JBEI Performance Metric for FY16: Develop an improved metabolic engineering method for modifying microorganisms for biofuel production from cellulosic sugars

Q4 Metric: Methods to improve yields of biofuels from engineered microorganisms.

1. BACKGROUND

JBEI has leveraged our expertise to greatly expand the types of biofuels that microorganisms can produce from sugars derived from lignocellulosic biomass. We have synthesized and engineered many metabolic pathways in microbial hosts to produce useful biofuel molecules that could serve as replacements or blending agents for gasoline, diesel fuel, or jet fuel. Target molecules have included fatty acid-derived compounds (such as fatty acid ethyl esters and diesel-range methyl ketones) and isoprenoids (such as bisabolene, limonene, pinene, and isopentenol/isopentanol). Increasing yields to economically viable levels is an indispensable requirement for large-scale deployment of cellulosic biofuels (Van Dien, 2013). We have used a range of approaches to improve biofuel production yields, which are summarized in this report. Here, we discuss approaches for improving biofuel yield including a dynamic sensor-regulator system, 2-scale ^{13}C-metabolic flux analysis, rational metabolic engineering, principal component analysis of proteomic data, and other omics-informed methods.

2. IMPROVING YIELD IN FATTY ACID-DERIVED BIOFUEL PATHWAYS

Fatty acid biosynthesis is a widely distributed and essential metabolic capability found throughout the Bacterial and Eukarya domains of life. As such, fatty acids are readily available, potentially renewable compounds that have been targeted by the biofuel industry because they include highly reduced, aliphatic moieties that mimic the high-energy-density properties that characterize hydrocarbon components of petroleum-derived fuels. However, the carboxylic acid moiety has characteristics that are not compatible with model fuels (such as polarity, reactivity, and oxygen content) and needs to be modified for use in biofuels, for example by esterification, reduction, or decarboxylation. Accelerated research-and-development activity in biofuels over the past decade has facilitated the discovery of a variety of enzymes and metabolic pathways that enable biochemical conversion of fatty acids (and intermediates of fatty acid biosynthesis) to a range of industrially relevant compound classes, such as fatty acid alkyl esters, fatty alcohols, aliphatic methyl ketones, alkanes, and alkenes (α-olefins and long-chain internal alkenes), as has been described in recent reviews (e.g., (Beller et al., 2015)). Here, we present examples of several successful approaches for improving production of free fatty acids and fatty acid-derived fuels, namely diesel-range methyl ketones and fatty acid ethyl esters, in *E. coli* and *S. cerevisiae*.

2.1. Using rational metabolic engineering to improve methyl ketone production

We have engineered a novel metabolic pathway in *Escherichia coli* to overproduce saturated and monounsaturated aliphatic methyl ketones in the C_{11} to C_{17} (diesel) range. The host was a fatty acid-overproducing *E. coli* strain that overexpressed a truncated, cytoplasmically directed version of a native thioesterase (‘*tesA*) and had a chromosomal deletion of the acyl-CoA dehydrogenase *fadE*. Features of the first generation of methyl ketone-overproducing *E. coli* (Goh et al., 2012) included the following: (a) overproduction of β-ketoacyl-coenzyme A (CoA) thioesters achieved by modification of the β-oxidation pathway (specifically, overexpression of a heterologous acyl-CoA oxidase from *Micrococcus luteus* and native FadB, and chromosomal deletion of *fadA*) and (b) overexpression of a native thioesterase (FadM). FadM was previously associated with oleic acid degradation, not methyl ketone synthesis, but overexpression of this thioesterase enhanced methyl ketone titers ca. 10-fold. Whole-genome transcriptional studies of modified fatty acid-overproducing strains led to the discovery that FadM is a valuable catalyst for enhancing methyl ketone production (Goh et al., 2012).

Recently, we have made a series of pathway and host genetic modifications to the first-generation *E. coli* strain that improved methyl ketone titer and yield 160-fold, as shown in Figure 1 (Goh et al., 2014). Pathway modifications made to increase fatty acid flux into the pathway included overexpression of *fadR*, a fatty acid-responsive transcription factor that has been used to enhance fatty acid production,(Zhang et

al., 2012b) and *fadD*, a fatty acyl-CoA synthetase that is the first committed step of β-oxidation and the gateway for entry of fatty acids into our methyl ketone pathway. Successful enhancement of methyl ketone production by overexpression of *fadR* and *fadD* required screening of multiple combinations of promoters and inducer concentrations, and overexpression of just one of these genes alone did not improve performance (Goh et al., 2014). Another pathway modification that resulted in increases in methyl ketone production was consolidation of the pathway from two plasmids into one; unexpectedly, this modification was only successful when the *M. luteus* acyl-CoA oxidase was codon optimized, even though codon optimization was not beneficial in the two-plasmid system (Goh et al., 2014). Host engineering modifications included chromosomal deletions to remove key acetate production pathways (*poxB*, *ackA*, and *pta*), as acetate release was observed in earlier methyl ketone-producing strains and represented an obvious metabolic inefficiency (Figure 1).

