bacterial colonies to ensure a single genome is selected. Plates were then put in an anoxic chamber, with Argon atmosphere, under light at 32° Celsius until colonies appeared after 2-3 days. Isolated colonies were used to streak more antibiotic plates, and this growth was used to inoculate liquid cultures. Colonies were streaked onto new plates before transfer to liquid media to increase amount of cells being transferred to liquid.

To test for homologous recombination in successful CBS transformants, colony PCR was first attempted. If this was unsuccessful, isolated colonies were grown in liquid culture and a genomic DNA was extracted. Genomic DNA was extracted using the GenElute Bacterial Genomic DNA kit (Sigma Aldrich) and subsequently used with PCR to detect integration into the genome.

Detecting Gene Expression of *ispS*

Gene expression was evaluated by reverse transcription-polymerase chain reaction (RT-PCR). RNA was first extracted from cells using a RNeasy Mini Kit (Qiagen). Superscript reverse transcriptase (Invitrogen) was used to generate cDNA from RNA using a reverse primer from the amplification of *ispS*, following the manufacturer's protocol. The recovered cDNA was used as template DNA in a PCR reaction, following guidelines discussed above for PCR reactions. PCR products were then visualized using gel electrophoresis.

GCMS Analysis

Isoprene emission was detected using a GC-MS (Shimadzu GCMS-QP2010) with a 30 m SH-Rxi-624Sil Shimadzu column (221-75962-30). The following temperature program was applied: oven temp was set to 45° C to give an initial temperature of 45°C, then an increase of 15° C/min is applied until 90° C is reached, then rising from 90°C to 220°C at a rate of 60° C/min. The injector temperature was set to 200° C and isoprene was eluted with this program at 2.6 minutes. Cultures were placed in a dry bath at 90°C for 10 minutes before a 200 μL headspace sample was injected into the GC-MS. Fluoroelastomer stoppers were used for isoprene emission tests because butyl rubber stoppers may produce some isoprene via monomer leaching.

HPLC Analysis

A Shimadzu (LC-20AD) HPLC was used to identify end products of growth on carbon monoxide. A Rezex Organic Acid column with dimensions 300 mm x 7.8 mm was used with a mobile phase composed of .005 M H_2SO_4 with flow rate .5 mL/min. Column temperature was 55°C and detector PDA (Photodiode Array Detector) temperature was 40°C. Absorbance was measured at 210 nm and method ran for 32 minutes. 100 μL injections were performed using samples that had been sterile filtered prior to injection.

Results

Isoprene Production in *R. gelatinosus* CBS

A plasmid was designed to insert the isoprene synthase gene, *ispS*, into the CBS genome at a neutral site, *hypE1* (Figure 2A). The isoprene synthase gene used is from *Pueraria montana* (kudzu vine) and was codon optimized for CBS. This sequence was chosen based on previous work (Zhao 2010, Lindberg 2010). A kanamycin gene was also included in the plasmid design for selection of cells that had been transformed with the recombinant DNA. Both the *ispS* and kanamycin resistance genes were placed under the control of a single eukaryotic chloroplast *psbA* promoter, known for strong constitutive expression. (Brixey 1997, Ungerer 2012). This cassette was flanked with approximately 1000 bp of upstream and 1000 bp of downstream flanking sequence of the *hypE1* gene to allow homologous recombination and replacement of the native *hypE1* gene with the recombinant DNA. Primer sequences used to synthesize plasmid fragments are listed in Figure 2A, and the plasmid was assembled using Gibson assembly to generate the pIspS plasmid. The constructed plasmid was then transformed into *E. coli* for amplification.

The plasmid was confirmed by restriction digest (Figure 2B), and CBS was transformed with pIspS. Colonies that did appear were screened with colony PCR to test for homologous recombination. Genomic material was collected from three colonies (1, 2, and 3) to confirm recombination.

Primers designed to test insertion of *ispS* and *kanR* into the genome were used in PCR reactions with the newly extracted genomic DNA, without success. Another PCR test, designed to amplify the newly modified *hypE1* region suggested no insertion. (Figure 3) This, paired with the failure of the other primers to confirm insertion suggests that the gene was not inserted into the genome. However, a RT-PCR was also performed that confirmed expression of *ispS*. (Figure 4) Lastly, GC-MS analyses of headspace samples were used to quantify isoprene production. (Figure 5)

Headspace samples taken from cultures at OD ~1.3 showed 2e-5 µg/mL of isoprene in the headspace. This value is difficult to compare to other studies which observe isoprene emission over a longer period of time and using larger vessels.

Addition of Mevalonate (MVA) Pathway

Plasmids for introducing the mevalonate pathway genes into CBS were designed. The purpose of these plasmids are to insert MVA pathway enzymes and delete PHB synthesis enzymes to direct additional carbon flux toward isoprene production. First, the genes being targeted for deletion were selected. Using Archetype to search through CBS's metabolic pathways, a location for a possible bottleneck in PHB synthesis was found. (Figure 6) CBS contains two genes that can be used for the last step in PHB synthesis. These genes are polyhydroxybutyrate polymerase (PHB) and polyhydroxyalkanoate polymerase (PHA). Trying to delete any other part of this pathway would require more than two deletions, so these enzymes were prime targets for deletion. 5 genes would be inserted into CBS using two plasmids, each with a different antibiotic resistance. (Figure 7) Antibiotics were selected based on availability and previous use with CBS. Primers were designed after creating the plasmids in Snapgene and various restriction sites were included to create sites for future manipulation. Gene sequences were selected from *Streptococcus pneumoniae* R6 and codon optimized for CBS in Archetype before ordering from BioBasic. Genes were selected from *S. pneumoniae* as a control, as previous studies used these genes. (Bentley 2014, Yoon 2008, 2012) Plasmids were constructed via

Gibson reaction and confirmed by restriction digest. Only two plasmids have been completed and confirmed; pΔHypF1-PMD-FNI, and pΔPHB-PMD-FNI.

The Hmg-CoA reductase gene of the mevalonate pathway was left out of initial designs by accident. It was decided to incorporate this gene into the plasmids containing the *MK* and *PMK* genes. This would result in 2 modified plasmids, pΔPHA-MK-PMK-HmgR and pΔHypE1-MK-PMK-HmgR. To achieve this goal, an *hmgR* gene from *S. pneumoniae* was codon optimized for CBS and ordered from BioBasic. Primers were designed that would amplify the *hmgR* gene and also create flanking regions homologous to *PMK* and *ampR*. (Figure 8) First, a restriction digest using BamH1 would be performed on pΔPHA-MK-PMK. Then, a Gibson reaction would be performed on the *hmgR* fragment, along with the BamH1 digested pΔPHA-MK-PMK to create the pΔPHA-MK-PMK-HmgR plasmid.

Another issue that was encountered was with amplification of the gene inserts. PCR reactions attempting to amplify MK-PMK and PMD-FNI were unsuccessful. Initially, forward primers were designed that contained the entire *psbA* promoter. This resulted in a long primer that was capable of annealing to itself. This annealing prevented PCR amplification of the gene inserts and new primers had to be designed. To fix the problem, the PCR amplification was split into two separate amplification reactions. For the first round, a forward primer, with the first half of *psbA* was used with a reverse primer. (Figure 9) Using this PCR product, another amplification was performed using the second forward primer, which contained the remaining half of the *psbA* promoter with the same reverse primer. This resulted in amplified gene fragments with a full *psbA* promoter attached.

Towards Increasing Growth Rates on Syngas

Growth tests using solely CO as a carbon source were conducted on *rcoM* overexpression and *rcoM* deletion strains. The tests included WT, an *rcoM* overexpression strain, and the $\Delta rcoM$ strain. Cultures were first grown anaerobically in BG media to an OD of 1. Then, 0.5 mL of a culture was added to 9.5 mL of M1 w/ YE in anaerobic culture tubes with 17 mL of headspace. Headspace was exchanged with argon and tubes were placed under light at 32° Celsius, shaking at 215 RPM. Once growth had stopped, 3 mL of CO was added to the headspace and growth was recorded by OD. To determine growth in the M1 w/YE mixture had stopped, ODs were taken at 2 hour intervals until no change in OD between a 2-hour period was observed. This was necessary to ensure that CO was the only carbon source being utilized. The results of this experiment showed an enhanced growth rate for the *rcoM* overexpression strain, and very slow growth for the $\Delta rcoM$ strain. (Figure 11)

Redirecting Metabolic Flux

An experiment was designed to test the fermentation end products of growth on solely carbon monoxide. CBS was first adapted to CO in a serum vial using M1 w/ YE and CO (30%) in the headspace. This culture was transferred to a serum vial with M1 w/ CO (30%) in the headspace and grown to an OD > 1. Three mL of this culture was transferred to 6 anaerobic culture tubes with 7 mL M1 to give a culture volume of 10 mL and starting OD of ~.3. Three controls were immediately centrifuged at 4700 RPM (4816 G) for 10 minutes and the supernatant was collected and filtered with a .22 μ m syringe filter. These controls are meant to show what byproducts are produced prior to CO injection. The remaining three culture tubes were injected with 3 mL of CO and placed under lights with shaking. After growth had been observed via an OD doubling, GC measurements were taken to confirm consumption of CO. After growth had been confirmed, cultures were spun down and supernatant collected and

filtered. The samples were analyzed via HPLC and several end products were observed. (Figure 12)

Additional Work

Another plasmid, p Δ CooA was also constructed. (Figure 10) This plasmid was designed to delete the *cooA* gene in CBS, and mark this deletion with Ampicillin resistance. This plasmid had already been designed and a Gibson reaction had been attempted, but transformation in *E. coli* was unsuccessful. The problem was thought to be in the T7 terminator region. Without a proper transcriptional terminator, cells are not likely to produce functional RNA transcripts. The solution was to design new primers for amplification of the T7 region. Next, all components of the plasmid were re-synthesized and another Gibson reaction was performed, which was successful. Transforming this strain into CBS was difficult, but colonies were isolated and screened for recombination. The results showed no recombination, however it was discovered the problem was related to the primers designed. The reverse primer was designed incorrectly and annealed to the same strand as the forward primer. With two forward primers, no PCR product could have been achieved. No further confirmation has been done on this strain.

