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

This Summary Report integrates the four FY16 quarterly reports:

- **Q1**: Metabolic pathways for converting cellulosic sugars to biofuels
- **Q2**: Modification and control of microbial metabolic biofuel pathways
- **Q3**: Tools for designing microbial metabolic biofuel pathways
- **Q4**: Improving yields of microbial metabolic biofuel pathways

and presents a future outlook for the further development of improved metabolic engineering methods for modifying microorganisms for biofuel production from cellulosic sugars.

1. BACKGROUND

JBEI's Fuel Synthesis Division (in collaboration with the Combustion Research Facility at Sandia National Lab) has identified high-performing advanced biofuels compatible with existing engines and fuel-distribution infrastructure. JBEI has developed metabolic routes to these advanced biofuel targets by leveraging well known (isoprenoid, fatty acid, polyketide) biosynthetic pathways and supplementing them with additional, sometimes novel, enzymatic activities. Combinatorial libraries of promoters, ribosomal binding sites, and intergenic regions have been used to optimize metabolic pathways for the efficient conversion of sugars to fuel products. In parallel, complementary approaches such as the elimination of metabolic flux-diverting side reactions, and biosensors for dynamic pathway control, have also been pursued.

While indispensable for the large-scale deployment of cellulosic biofuels[7], engineering microbial strains to achieve economically viable yields has remained a long, labor-intensive, and host/pathway-specific process. JBEI has developed tools to facilitate and improve the design of engineered pathways and microorganisms (as well as plants) for the production of biofuels from cellulosic sugars. Representative tools include a (digital as well as physical) repository of microorganisms, sequences, parts, and seeds; a visual computer-aided design canvas for DNA constructs along with the automatic generation of optimized protocols to build them; a repository for the systematic storage and visualization of complex functional-genomics data; and models that predict the phenotypic outcomes that would result from genetic modifications. Taken together, these approaches and tools have contributed to the development of an improved metabolic engineering method for modifying microorganisms for biofuel production from cellulosic sugars.

2. METABOLIC PATHWAYS FOR CONVERTING CELLULOSIC SUGARS TO BIOFUELS

2.1 Fatty acid-derived biofuels

Fatty acids include highly reduced, aliphatic moieties that mimic the high-energy-density properties that characterize hydrocarbon components of petroleum-derived fuels. 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 (alpha-olefins and long-chain internal alkenes)[8].

2.1.1 Fatty acid ethyl esters (FAEE) and long-chain alkenes

FAEE have similar fuel properties to fatty acid methyl esters (FAME), which are the components of "biodiesel" fuel. *E. coli* strains have been engineered to carry out multiple metabolic reactions to produce FAEE[9]: (1) production of ethanol, (2) production of acyl-CoAs, and (3) esterification of the ethanol and acyl-CoAs. A substantial improvement in FAEE production in *E. coli* was achieved by introducing a dynamic sensor-regulator system[4], resulting in a FAEE titer of 1.5 g/L, representing 28% of maximum theoretical yield, which is the best performance reported for FAEE to date. In other efforts related to fatty acid-derived fuels, we discovered enzymes needed for long-chain alkene biosynthesis by head-to-head condensation of fatty acids in the actinobacterium *Micrococcus luteus*. We identified OleABCD as being essential for long-chain alkene biosynthesis in *M. luteus*[10]. Heterologous expression of *M. luteus oleABCD* in *E. coli* resulted in production of C_{27} and C_{29} alkenes, which are too long to serve as diesel fuels, but could be cracked to shorter hydrocarbons suitable for use as fuels.