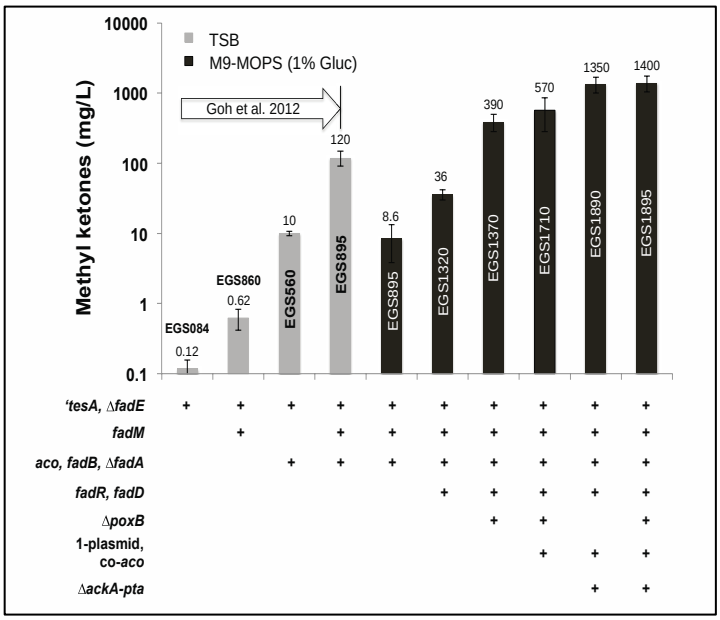

Figure 1. Summary of improvements in performance resulting from engineering modifications to E. coli *strains bearing the methyl ketone biosynthesis pathway. More details are given* by *(Goh et al., 2014)*.

In our best strain to date, a titer of 1.4 g/L of C_{11} to C_{17} methyl ketones was produced in minimal growth medium with 1% glucose, representing 40% of maximum theoretical yield (Goh et al., 2014). Fed-batch incubations with glucose resulted in ~4.8 g/L methyl ketones (Goh, Beller, *et al.*, unpublished data). These titer and yield values are among the highest reported for any fatty acid-derived fuels or bioproducts.

2.2. Improving fatty acid ethyl ester (FAEE) production in *E. coli* with a Dynamic Sensor-Regulator System (DSRS)

A valuable synthetic biology tool that has been used at JBEI to improve production is the Dynamic Sensor-Regulator System (DSRS), whereby a metabolite-responsive transcription factor is used to regulate selected pathway genes based on concentrations of the cognate metabolite. This can be a more efficient means of regulation than, for example, use of inducers like IPTG (isopropyl β-D-1-thiogalactopyranoside), because the DSRS will upregulate pathway genes *only* when important metabolites are being produced, as opposed to a more non-specific regulation scheme that does not account for optimal timing of pathway gene expression. Here, we present an example of using DSRS in the context of FAEE, a renewable diesel substitute.

2.2.1 FadR as a regulator of fatty acid biosynthesis and metabolism

Regulation of fatty acid biosynthesis and β-oxidation is complex, as described elsewhere (Handke et al., 2011; Janssen and Steinbuchel, 2014; Magnuson et al., 1993). One important regulatory element in *E. coli* is the transcription factor, FadR. FadR is a negative transcriptional regulator of the *fad* (fatty acid degradation, or β-oxidation) operon. When fatty acyl-CoAs are at very low intracellular concentration, FadR binds to promoters of all β-oxidation genes and blocks access for RNA polymerase, thus preventing transcription. Conversely, when acyl-CoAs are present, they bind with FadR and it is released from its binding site on the promoter, thus allowing (de-repressing) transcription. FadR can also act as a positive regulator (transcriptional activator) of certain fatty acid biosynthesis genes, such as *fabA* and *fabB*.