Discussion

For this work, parts of the mevalonate pathway will be heterologously expressed in conjunction with an isoprene synthase gene in CBS in an attempt to increase isoprene production. Also being investigated is whether carbon flux from an energy storage pathway can be redirected towards isoprene production via gene knockouts. Last, we aim to develop a better understanding of CO metabolism in CBS by studying transcriptional regulators and fermentation end products tied to growth on CO.

ispS Expression in a PNSB (purple non sulfur bacteria)

The isoprene synthase gene *ispS* from *P. montana* (kudzu vine) has been shown previously to catalyze the conversion of DMAPP to isoprene in cyanobacteria. (Lindberg 2009, Zurbriggen 2012, Bentley 2014). Previous studies show that codon optimization of this gene improved its efficiency. (Zurbriggen 2012, Bentley 2014) For this reason, the *ispS* gene from *P. montana* was codon optimized for CBS. Successful expression and optimization of this gene had not been observed in a PNSB like CBS before. To express this gene, a *psbA* promoter from pea plant chloroplasts was used. Previous studies have used this promoter to drive foreign gene expression in cyanobacteria, (Ungerer 2012) but it has not been used for expression in a PNSB like CBS before. Previous studies looking to increase isoprene production in cyanobacteria have used the cyanobacterial *psbA2* promoter to drive expression of foreign genes, not the *psbA* promoter. (Bentley 2014, Lindberg 2010, Mulo 2012)

In this work homologous recombination of *ispS* was not verified in any of the transformants. The *ispS* strain was found to produce $2e-5$ $\mu\text{g/mL}$ isoprene in the headspace at an OD of 1.3 and WT samples did not produce a detectable amount of isoprene under similar conditions. It is hard to compare the amount of isoprene produced in this work to others because this was preliminary work. Other works have designed reactors and experiments specifically for maximizing the amount of isoprene produced. It is clear however, that the amount of isoprene produced in this work is far less than similar works have displayed. One comparison is with Zurbriggen et al, 2012. In this work, after expression of kudzu *ispS* in *E. coli* 1.25 mg/L broth of isoprene were produced over a 30 hour incubation period. (Zurbriggen 2012) Scaling up our own isoprene emission data gives a theoretical value of .34 $\mu\text{g/L}$ broth of isoprene for our *ispS* CBS

strain. From this, it appears that expression of *ispS* in CBS did not produce near as much isoprene as expression of *ispS* has in a comparable work. The work of Lindberg et al, 2009 showed 50 µg of isoprene per gram of dry cell weight per day with a cyanobacteria host. Using a conversion factor (Ren 2013) to convert our OD to dry cell weight gives .072 µg of isoprene per gram of dry cell weight. This value is also much lower than previous works have shown. However, this is the first work to use CBS as a platform for the production of a commodity chemical.

Because the expression of *ispS* was verified by RT-PCR but insertion into the genome could not be confirmed, it is assumed that the plasmid did not undergo homologous recombination with CBS. Homologous recombination is a rare event unless the cell has pressure to do so. Our plasmid system was designed to increase the chances of this event by keeping plasmid copy number low via expression of the *rom* gene, (Tomizawa 1984) but this was unsuccessful. An alternative technique would be to use suicide vectors (plasmids) to increase the chances of a recombination event in CBS. A suicide vector increases the chances of a recombination event by preventing the cell from continually expressing genes from the plasmid. With this pressure, only cells that have undergone a homologous recombination event will be selected for and survive.

Partitioning Carbon Away from an Energy Storage Pathway

Previous efforts to increase flux through terpenoids pathways have focused on the MVA and MEP terpenoid pathways. Efforts towards optimizing the MEP pathway, to improve DMAPP production have been attempted, (Banerjee 2013) but higher amounts of DMAPP production have been observed by expressing an additional terpenoid pathway, the MVA pathway. (Lindberg 2010, Bentley 2014). The MEP pathway, which is native in CBS has regulatory controls that inhibit high flux through this pathway. (Banerjee 2013) Previous works have explored the efficiencies of MVA pathway genes from various organisms, and codon optimized MVA pathway genes from *S. pneumoniae* were found to result in the most flux through this pathway. (Yoon 2009). The insertion of this entire pathway into *Synechocystis* PCC 6803, (Bentley 2014), was the first instance of an entire biosynthetic pathway being introduced into a photosynthetic organism. Previous works like this have only looked at the introduction of this foreign pathway and not the simultaneous redirection of carbon flux towards this pathway. However similar strategies, that involved the simultaneous knockout and introduction of genes has been performed on photosynthetic bacteria successfully. (Liu 2011) This work would be the first to attempt to eliminate a major carbon storage pathway in bacteria to increase isoprene production. It is also the first work to study isoprene production from syngas in a photosynthetic organism. If the strategy employed in this work is shown to be successful it would be beneficial to both platforms for isoprene production and production of other isoprenoids that use DMAPP as a starting material.

Transcriptional Regulation of CO Metabolism

In determining transcriptional controls of growth on CO, growth tests comparing WT, a *rcoM* overexpression strain, and the $\Delta rcoM$ strain clearly show that growth on CO is improved by overexpression of *rcoM*. This could mean that basal amounts of this protein are not enough to maximize the efficiency of this system. Also important is that CO growth *is* possible without this transcription factor, but is very slow. This is evidence for alternative CO transcription factors, possibly *cooA*. However, *cooA* is speculated to control the regulation of other genes as well.

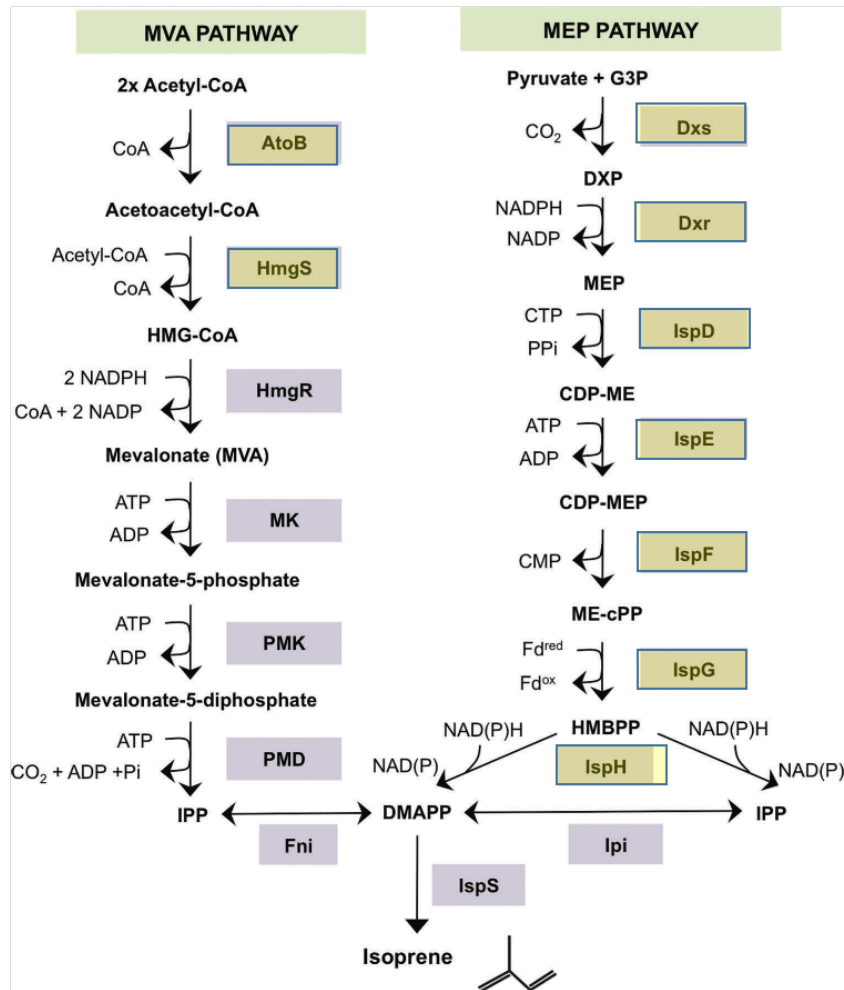
In CBS, the *cooA* gene is adjacent to the *cowN* gene in the genome. (Wawrousek 2014) In the PNSB *Rs. rubrum*, the *cowN* gene has been observed to protect nitrogenase from inhibition by CO. (Kerby 2011) It is known that CBS can fix nitrogen in the presence of CO, (Maness 1994) but the mechanism for protecting nitrogenase from CO is unknown. (Wawrousek 2014) It is hypothesized that *cooA* may regulate *cowN* expression in CBS due to these genes' proximity to in the genome of CBS. The plasmid, p Δ CooA, could be used to investigate this in CBS. Developing a better understanding of the control of CO metabolism in CBS will allow us to better forward engineer this strain as a platform for fuel and chemical production using syngas as a feedstock.