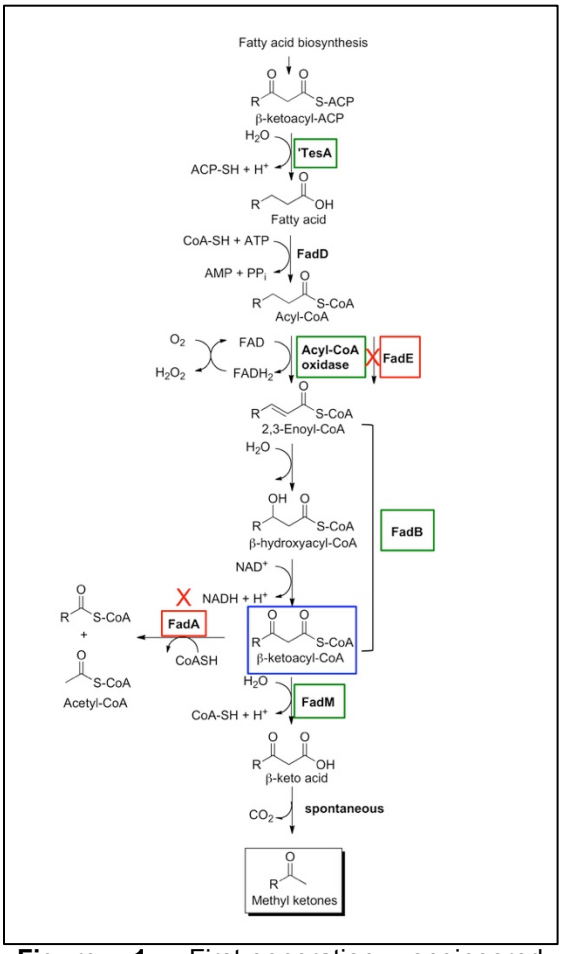

Figure 1. First-generation engineered pathway to convert fatty acids to methyl ketones in *E. coli* DH1. Green boxes indicate overexpressed genes and red boxes indicate chromosomal deletions. The blue box indicates the putative substrate for FadM (producing free β-keto acids). The final step, decarboxylation of β-keto acids to form methyl ketones, is shown as "spontaneous"; this has been demonstrated by *in vitro* reconstitution studies[3].

2.1.2 Methyl ketones

We have proposed[11] medium-chain methyl ketones as potential diesel fuel blending agents, and have shown that they have cetane numbers that compare favorably with those of typical U.S. diesel fuel. We have engineered a novel metabolic pathway in *E. coli* to overproduce saturated and monounsaturated aliphatic methyl ketones in the C_{11} to C_{17} (diesel) range. Features of the first generation methyl ketone-overproducing *E. coli*[11] are shown in Figure 1. 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[11]. We have transferred the methyl ketone pathway into *Ralstonia eutropha*, which produced up to 180 mg/L of methyl ketones under chemolithoautotrophic growth conditions with CO_2 and H_2 as the sole carbon source and electron donor, respectively[12]. Recently, we have made genetic modifications to the first-generation *E. coli* strain that improved methyl ketone titer and yield 160-fold[3]. Host and pathway modifications included balancing overexpression of *fadR* and *fadD* to increase fatty acid flux into the pathway, consolidation of the pathway from two plasmids into one, codon optimization, and knocking out key acetate production pathways (*poxB*, *ackA*, and *pta*). 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, and 3.4 g/L methyl ketones was produced after 45 hr of fed-batch fermentation with glucose. These titer and yield values are among the highest reported for any fatty acid-derived fuel or bio-based chemical.

2.2 Isoprenoid-derived biofuels

Isoprenoids have a number of structural attributes that give them favorable fuel properties. For example, their carbon chain lengths (C_5, C_{10}, and C_{15}) are appropriate for a range of transportation fuel types, and their methyl branching, which is a common structural feature of isoprenoids, lowers the freezing point significantly and endows them with excellent cold weather properties as fuels. In recent years, several isoprenoids have been developed and tested as potential gasoline, diesel, and jet fuels with favorable energy contents, cold weather properties, and octane/cetane numbers[13]. At JBEI, we have engineered isoprenoid biosynthetic pathways to achieve microbial production of biofuels; the pathways and products are summarized in Figure 2. Isoprenoid biosynthesis is initiated by the formation of two universal C_5 precursors, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). The starting C_5 precursors can be synthesized by two routes: the 2-methyl-D-erythritol-4-phosphate (MEP) or 1-deoxy-D-xylulose 5-phosphate (DXP) pathway, and the mevalonate (MVA) pathway. We have exploited synthetic biology to allow these heterologous pathways to work efficiently in new hosts, and also employed multiple omics tools to monitor and optimize the pathway enzymes and metabolites to improve yields and titers of isoprenoid production[2, 14].