2.2.2 Using synthetic FadR promoters and DSRS to efficiently regulate and improve FAEE biosynthesis

JBEI researchers have developed and used the DSRS approach to successfully increase production of the fatty acid-derived FAEEs (Zhang et al., 2012a). In this study, certain modules of the FAEE biosynthetic pathway were placed under the regulatory control of synthetic hybrid promoters that included FadR-binding sites as well as LacI-binding sites. The concept behind the hybrid promoters was to make them responsive to fatty acyl-CoAs (which are key metabolites in the FAEE pathway) as well as to IPTG, which introduced additional stringency, as endogenous fatty acids could cause leaky expression if only FadR-binding sites were present. A schematic of the approach is shown in Figure 2a. As shown, synthetic hybrid promoters containing FadR-binding sites were used to control expression of genes in Module B (*pdc* and *adhB*, catalyzing ethanol production) and Module C (*fadD*, an acetyl-CoA synthetase, and *atfA*, a wax-ester synthase that produces FAEEs from acyl-CoAs and ethanol). As shown in Figure 2b, the approach was highly successful, resulting in a 3- to 4-fold increase in FAEE titers to attain a best titer of 1.5 g/L (Zhang et al., 2012a). Not only did use of the synthetic promoters increase titers, but plasmid stability in this 3-plasmid system also increased dramatically.

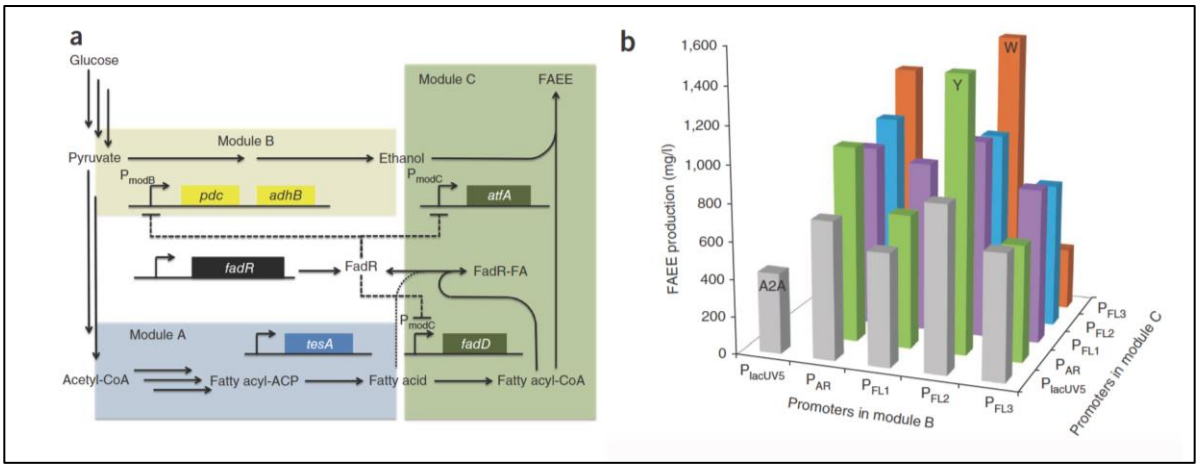

Figure 2. Use of a Dynamic Sensor-Regulator System (DSRS) to improve FAEE titers. (a) Three modules for dynamic FAEE production (b) FAEE production yield from different strains. From (Zhang et al., 2012a).

2.3. Improving fatty acid production in *S. cerevisiae* guided by combining ^{13}C labeling experiments with genome-scale models.

It is imperative to develop systematic methods (i.e., generalizable to more than one pathway or host) for yield improvement, given that obtaining commercially viable yields is a commonly unmet need for biofuels (Van Dien, 2013). Flux-based metabolic modeling approaches are particularly well-suited for this endeavor since metabolic fluxes describe how carbon flows from feed to final product. Indeed, Flux Balance Analysis (FBA) has previously successfully leveraged comprehensive genome-scale models for this purpose (Asadollahi et al., 2009; Park et al., 2009). Two-scale ^{13}C Metabolic Flux Analysis (2S-^{13}C MFA) improves on FBA by eliminating evolutionary assumptions and determining fluxes for genome-scale models using the informative constraints obtained from ^{13}C labeling experiments (Garcia Martin et al., 2015).