Fermentation end products of growth on CO

HPLC analyses of CO fermentation end products show that there are metabolic end products that are produced during growth on CO. Previous works have shown that carbon flux may be redirected from fermentation pathways, (Li 2012) but this has not been investigated with respect to syngas fermentation in a photosynthetic microorganism. After identification of these metabolites is performed, options for deleting pathways involved with these metabolites can be explored. Identification of these metabolites can be done via LC-MS, of which, one is available on campus. Efforts to perform this analysis were unsuccessful due to time constraints, but future work on this is ready to be performed.

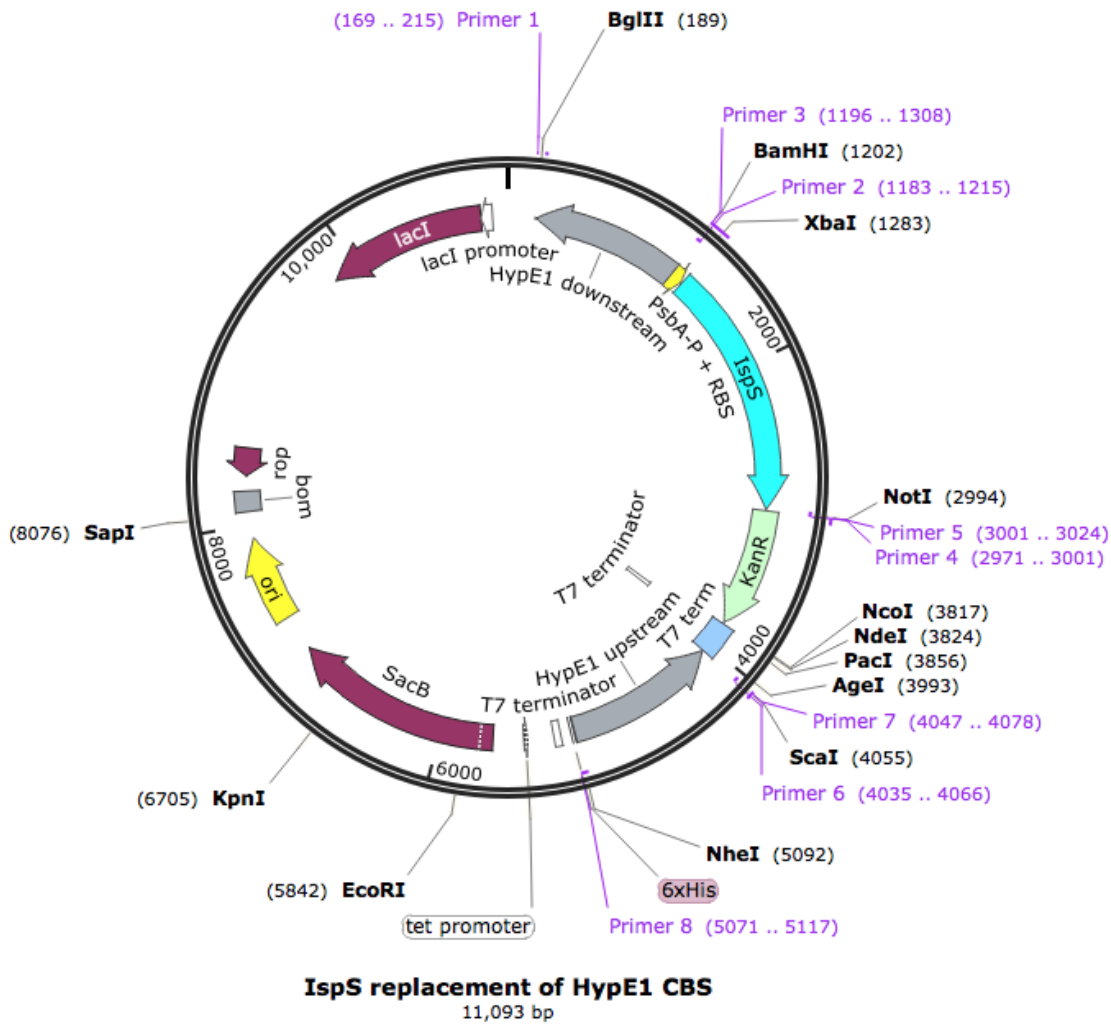
There are considerable problems with syngas fermentation that still need to be worked around. Such issues include the low mass transfer rate of syngas into liquid culture and the energetic constraints of syngas fermentation. Improvements in reactor design and implementation of new and unexplored aiding technologies may improve the issue of mass transfer into liquid culture. Using photosynthetic organisms can reduce the energetic constraints of syngas fermentation by harnessing energy from sunlight, however using solar as an energy source has its own issues. Issues that plague traditional solar energy, mainly the unavailability of sunlight 24 hours a day is an issue that will reduce productivity. However, continued research in this field will help make future production schemes, that will satisfy global demand, more feasible.

Figure 1: Diagram showing the MVA and MEP terpenoids pathways. Enzymes that are already naturally present in the CBS strain are highlighted and outlined. This figure was taken from Bentley et al, 2014.



(Bentley 2014)

Figure 2A: pIspS Plasmid. This plasmid was constructed from 5 individual fragments: *hypE1* downstream, *ispS*, *kanR*, *hypE1* upstream, and the backbone region (including an origin (*ori*) of replication). Primer locations are noted on the diagram and complete sequences are listed below.



Sequences of Primers Used

Primer 1 (Forward) - 5'-GCGTCCGGCGTAGAGGATCGAGATCTCATCGACAAAGGTGTGCGCCT-3'
 Primer 2 (Reverse) - 5'-TTGAGATCGGATCCCAGCTGCCGCGCATCTGCT-3'
 Primer 3 (Forward) - 5'-CAGCTGGGATCCGATCTCAATGAATATTGGTTGACACGGGGCGTATAAGACATGTTATA
 CTGTTGAATAACAAGGAAAGGAGAAGAAATCTAGAATGCCCTGGATCTGTGCCAC-3'
 Primer 4 (Reverse) - 5'-CTTAAGCTCCTTGCGCCGCTCAGACGTACATCAGCTGGTTG-3'
 Primer 5 (Forward) - 5'-GCGGCCGCAAGGAGCTTAAGATGAGCCATATTAACGGGAAACG-3'
 Primer 6 (Reverse) - 5'-CCTAGAACAGTACTGGGACCCGTTTCAGGGCAG-3'
 Primer 7 (Forward) - 5'-GGTCCCAGTACTGTTCTAGGCGGCTCGCGGAT-3'
 Primer 8 (Reverse) - 5'-CAATGGTGATGGTGATGATGGCTAGCGCTGCAGGTGATGGAAGTCTG-3'

Figure 2B: Restriction Digest Confirmation of pIspS. PCR was used with restriction digestion to confirm the constructed plasmid. Lanes 1 and 2 were PCR reactions used to verify the presence of *hypE1* downstream and upstream regions and to serve as positive controls for the PCR reactions. Lanes 3 and 4 are restriction digests of portions of pIspS. The 2117 bp band encompassing *kanR* and upstream *hypE1* region was digested with *Nco*I and bands at 1270 bp and 816 bp were confirmed in lane 3. These bands represent the *hypE1* downstream region and Kanamycin resistance respectively. The 2803 bp band was digested with *Bam*H1, and bands were expected at 1013 bp and 1790 bp. Both of these bands are observed in Lane 4, where the upper band corresponds to *ispS* and the lower band is the downstream *hypE1* Region.

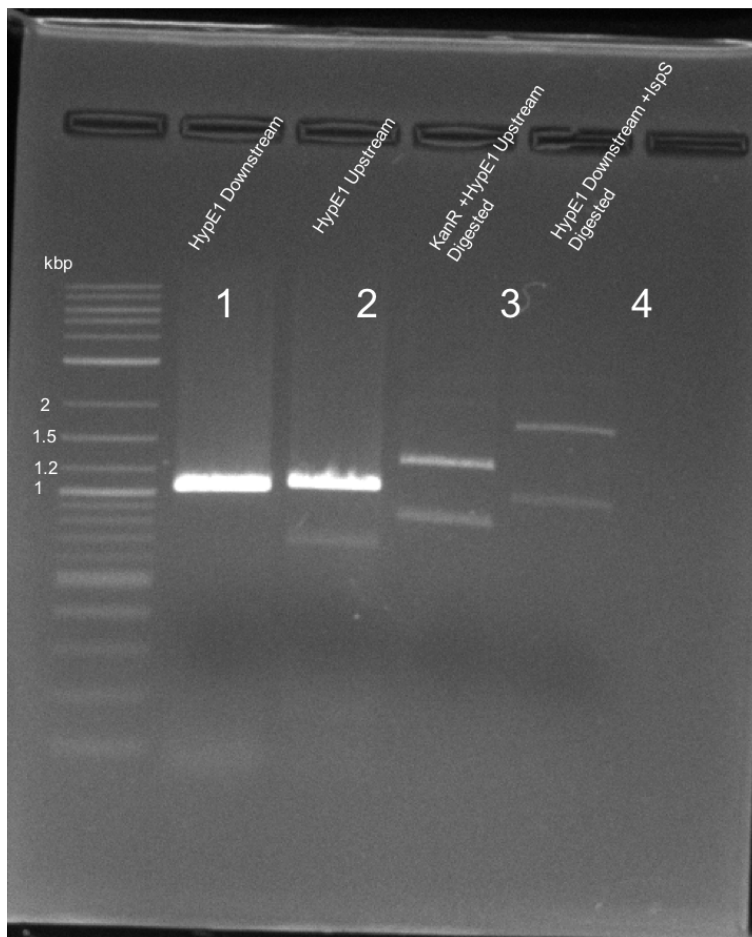


Figure 3: *hypE1* Region Amplification. If insertion into the *hypE1* site had occurred, amplification from genomic CBS DNA would produce bands at 4949 bp, like in the control. Amplification of ~3000 bp suggests no insertion has occurred. PCR attempt using colony 1-3 are seen in lanes 1-3, and PCR attempt using pIspS is in lane 4 as a positive control. Small “5” and “3” represent ladder bands corresponding to 5000 bp and 3000 bp.