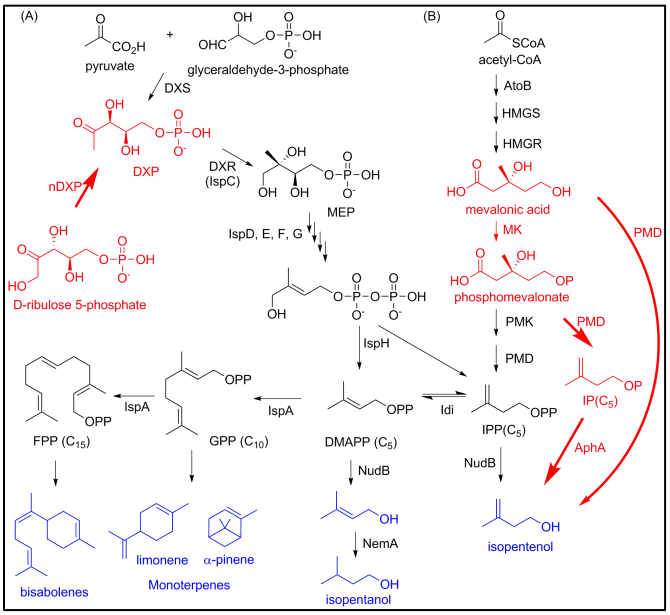

Figure 2. Pathways for isoprenoid-based biofuels and examples of JBEI isoprenoid biofuels. (A) MEP (or DXP) pathway with a variant (nDXP) pathway for DXP from D-ribulose 5-phosphate. (B) MVA pathway with variant pathways for isopentenol. Biofuel products (in blue) are bisabolene (C_{15}), limonene (C_{10}), pinene (C_{10}), isopentanol (C_5), and isopentenol (C_5). Novel isoprenoid pathways invented at JBEI are shown in red with bold arrows.

2.2.1 Bisabolene

Bisabolane has a cetane number in the range expected for diesel fuels (40~60), displays better cold-temperature properties than D2 diesel (freezing point of -78°C for bisabolane vs. -3°C for D2 diesel), and has reasonable fuel density (0.88 g/mL). Using a bisabolene synthase from *Abies grandis*, we achieved 1.2 g/L of bisabolene production in batch culture (35% of maximum theoretical yield[2]). Bisabolene protects yeast against Tween 20 (T20), and cultures grown in the presence of T20 for 14 days produced bisabolene at titers up to 4-fold higher than cultures grown with an overlay of dodecane, and 20-fold higher than cultures grown without dodecane[15].

2.2.2 Monoterpenes – limonene and pinene

We have achieved the production of the monoterpene limonene in *E. coli* with a limonene synthase from *Mentha spicata*[16]. Principal Component Analysis of Proteomic data (PCAP)-guided metabolic pathway engineering resulted in over a 40% titer improvement (to 650 mg/L) of limonene in a batch culture[2]. This limonene production system has also been coupled with ionic liquid-tolerant cellulases[17] to convert unsaccharified cellulose to glucose and convert the glucose to limonene, in the presence of ionic liquids, setting the stage for processes that use fewer unit operation to go from lignocellulose to final products[18]. Recently, hydrogenated dimers of α- and β-pinene have been synthesized chemically and have been shown to have a high energy density similar to that of JP-10[19]. Microbial production of pinene was demonstrated in *E. coli* with a pinene synthase from *Abies grandis*[20], producing 32.4 mg/L of pinene through the use of protein fusions that reduce product inhibition/toxicity through substrate channeling.

2.2.3 Isopentenol/isopentanol

Branched C_5 alcohols [isopentanol (3-methyl-1-butanol) and isopentenols (3-methyl-3-butenol and 3-methyl-2-butenol)] have higher energy contents and better gasoline-like properties than ethanol, as well as high octane numbers (RON, or research octane number, of 92-102)[21]. Isopentenols have been produced in engineered *E. coli*[14, 22, 23], and a titer of 2.2 g/L (up to 70% of apparent theoretical yield) has been achieved[13]. To overcome limitations intrinsic to IPP accumulation and to relieve "unnecessary" consumption of ATPs for isopentenol production, we designed "IPP-bypass" pathways for isopentenol production (Figure 2), and thereby achieved considerable isopentenol titers[24].