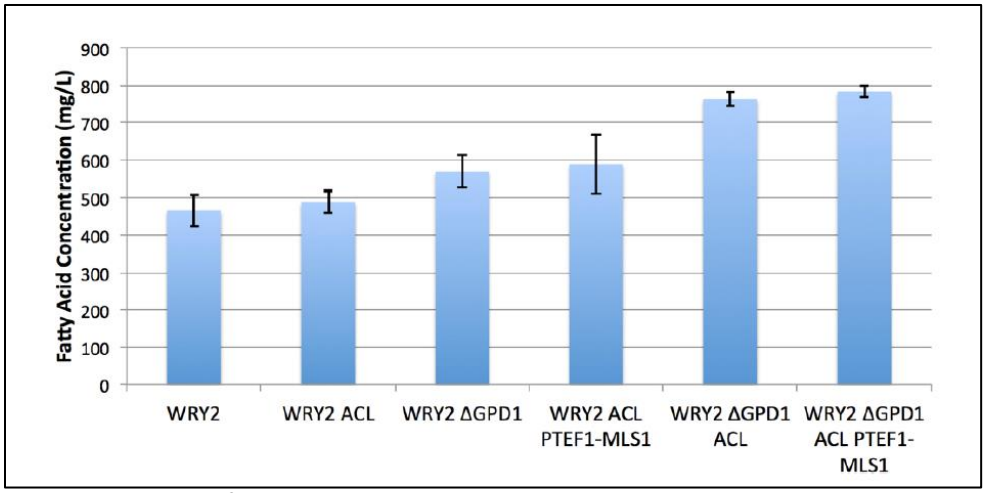

Figure 3: Use of 2S-^{13}C MFA showed why the expected increase in acetyl-CoA originated by ACL did not result in increasing fatty acid production unless MLS1 was downregulated (Figure 4). Knocking out GPD1 and downregulating MLS1 resulted in a 70% increase in production, starting with a strain displaying a significant production.

JBEI researchers used 2S-^{13}C MFA to guide a systematic improvement of fatty acid yields from an initial *S. cerevisiae* strain producing a titer of 460 mg/L (Ghosh *et al*, in review). In this strain, the inclusion of an ATP citrate lyase (ACL) was expected to increase acetyl-CoA supply and overall fatty acid production. However, 2S-^{13}C MFA showed that while acetyl-CoA supply seemed to increase, this did not result in fatty acid production increase (Figure 3): any extra acetyl-CoA gained was diverted into the malate synthase (MALS) reaction (Figure 4). This analysis suggested a downregulation of MALS in order to increase production, which ultimately resulted in an increase in production of 21% (*MALS* knockout created a very slow growing strain). Furthermore, 2S-^{13}C MFA showed that the glycerol-3-phosphate dehydrogenase (*GPD1*), which competes for carbon with the acetyl-CoA production pathway, was acting as a large carbon sink. Knocking out *GPD1* resulted in an extra increase in production of 33%. The final strain produced 780 mg/L of fatty acid, representing an increase of 70% over the initial titer (Figure 3).

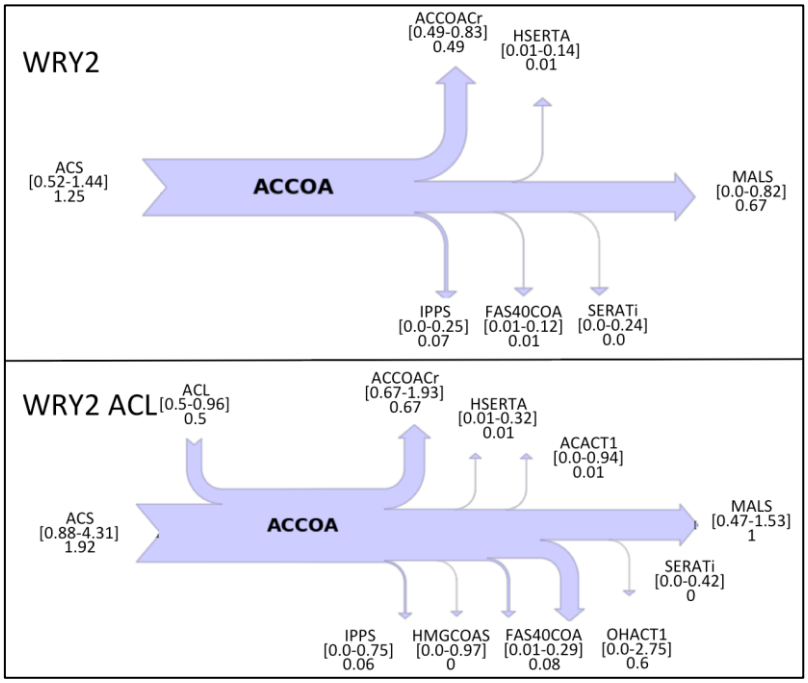

Figure 4: Sankey diagrams obtained from 2S-^{13}C MFA showing all the reactions producing and consuming acetyl-CoA, along with their fluxes. The addition of ACL increases the amount of acetyl-CoA being produced but this extra flux, instead of significantly increasing fatty acid production (ACCOACr), is diverted into the malate synthesis (MALS).