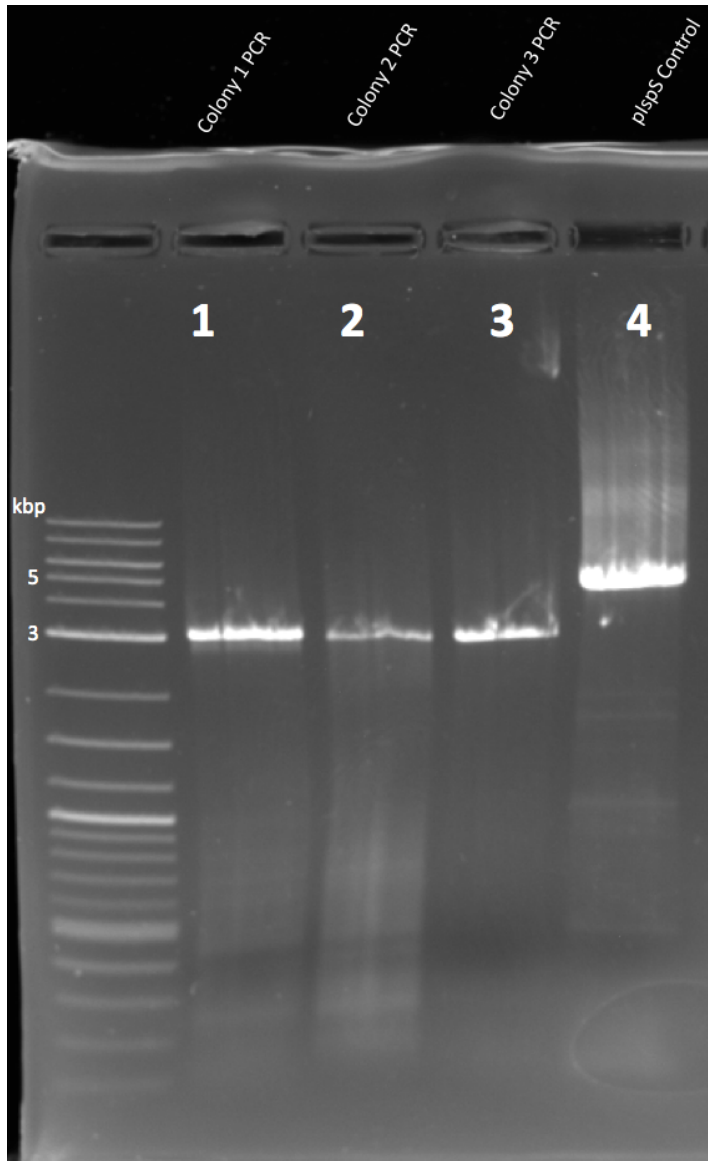


Figure 4: Reverse Transcriptase-PCR Reaction Confirms Expression of *ispS*. This experiment was ran using colony 1 from figure 2. Lane 1 is a positive control for the PCR reaction used in lane 2 which used pIspS template DNA. The band present in lane 1 represents the correct amplification length of the *ispS* gene. Lane 2 is the band produced from the RT-PCR reaction, which confirms that *ispS* is expressed by the pIspS CBS mutant. Lane 3 is a negative control for the RT-PCR reaction. It was necessary to confirm that results in lane 2 were not due to DNA contamination. There would be a band present in lane 3, as seen in other lanes, if DNA contamination had occurred.

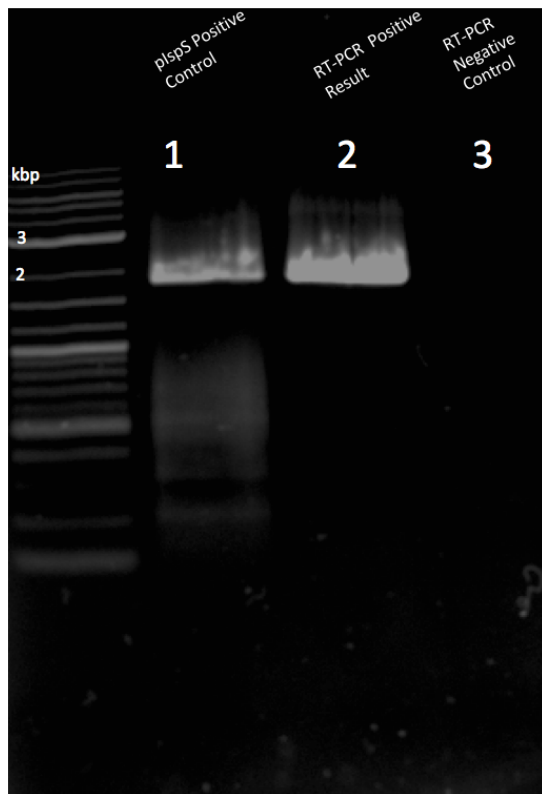
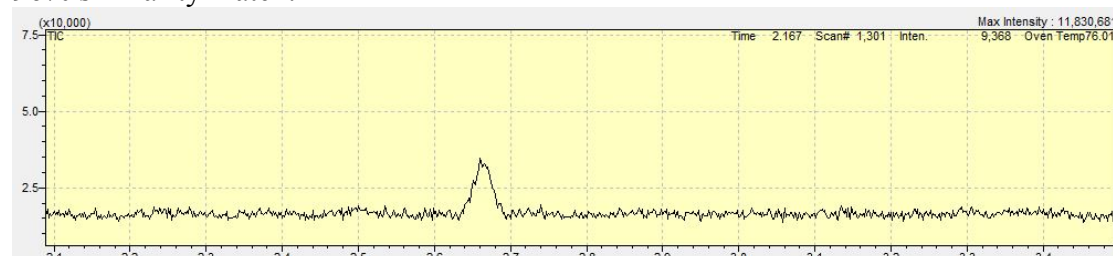
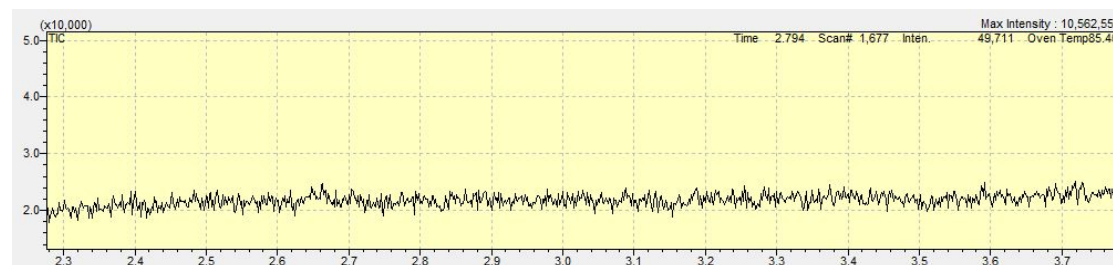


Figure 5: Isoprene Emission and Mass Spectra Identification of Isoprene. Below are the GC chromatograms and mass spectra of isoprene which show isoprene emission and identification from the *IspS* mutant, but not from a control Wild Type. Headspace samples were taken from cultures at OD ~1.3. This signal strength correlated with 2e-5 µg/mL isoprene in the headspace at a retention time of 2.67 minutes. Below that is the Mass-Spec identification of isoprene, with a 96% similarity match.