2.2.4 MEP (or DXP) pathway engineering

In *E. coli*, a hypothetical novel route from a pentose phosphate to DXP could enable a more direct pathway from C5 sugars to terpenes, and circumvent regulatory mechanisms. We have discovered two enzymes that can convert ribulose 5-phosphate directly to DXP[25], as shown in Figure 2. We have demonstrated the utility of these genes for engineering isoprenoid production in *E. coli*, and further improved flux by fusing the novel DXP proteins to DXP reductase, improving bisabolene titers more than 4-fold and alleviating the accumulation of intracellular DXP.

2.3. Polyketide-derived biofuels

Polyketides are some of the most diverse molecules known. Polyketide synthases (PKSs) perform Claisen condensation reactions between a loaded acyl-ACP intermediate and an alpha-substituted (H, CH_3, C_2H_5, etc.) malonyl extender unit, analogous to fatty acid biosynthesis. We have determined that approximately 20 of the roughly 150 commodity chemicals tracked by the petrochemical market information provider ICIS (http://www.icis.com/chemicals/channel-info-finder/) could be produced by mixing and matching naturally occurring polyketide synthase domains. To form these chemicals, engineered polyketide synthases would load acyl-CoAs, perform a programmed number of extension reactions, and then release products. We have begun work on engineering PKSs for the production of fuels (Figure 3), and have demonstrated the production of 3-hydroxyacids[26], methyl ketones (MKs), ethyl ketones (EKs), and alcohols, by constructing hybrid PKSs. The MKs that we have produced are in the range of molecules useful as gasoline replacements. The lipomycin PKS is useful for producing short, highly branched molecules; the borrelidin PKS is useful for producing longer molecules that would be useful as diesel or jet fuel.

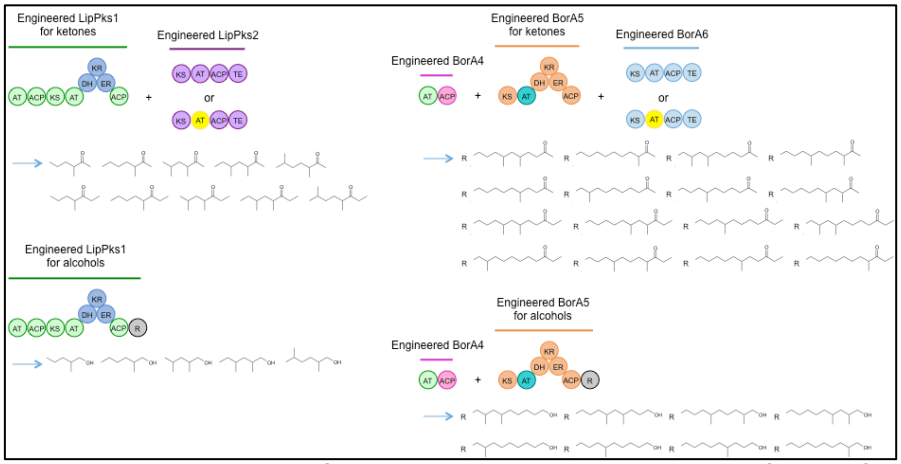

Figure 3. Engineered PKS systems to produce spark ignition fuels (left) and compression ignition fuels (right). Circles of the same color indicate domains derived from the same natural source.

3. MODIFICATION, CONTROL, AND YIELD IMPROVEMENT OF BIOFUEL PATHWAYS

3.1. Engineering fatty acid-derived biofuel pathways with fatty acid-responsive regulators

The negative transcription factor FadR is an important fatty acid biosynthesis and β-oxidation regulatory element in *E. coli*. When fatty acyl-CoAs are present at very low intracellular concentrations, FadR binds to promoters of β-oxidation, repressing transcription. Conversely at moderate concentrations, acyl-CoAs bind to FadR, releasing FadR from its promoter binding site, de-repressing transcription. JBEI researchers have exploited FadR in the development of a Dynamic Sensor-Regulator System to successfully increase the production of FAEEs[9]. In this study, modules of the FAEE biosynthetic pathway were placed under the regulatory control of synthetic hybrid promoters that included FadR-binding sites. A schematic of the approach is shown in Figure 4a. The approach was highly successful (Figure 4b), resulting in a 3 to 4-fold increase in FAEE titers to 1.5 g/L[4], as well as a dramatic increase in the stability of the 3-plasmid system.