3. IMPROVING YIELD IN ISOPRENOID-DERIVED BIOFUEL PATHWAYS

Isoprenoids have a number of favorable fuel properties, and recently we have developed several isoprenoids as potential gasoline, diesel, and jet fuels. In past years, we have primarily engineered the heterologous MVA pathway in *E. coli* for isoprenoid biofuel production. The functional expression of the MVA pathway enzymes and terpene synthases in a heterologous host has been achieved for the production of various terpene targets at much higher yields than those achieved using native MEP pathway (Martin et al., 2003). Recently, we have exploited synthetic biology to allow this heterologous pathway to work more efficiently in new hosts, and also employed multiple omics tools to monitor and optimize the pathway enzymes and metabolites to improve isoprenoid yields and titers. In this section, we review our recent efforts to improve the production yields of a few selected isoprenoid biofuels in the past few years.

3.1. Improving bisabolene production in *E. coli* with proteomics-aided pathway engineering

Bisabolene is a precursor of bisabolane, the hydrogenated C_{15} isoprenoid that has favorable cetane numbers for diesel fuels and displays excellent cold-temperature properties (Peralta-Yahya et al., 2011). Plants are the natural source of bisabolene, but we have engineered microbial platforms for bisabolene to achieve convenient and cost-effective means to produce bisabolene (Peralta-Yahya et al., 2011).

By employing the MVA pathway and the terpene synthase from a plant, we achieved high-titer production of bisabolene in *E. coli*. First, we bioprospected various bisabolene synthases from plants and discovered that the synthase from *Abies grandis* has good and specific activity for bisabolene production. With the highest bisabolene-producing gene in hand (AgBIS), we set out to improve the *E. coli* production of FPP *via* metabolic engineering of the mevalonate pathway. From targeted proteomics data of the pathway protein levels, a few pathway enzymes (tHMGR, HMGS, MK, and PMK) showed quite low level of protein expression from the initial strain. We codon-optimized four of the five *S. cerevisiae* genes that showed low protein expression to match the *E. coli* codon usage, and in addition, we placed bottom portion genes (*MK*, *PMK*, *PMD*, *idi*, and *ispA*) under control of a second promoter (P_{trc}). This engineering improved the yield 2.5-fold higher from the initial strain with the terpene synthase, and reached a titer of ~900 mg/L of bisabolene (Peralta-Yahya et al., 2011). We further engineered the pathway to improve the bisabolene yield in both *E. coli* and yeast.

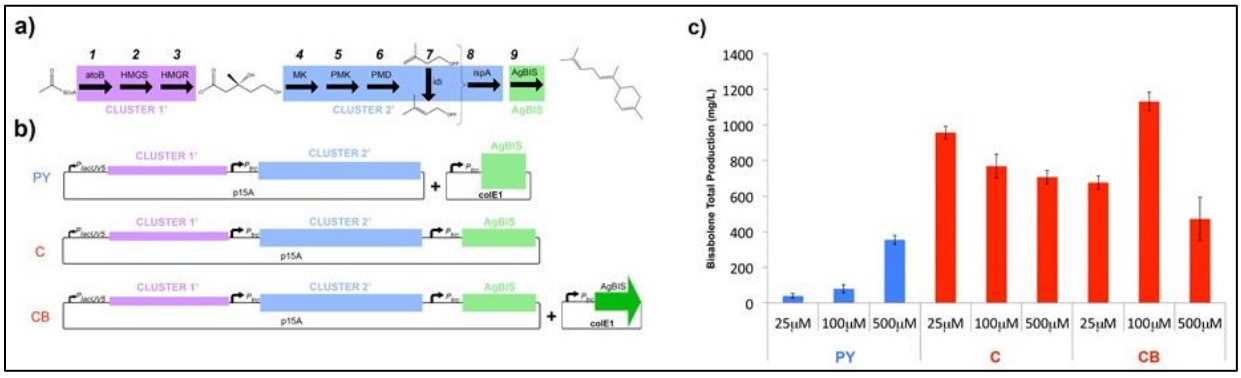

Figure 5. Bisabolene yield improvement (a) pathway description (b) PY, C, CB strains plasmid schematics (c) bisabolene yield from three strains at various IPTG concentrations. From (Alonso-Gutierrez et al., 2015)

A proteomics data-mining approach was used to further increase production for this strain. We generated a set of strains with different promoter strengths and induced them at different times and with

different levels of inducer. For each of these scenarios involving a different strain and induction strategy, we collected limonene production and targeted proteomics data, and applied Principal Component Analysis (PCA) to these data sets (Figure 5). This PCA of proteomics data (PCAP) resulted in metabolic engineering suggestions that resulted in more than a 200% increase in bisabolene production, for a total of 1.2 g/L of bisabolene in batch culture, which represents 35% of the maximum theoretical yield (Alonso-Gutierrez et al., 2015) (Figure 5).