pIspS Strain



WT control

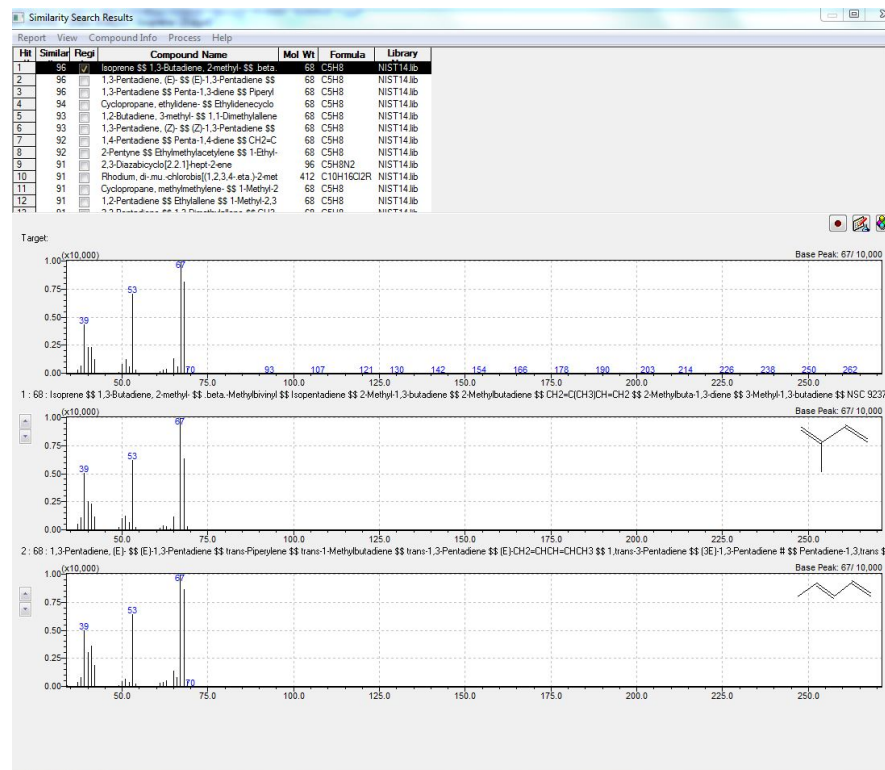
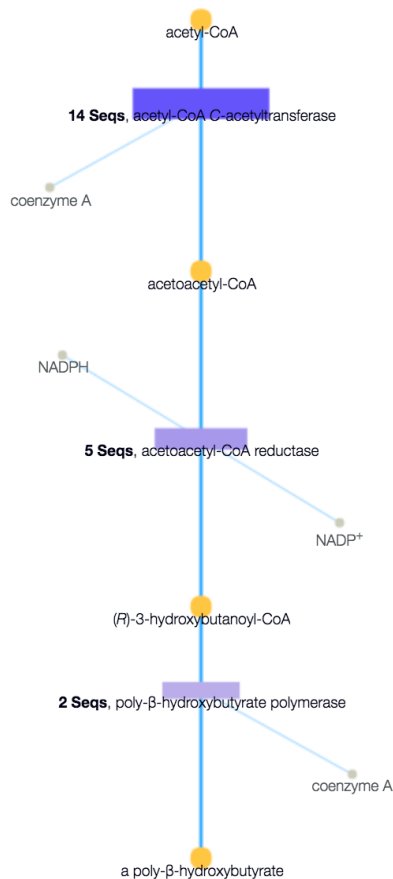


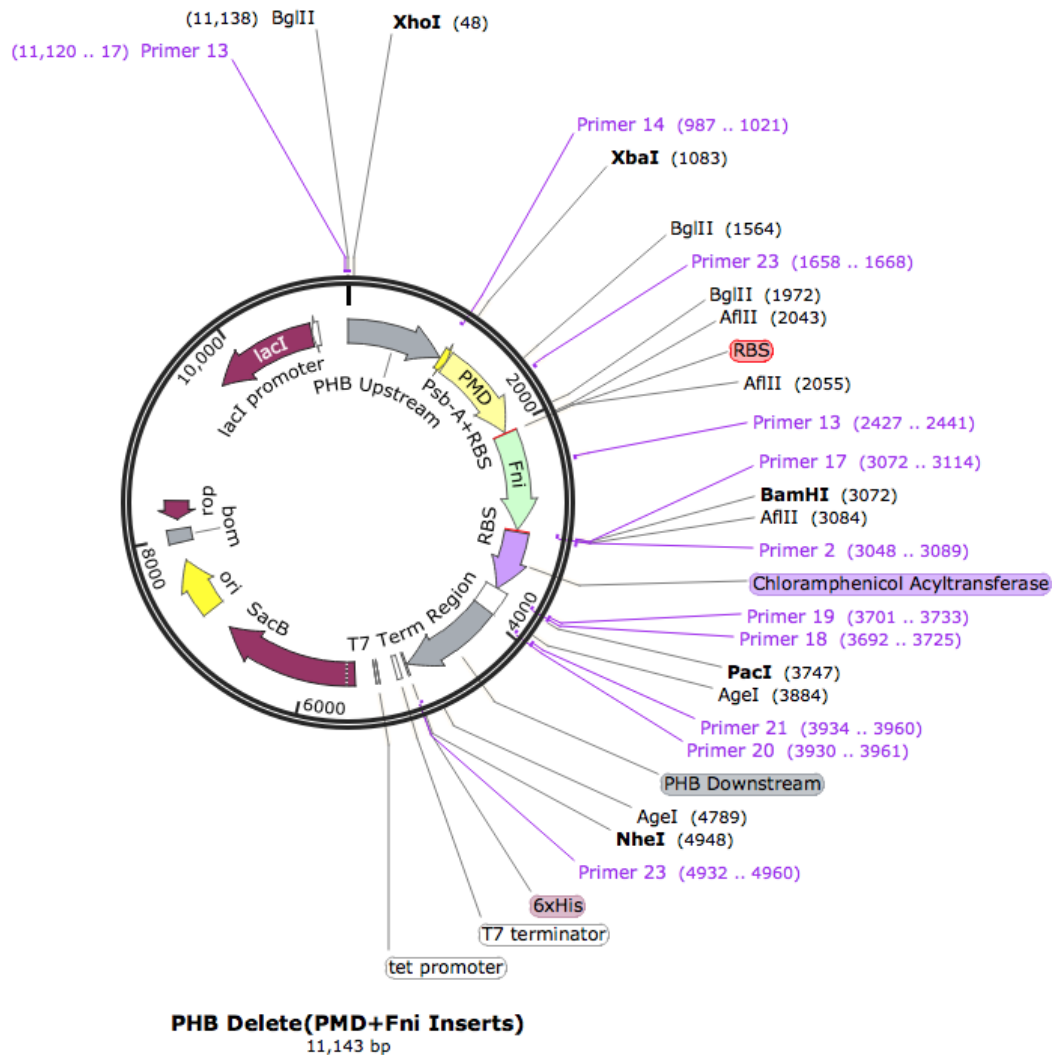
Figure 6: Poly- β -hydroxybutyrate Biosynthesis Pathway in CBS. Below is a representation of gene sequences harbored by CBS for the production of poly- β -hydroxybutyrate. The three genes in this pathway that could be knocked out in an attempt to cause a build up of acetyl-CoA are acetyl-CoA-acetyltransferase, acetoacetyl-CoA reductase, or poly- β -hydroxybutyrate polymerase genes. Of these three genes, the poly- β -hydroxybutyrate polymerase is a good choice of gene to target because CBS contains only two copies of this gene. To target another enzyme in the pathway would require up to 14 different deletions. Furthermore, there are likely multiple copies of the other genes in this pathway because they are required for primary cellular functions.



(Screenshot, <https://archetype.syntheticgenomics.com>)

Figure 7: Plasmids Designed for MVA Pathway Addition and PHA Pathway Gene Knockout. Below are the 6 plasmids designed to test the MVA pathway addition. Also included is pΔPHA-MK-PMK which is required for making pΔPHA-MK-PMK-HmgR. Below each plasmid are the primer sequences used. For plasmids with gene inserts, primers used for these portions are separated from the rest. If no primers are listed, plasmid construction is not dependent on primers. Next to each is also a description of the plasmids importance, and a plan for construction.

A.



Description: pΔPHB-PMD-FNI is the first of two ‘insert and delete’ plasmids which are designed to test the effects of both deleting the PHB pathways and insertion of the MVA pathway. The plasmid was constructed, in pieces via PCR. Primer pairs used for each fragment can be seen in the diagram above. In total, there are six fragments: PHB upstream, PMD-FNI insert, chloramphenicol resistance, T7 Terminator Region, PHB downstream, and the backbone region. The backbone region is prepared by digesting pIspS with BglII and NheI and collecting the fragment that is 6190 bp.

Primers Used

Primer 13 (Forward) - 5'-GTCCGGCGTAGAGGATCGAGATCTAACCCGGCGAGATGGGC-3'
Primer 14 (Reverse) - 5'-CATTGAGATCGGATCGCGTGGCAGACGG-3'

Primer 17 (Forward) - 5'-GGATCCAGGAGGCTTAAGATGGTATTTGAAAAAATTGATAAAA-3'
 Primer 18 (Reverse) - 5'-GGACCCCATATGTAACTATTATCAATTCCTGC-3'
 Primer 19 (Forward) - 5'-GATAAATAGTTAACATATGGGGTCCCCGTCTAC-3'
 Primer 20 (Reverse) - 5'-TGCCGGCGCTGCTGGACTGGGACCCGTTTCAGG-3'
 Primer 21 (Forward) - 5'-AACGGGTCCCAGTCCAGCAGCGCCGGC-3'
 Primer 23 (Reverse) - 5'-GATGATGGCTAGCAAGCCGCTGGGCGTTCG-3'

PMD-FNI Amplification

First Half Forward Primer (First half of PsbA)

5'-AAGACATGTTATACTGTTGAATAACAAGGAAAGGAGAAGAAATTCTAGAATGGACCGGAG-3'