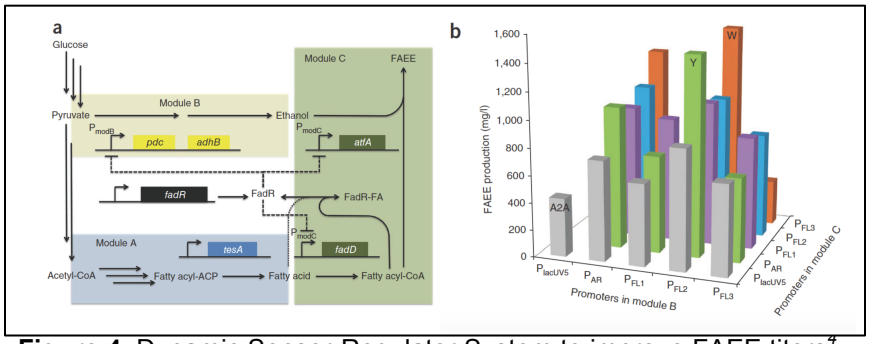

Figure 4. Dynamic Sensor-Regulator System to improve FAEE titers[4].

3.2. Alcohol (1-butanol) sensors

The majority of short-chain alcohols cannot be directly screened or selected for. We have developed a generalized approach to screen or select for improved small-molecule biosynthesis using transcription

factor-based biosensors[27]. We have coupled host antibiotic resistance to either small-molecule concentration or a small-molecule production phenotype. We constructed biosensors for alcohols, using transcription factor-promoter pairs selected for specific activation by 1-butanol. The 1-butanol biosensor was applied to optimize 1-butanol biosynthesis in engineered *E. coli*, yielding a 35% increase in 1-butanol specific productivity.

3.3. Isoprenoid pathway regulation

We employed microarray analysis to identify *E. coli* genes that are up- or down-regulated by the accumulation of the toxic isoprenoid pathway intermediate farnesyl diphosphate (FPP). We then screened a library of 35 FPP-responsive promoters for their ability to dynamically regulate the production of FPP *en route* to the sesquiterpene, amorphadiene. Dynamic FPP regulation resulted in 2-fold higher amorphadiene yields than comparable inducible or constitutive pathway expression systems[28]. Moreover, the dynamic system eliminated the need for expensive inducers, reduced acetate accumulation, and improved growth. Previous studies have implicated IPP accumulation as a cause of reduced growth and decreased glucose uptake. We hypothesized and subsequently verified that an adaptive-control system, Feedback-Regulated Evolution of Phenotype (that increases the mutation rate with intracellular IPP concentration, and decreases the mutation rate with isoprenoid concentration), could be used to evolve increased isoprenoid production[29].

3.4. Synthetic transcription factor (TF) / hybrid promoters for yeast metabolic engineering

We have constructed a library of hybrid promoters that are regulated by a synthetic TF (Figure 5). The resulting system does not affect the transcription of native *S. cerevisiae* genes, and the hybrid promoters (driving genes of interest) can be induced using estradiol, a compound with no detectable impact on *S. cerevisiae* physiology. A series of hybrid promoters (a library of 240 was designed, constructed, and tested) can be induced to different levels with the same synthetic TF at the same level of exogenously supplied estradiol.