3.2. Improving monoterpene production in *E. coli* with proteomics-aided pathway engineering and protein engineering

Monoterpenes are produced from geranyl diphosphate (GPP, C_{10}) by various monoterpene synthases, and the hydrogenated products of mycene and ocimene (2,6-dimethyloctane) and the hydroxylated compounds such as linalool and geraniol have been suggested as potential biofuel precursors. Recently, hydrogenated dimers of α- and β-pinene have been synthesized chemically and showed a high energy density similar to that of JP-10 (Harvey et al., 2010).

By employing the MVA pathway, we achieved high titer production of the monoterpene limonene in *E. coli* (Alonso-Gutierrez et al., 2013). A step-by-step optimization of limonene production was performed in *E. coli* strains with the heterologous MVA pathway, GPP synthase (GPPS) from *Arabidopsis thaliana*, and limonene synthase from *Mentha spicata* (Alonso-Gutierrez et al., 2013). A series of gene modifications to improve the enzyme availability and activity lead to increases in limonene titers to 430 mg/L of L-limonene (72 hours, 1% glucose), nearly 90-fold higher than previous reports (Carter et al., 2003; Misawa, 2011) (Figure 6).

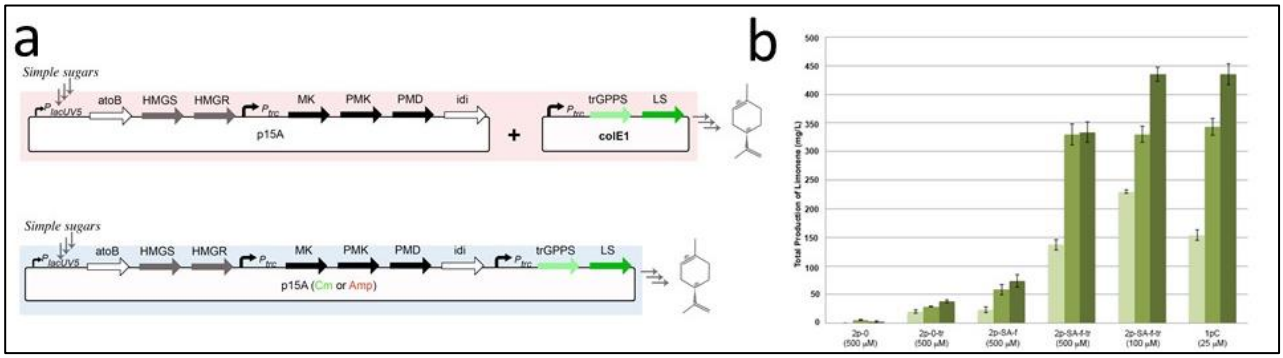

Figure 6. Strain engineering for limonene yield improvement (a) 2-plasmid and 1-plasmid systems (b) limonene yield improvement by strain engineering. From (Alonso-Gutierrez et al., 2013)

A targeted proteomics approach was used to suggest metabolic engineering strategies to increase limonene production (PCAP, as described above). This method provided suggestions for the metabolic pathway engineering which resulted in more than a 30% titer improvement to 650 mg/L limonene in batch culture (Alonso-Gutierrez et al., 2015) (Figure 7).

Microbial production of pinene was also demonstrated in *E. coli* with relatively low yield (Sarria et al., 2014). We combinatorially screened various pinene synthases (PSs) and GPPS enzymes to improve flux through the last two steps of the pathway. By combining expression of three PS and three GPPS from conifers, we achieved ~28 mg/L of pinene using GPPS and PS from *Abies grandis*, and we designed GPPS-PS protein fusions to reduce GPP product inhibition and toxicity by substrate channeling, producing 32.4 mg/L of pinene.