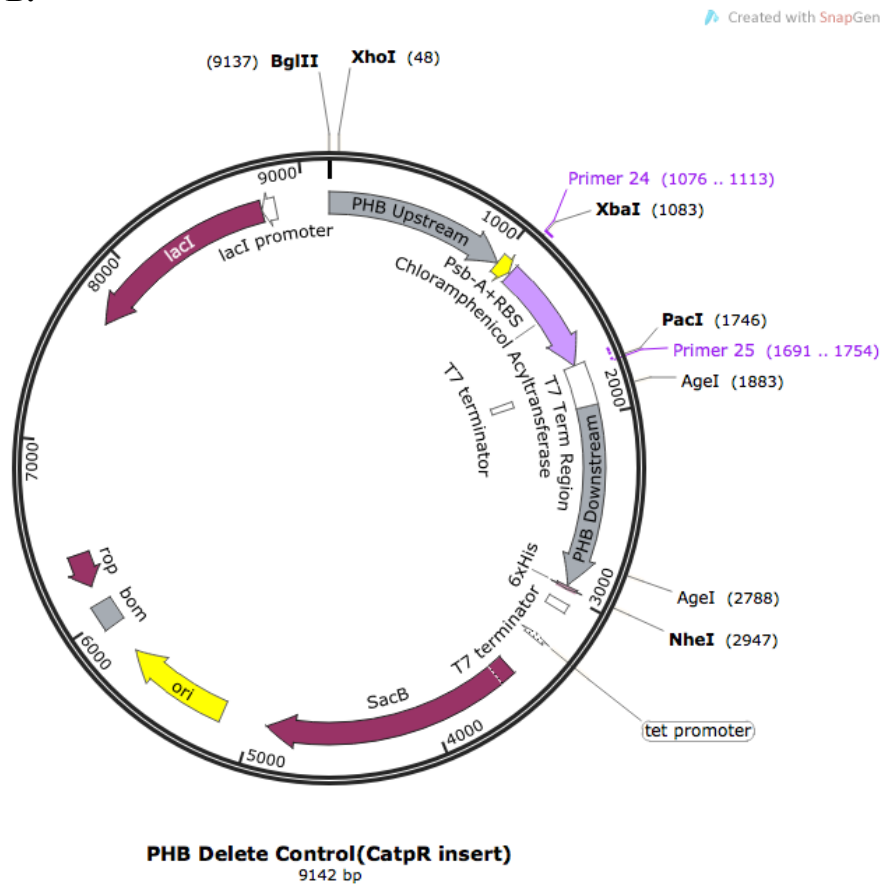
Second Half Forward Primer (Second half of PsbA)

5'-GATCCGATCTCAATGAATATTGGTTGACACGGGCGTATAAGACATGTTATACTGTTGA-3'

Reverse Primer (used with both First/Second Forward Primers)

5'-CTTAAGCCTCCTGGATCCTCAGGCCTTCTTCATC-3'

B.



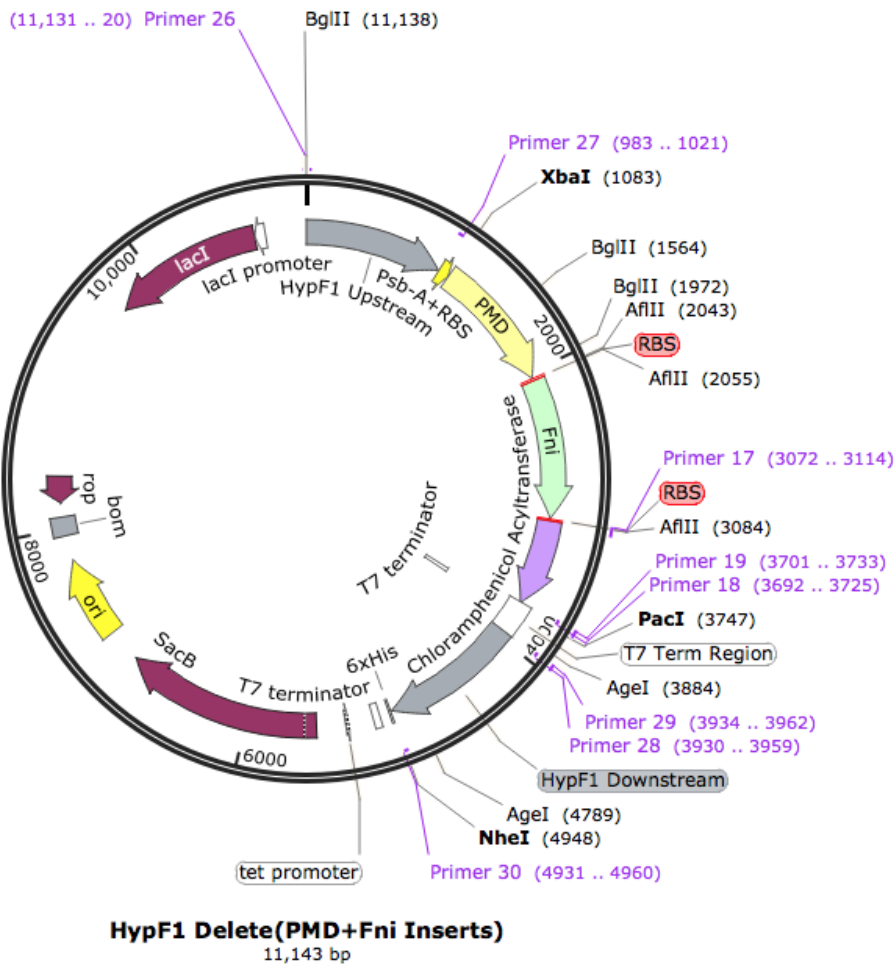
Description: p Δ PHB is a plasmid made to create a control mutant where only the PHB gene has been deleted. This control is needed to show if isoprene production is affected by simply deleting these enzymes, as opposed to inserting MVA pathway genes. Plasmid is constructed by first synthesizing a new Chloramphenicol resistance fragment. Next, p Δ PHB-PMD-FNI is digested with XbaI and PacI and the larger fragment, which includes the backbone and PHB regions is collected. The Chloramphenicol resistance fragment is also digested with XbaI and PacI. Next, a ligation reaction is performed to join these two pieces and create p Δ PHB.

Primers Used

Primer 24 (Forward) - 5'-AAGAAATCTAGAATGGTATTTGAAAAAATTGATAAAA-3'

Primer 25 (Reverse) - 5'-CTAGGTTAATTAATTAATACTATTTATCAATTCCTGC-3'

C.



Description: pΔHypF1-PMD-FNI is the last of the plasmids involved in testing the insertion of PMD and FNI. Here, the MVA pathway genes are inserted into a neutral site to test if increased isoprene production is coming from only the addition of MVA pathway genes, and not from deletion of PHB genes. This plasmid is constructed in the same way that pΔPHB-PMD-FNI was, except new Chloramphenicol resistance, T7 Terminator, and *hypF1* Upstream/Downstream regions must be synthesized using the primers listed.

Primers Used

Primer 26 (Forward) - 5'-AGGATCGAGATCTGCAAGACGACGCTGCTGTGC-3'
 Primer 27 (Reverse) - 5'-CATTGAGATCGGATCGTTCGGCCATTACCGCGGC-3'
 Primer 17 (Forward) - 5'-GGATCCAGGAGGCTTAAGATGGTATTGAAAAAATTGATAAAA-3'
 Primer 28 (Reverse) - 5'-CCGGCGCTGCTGGACTGGGACCCGTTTCAGG-3'
 Primer 29 (Forward) - 5'-AACGGGTCCCAGTCCAGCAGCGCCGGCAG-3'
 Primer 30 (Reverse) - 5'-GATGATGGCTAGCAAGCCGCTGGGCGTCGG-3'

primers below. pΔPHA-MK-PMK is digested by BamH1 and is subjected to a Gibson reaction with the *hmgR* fragment. This will yield pΔPHA-MK-PMK-HmgR.

Primers Used

Primer 1 (Forward) - 5'-TCCGGCGTAGAGGATCGAGATCTGCGGGCCTGTGCGAG-3'

Primer 2 (Reverse) - 5'-CATTGAGATCGGATCGCGTGCGCGGTTCAAG-3'

Primer 5 (Forward) - 5'-GGATCCAGGAGGCTTAAGATGAGTATTCAACATTTCCGTGT-3'

Primer 6 (Reverse) - 5'-GGACCCATATGTTACCAATGCTTAATCAGTGAGG-3'

Primer 7 (Forward) - 5'-TAAGCATTGGTAACATATGGGGTCCCCGTCTAC-3'

Primer 8 (Reverse) - 5'-AGCAGCGAACGGAGACTGGGACCCGTTCAAG-3'

Primer 9 (Forward) - 5'-AACGGGTCCCAGTCTCCGTTCCGCTGCTGAGC-3'

Primer 10 (Reverse) - 5'-GATGATGGCTAGCTCTTGGCGTTGATGTACTCGTCGG-3'

PMD-FNI Amplification

First Half Forward Primer (First half of *PsbA*)

5'-AAGACATGTTATACTGTTGAATAACAAGGAAAGGAGAAGAAATTCTAGAATGACCAAGAAG-3'

Second Half Forward Primer (Second half of *PsbA*)

5'-GATCCGATCTCAATGAATATTGGTTGACACGGGCGTATAAGACATGTTATACTGTTGA-3'

Reverse Primer (used with both First/Second Forward Primers)

5'-CTTAAGCCTCCTGGATCCTCAGCTCTTGTCTGTCGTGGC-3'