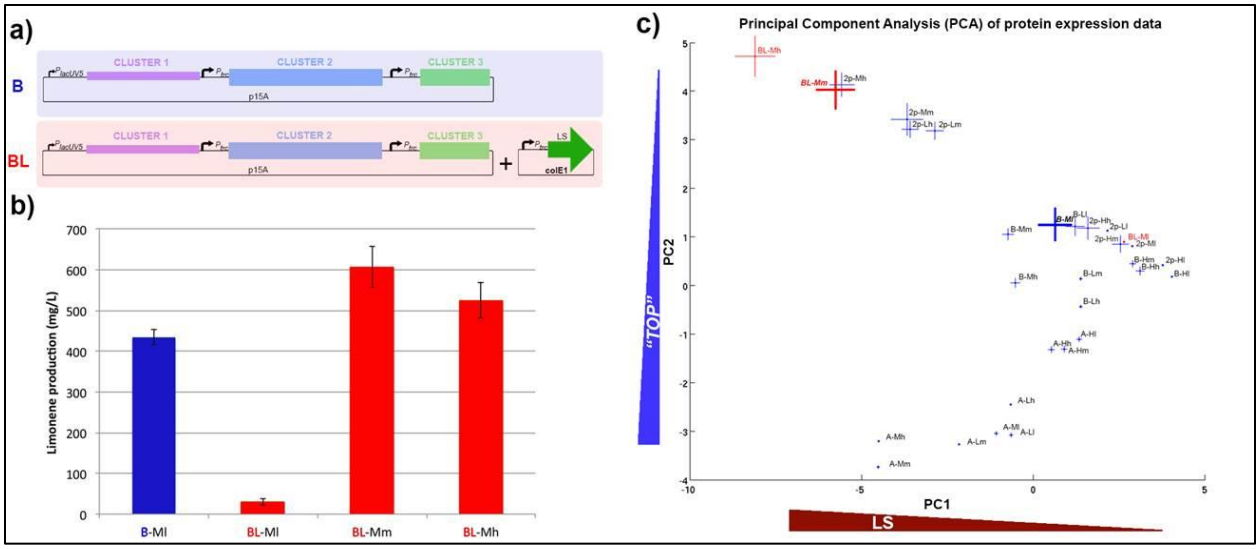

Figure 7. PCAP for limonene yield improvement (a) B and BL strain plasmid schematics (b) limonene yields of B (blue) and BL (red) strains (c) PCA plot with proteomic data and production titer (large cross means high production). B (blue) strain was engineered to improve yield toward BL (red) strain. From (Alonso-Gutierrez et al., 2015)

3.3. Improving isopentenol production in *E. coli* with multiple omics-aided pathway engineering and RBS engineering

Branched C_5 alcohols [isopentanol (3-methyl-1-butanol) and isopentenols (3-methyl-3-butenol and 3-methyl-2-butenol)] have higher energy contents than ethanol and high octane numbers (RON, or research octane number, of 92-102), which supports their use as gasoline replacements and as anti-knock additives (Mack et al., 2014). Among these C_5 alcohols, isopentenols were produced in engineered *E. coli* using a heterologous MVA pathway (Chou and Keasling, 2012; Withers et al., 2007).

A systematic approach using targeted proteomics and metabolomics led to higher isopentenol yield and titer (Figure 8). We generated pathway variants that expressed different amounts of mevalonate pathway proteins through changes in promoter identity, operon organization, and codon-usage. Then we analyzed proteomics data together with growth rates, titers, and key metabolite levels from microbial growth experiments. The omics data and the correlation analysis improved understanding of pathway dynamics and facilitated high-titer isopentenol production, and provided guidance on metabolic engineering approaches to improve biofuel production (George et al., 2014). This study also implicated prenyl diphosphate (e.g., isopentenyl diphosphate, or IPP) accumulation as a cause of reduced growth and decreased glucose uptake. The strong correlation between glucose consumption and IPP accumulation suggests that glucose uptake may be inhibited, though this may be a nonspecific consequence of reduced membrane integrity or inhibited cell metabolism (George et al., 2014). Further investigation to interrogate membrane integrity is an important first step towards addressing this uncertainty. Increasing the expression of the phosphatase NudB using a strong RBS whose strength was calculated using an RBS calculator facilitated an improvement in isopentenol titer and partially relieved IPP toxicity. Using this approach, a titer of 2.2 g isopentenol /L (up to 70% of apparent theoretical yield) was achieved in *E. coli* (George et al., 2015).

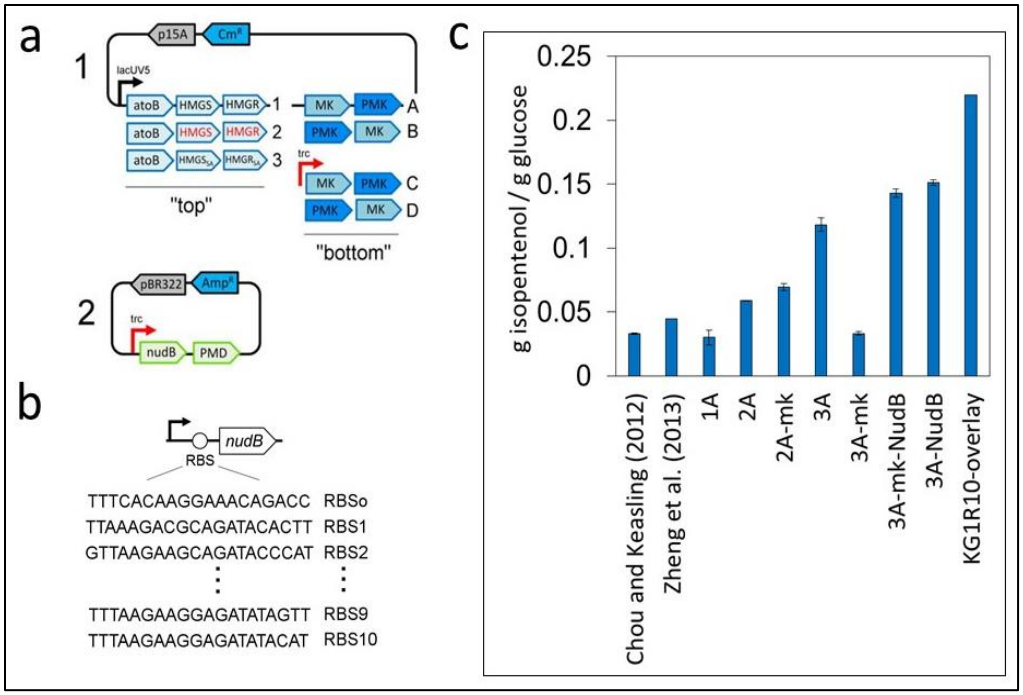

Figure 8. Isopentenol yield improvement (a) plasmid schematics (b) RBS variants for nudB (c) yield improvement.

Solvent-like products and inhibitory pathway intermediates remain bottlenecks in maximizing the efficiency of isopentenol production. Using array-based transcriptomic methods, we identified several candidate genes that were involved in isopentenol tolerance. An evaluation of several candidate genes, spanning multiple functional categories, led to the discovery of regulators, chaperones, and transporters that not only improve tolerance to exogenously added isopentenol but also improve production when co-expressed with production pathways (Foo et al., 2014). We co-expressed the tolerance-enhancing genes individually with an isopentenol production pathway, and expression of 6 of the 8 candidates improved the production of isopentenol in *E. coli*, with the methionine biosynthesis regulator MetR improving titer by 55%. Additionally, expression of MdlB, an ABC transporter, facilitated a 12% improvement in isopentenol production.

Even though the mechanism of IPP-related toxicity is currently unknown, we envision that sensing intracellular IPP level would be a good lever to dynamically regulate the IPP toxicity of the production strain and improve isoprenoid yield.

4. CONCLUDING REMARKS

As summarized above, we have used a wide variety of approaches for enhancing yields, including dynamic sensor-regulator systems that leverage metabolite-responsive transcription factors, 2-scale ^{13}C-metabolic flux analysis, rational metabolic engineering, principal component analysis of proteomic data, and other omics-informed methods. These approaches have led to yields that are among the highest reported in the literature for fatty acid-derived and isoprenoid fuels, for example, 40% of maximum theoretical yield for diesel-range methyl ketones, 55% for isopentenol, and 35% for bisabolene (Alonso-Gutierrez et al., 2015; Beller et al., 2015; George et al., 2015; Goh et al., 2014).

Although we have made considerable progress in improving biofuel yields for fatty acid- and isoprenoid-derived compounds, further developments will be needed for commercially viable, large-scale deployment of biofuel technologies. For example, successful yield improvement efforts are often host-

and pathway-specific, such that knowledge gained from one optimization effort is not necessarily applicable to new hosts or pathways. Hence, it is desirable to further develop systematic methods to optimize engineered microbial production; the 2-scale ^{13}C-metabolic flux analysis approach and omics-enabled approaches represent movement in this direction. Other JBEI efforts that will contribute to enhanced yields are bioengineering tools that will facilitate more efficient and rapid strain development cycles. Some of these tools were described in the previous (Q3) metric report, and include (1) ICE (Inventory of Composable Elements), a digital and physical repository of microorganisms, sequences, and parts, (2) DeviceEditor, j5, and DIVA – a powerful tool set for visually designing DNA constructs and automatically generating optimized protocols to build them, (3) EDD (Experiment Data Depot) – a repository of standardized experimental data, of use to improve predictability and repeatability, (4) jQMM (JBEI Quantitative Metabolic Modeling) library, which enables prediction of biological behavior and produces actionable items for yield increase, and (5) Arrowland, which can be used to provide an interactive, multi-scale visualization of omics data. JBEI will continue to develop and use these tools and systematic approaches to yield improvement to bring biofuels closer to commercialization.

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