HmgR Insert Primers

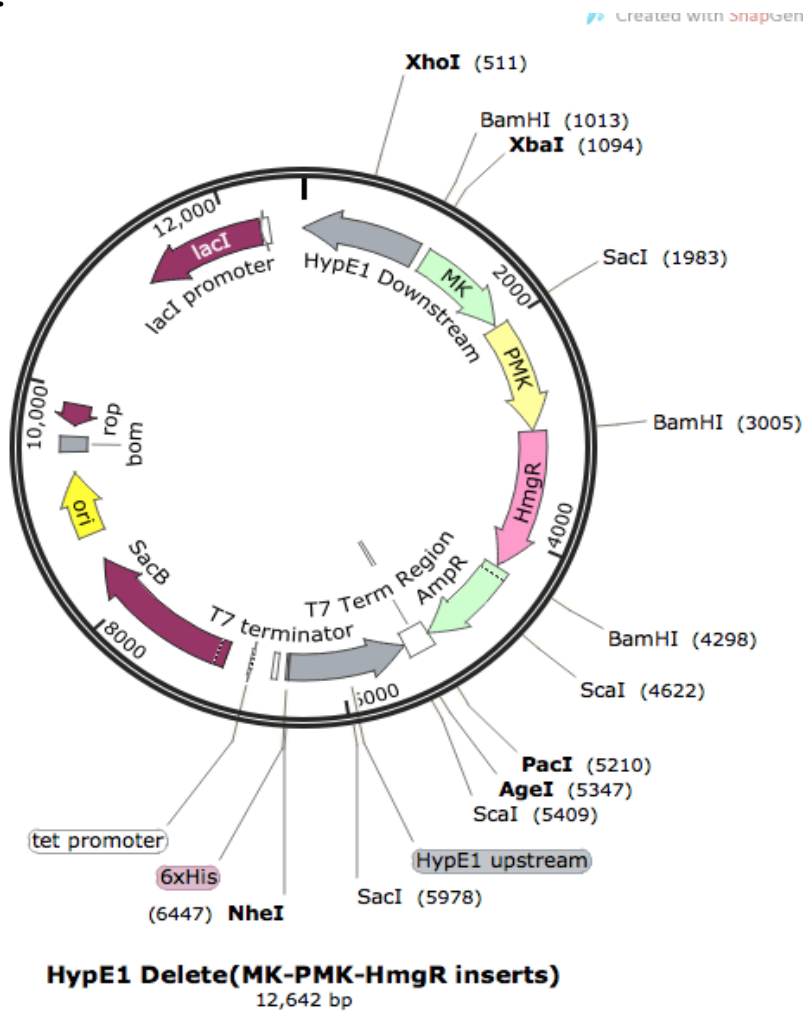
Forward Primer

5'-ACGACAAGAGCTGAGGATCCGGTACCAGGAGGCTTAAGATGAAGAT-3'

Reverse Primer

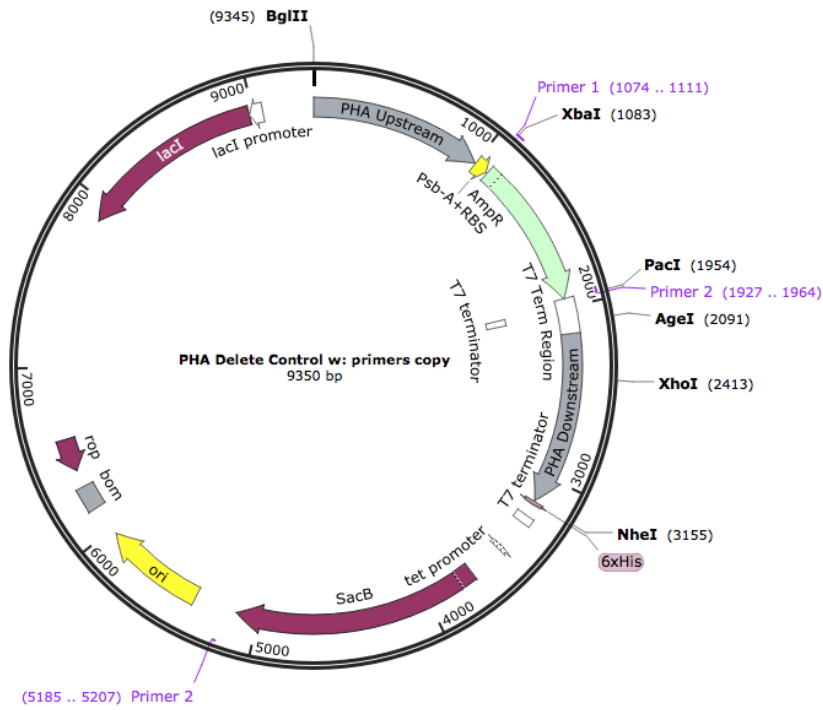
5'-ATCTTAAGCCTCCTGGATCCTCAGCTGCGCAGGTTCTCCA-3'

E.



Description: pΔHypE1-MK-PMK-HmgR is a control plasmid for testing the effects of only inserting the MVA pathway enzymes. It is constructed by digesting pIspS and pΔPHA-MK-PMK-HmgR with Xba1 and Pac1. Next, the backbone with HypE1 flanking regions (from pIspS) is ligated with the insert containing *MK*, *PMK*, *HmgR*, ampicillin resistance, and T7 Term (Terminator) Region (from pΔPHA-MK-PMK-HmgR).

F.



Description: pΔPHA is a control plasmid much like pΔPHB. It is constructed in the same manner as pΔPHB, except the primers below are used for the ampicillin resistance fragment.

Primers Used

Primer 1 (Forward) - 5'-AGAAGAAATTCTAGAATGAGTATTCAACATTCCGTGT-3'
 Primer 2 (Reverse) - 5'-GCCTAGGTTAATTAATTACCAATGCTTAATCAGTGAGG-3'

Figure 8: Primers Designed for Inserting *hmgR*. *hmgR* fragments that are created will have regions homologous to PMK and also *ampR*.

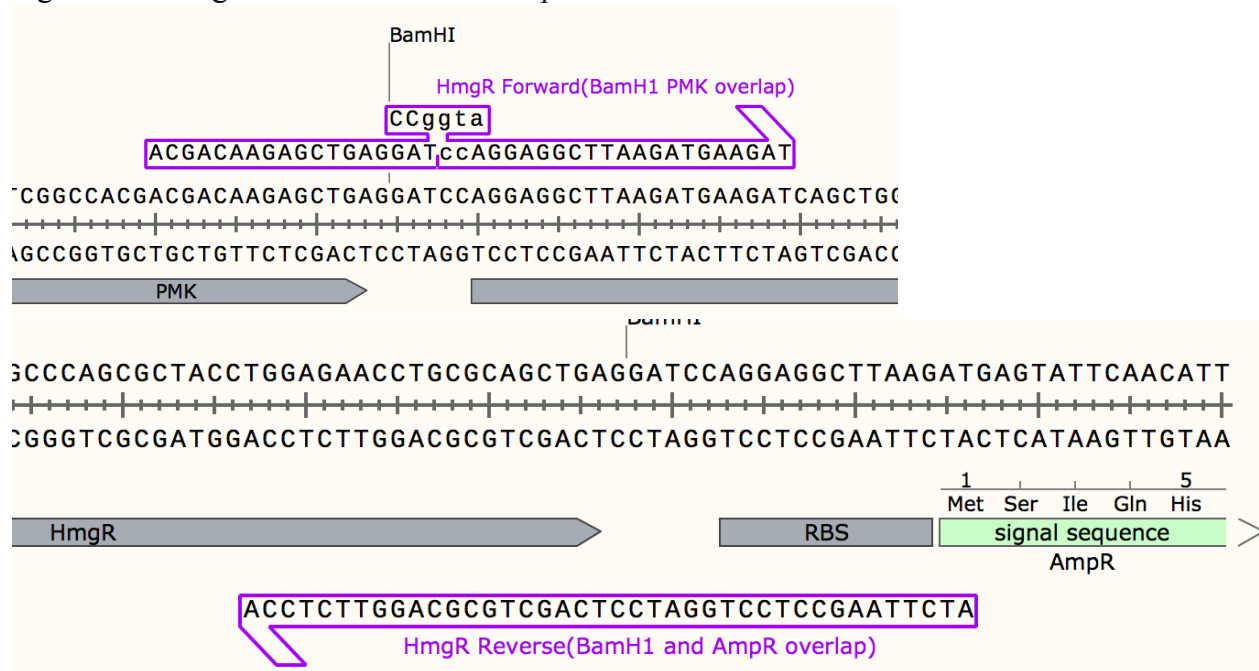


Figure 9: Primers Used for *psbA* Amplification. Below you can see the original “Old Forward Primer” that was very long and failed to produce fragments with PCR. New primers, splitting the *psbA* promoter in two, were designed.

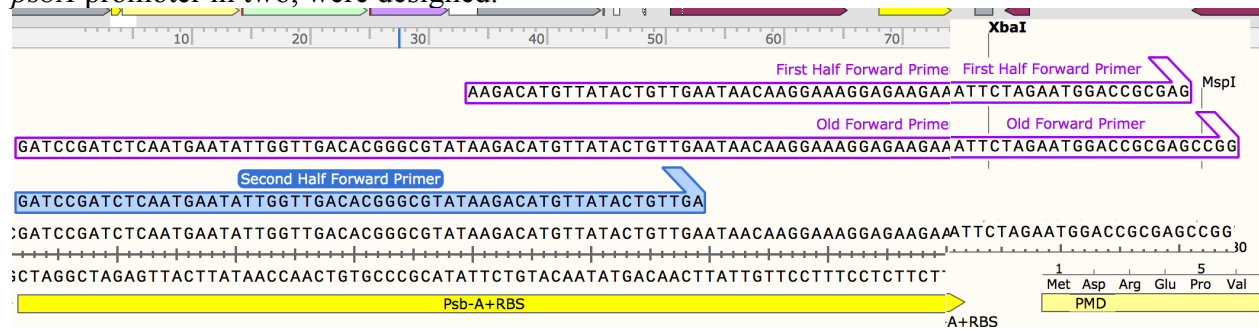
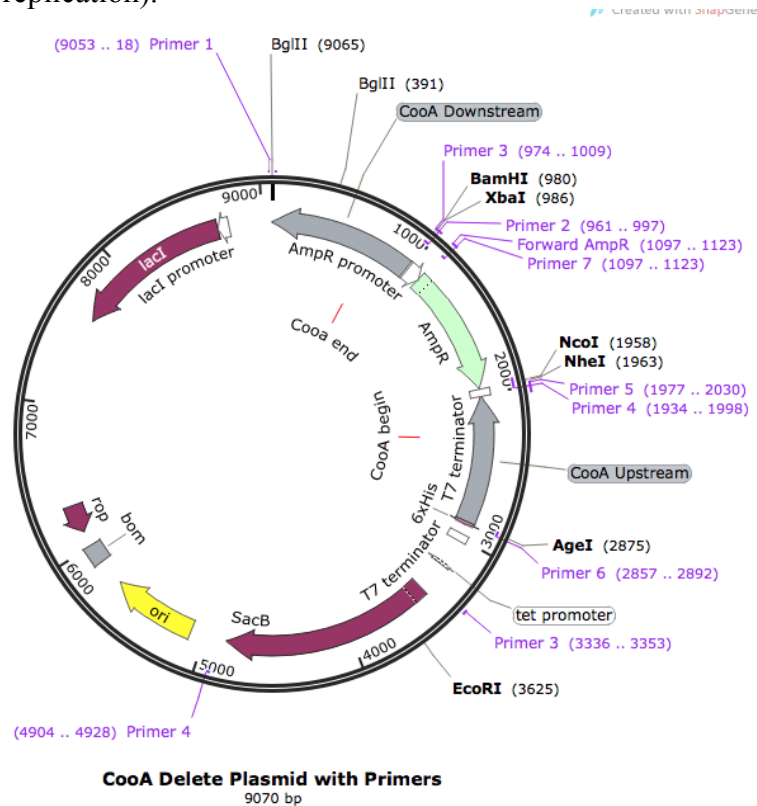


Figure 10: Constructed pΔCooA Plasmid. This plasmid was assembled from four fragments: *cooA* Downstream, *ampR*, *cooA* Upstream, and the backbone region (including an origin (ori) of replication).



Primers Used

- Primer 1 (Forward) - 5'- CGTAGAGGATCGAGATCTGGCATCGATCTTCTGGCG-3'
 Primer 2 (Reverse) - 5'- TCCGCGTCTAGAGGATCCCTGGACCTGAGCTTCGAGG-3'
 Primer 3 (Forward) - 5'- GTCCAGGGATCCTCTAGACGCGGAACCCCTATTTGT-3'
 Primer 4 (Reverse) - 5'- AAGACCCGTTTAGAGGCCCAAGGGGTTATGCTAGCCATGGTTACC
 AATGCTTAATCAGTGAGGC-3'
 Primer 5 (Forward) - 5'-TTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTGGGGTGTGCGATG
 GTCCAGAC-3'
 Primer 6 (Reverse) - 5'-ATGGTGATGATGACCGGTCGAGAAGATCGCCGAGA-3'

Figure 11: *rcoM* Overexpression Growth Test on CO. Below are results from growth tests on CO comparing growth rates of the WT, *rcoM* overexpression strain, and the $\Delta rcoM$ strain. Results show the *rcoM* overexpression strain consistently outperforms WT by both starting growth sooner and also by having a steeper growth rate. The $\Delta rcoM$ strain grew the slowest, as expected.

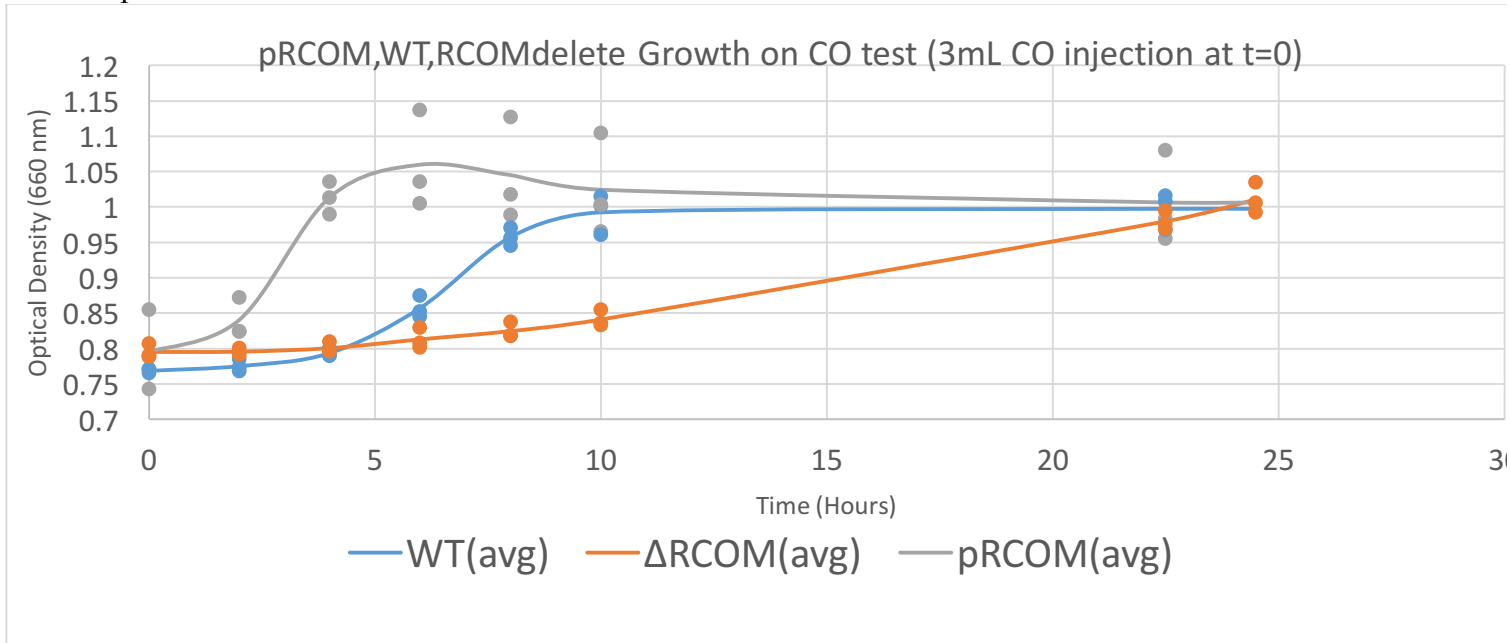
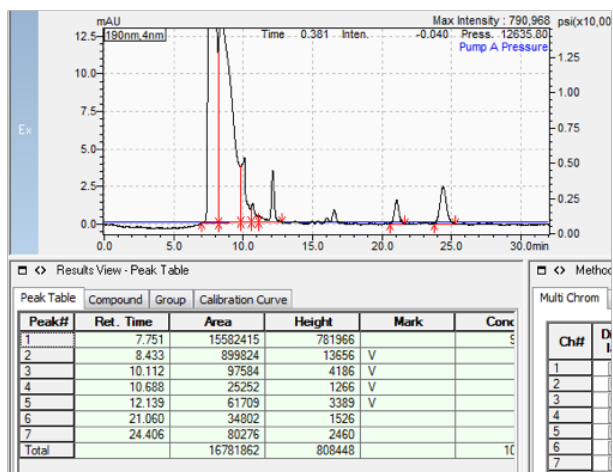
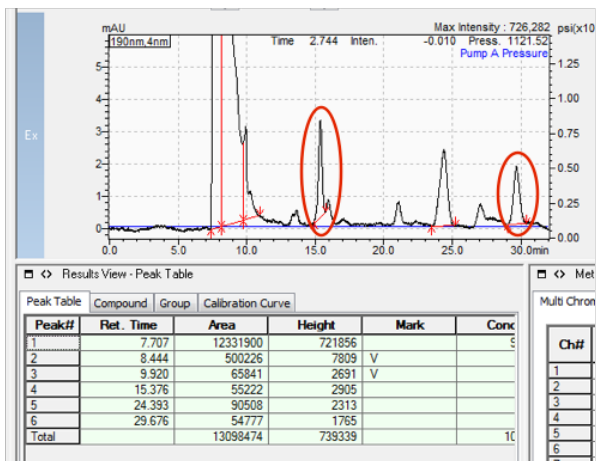


Figure 12: HPLC Results of CBS Growth on CO. This test was performed to analyze metabolites present in the media after growth on CO. Metabolites that have not been consumed are fermentation end product. There are clearly peaks present in the CO sample which were not present in the control, indicating that these are byproducts of growth on CO. Specifically, peaks near 15.4 and 29.7 minutes emerge. The identities of these peaks were not confirmed due to time constraints and issues with an LC-MS in another lab. Standards of various organic acids were eluted at the following times (minutes): pyruvic acid (10.8), malic acid (12.6), lactic acid (16.1), formic acid (12.2), acetic acid (18.9), propionic acid (22.2), butyric acid (26.9), and benzoic acid (87.0).

Control



Growth on CO



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