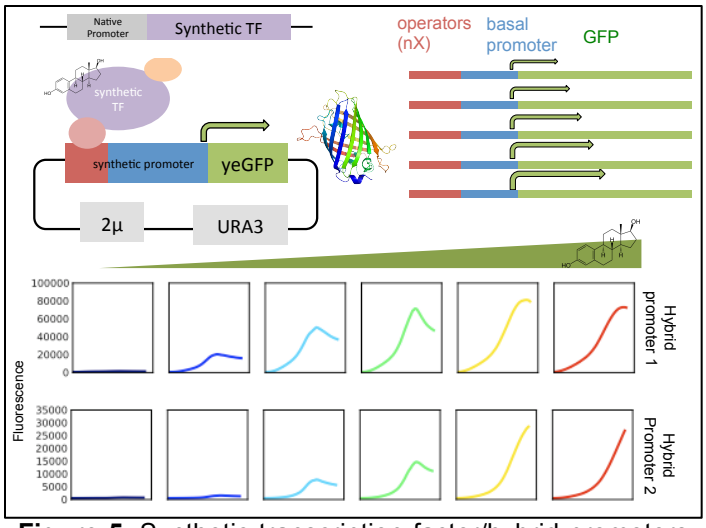

Figure 5. Synthetic transcription factor/hybrid promoters for yeast metabolic engineering. The chromosomally encoded synthetic TF is coupled with a library of hybrid promoters to regulate genes with differential inducibility and responsiveness in the same strain at the same level of inducer compound.

3.5. Global regulators and RNA-regulated devices

We have exploited the global regulator csrB, an endogenous non-coding RNA, to alter carbon flux in *E. coli* to improve the performance of the 1-butanol, mevalonate, and fatty acid pathways[30]. Predictable RNA-regulated devices can enable programmable pathway control[31]. We formulated a design-driven approach that uses mechanistic modeling and kinetic RNA-folding simulations to engineer RNA-regulated genetic devices that control gene expression[5] (Figure 6). We verified the models and design strategy by constructing 28 *E. coli* expression devices that gave excellent quantitative agreement between the predicted and measured gene expression levels (r = 0.94).

3.6 ^{13}C labeling combined with genome-scale models to improve fatty-acid production

Flux-based metabolic models describe how carbon flows from feedstock to final product. Flux Balance Analysis (FBA) has previously successfully leveraged comprehensive genome-scale models for yield improvement. 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 constraints obtained from ^{13}C labeling experiments[1] (see also Section 4.4, below). JBEI researchers used 2S-^{13}C MFA to

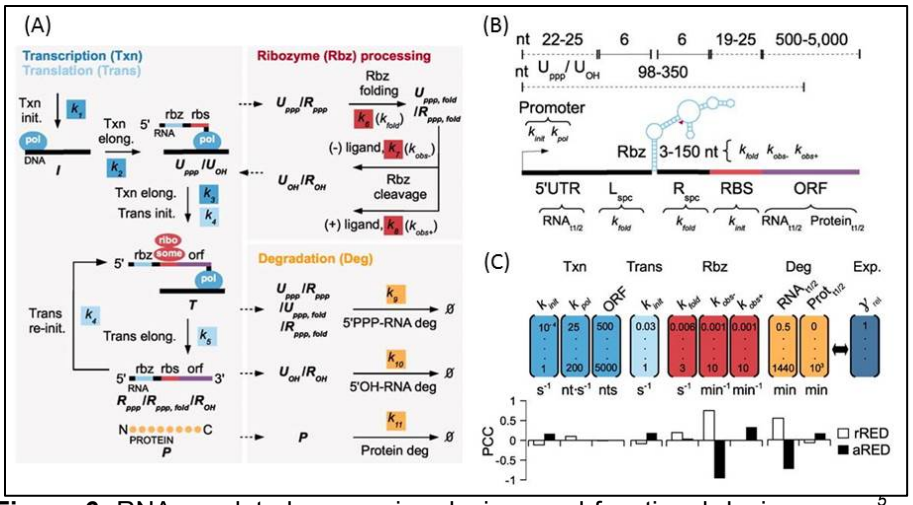

Figure 6. RNA-regulated expression devices and functional design space[5]. (A) Functions of rREDs and aREDs were simulated with a coarse-grained mechanistic model of effective rate constants. (B) Tunable components and design variables for static rRED and dynamic aRED genetic controllers. (C) Combinatorial design variable space was mapped to device outputs.

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: any extra acetyl-CoA gained was diverted into the malate synthase (MLS) reaction. This analysis suggested a downregulation of MLS in order to increase production, which ultimately produced an increase in production of 21% (MLS 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 of production of 33%. The final strain produced 780 mg/L of fatty acid, representing an increase of 70% over the initial titer.

4. TOOLS FOR DESIGNING MICROBIAL METABOLIC BIOFUEL PATHWAYS

4.1 ICE: Repository for strains, sequences, parts, and plant seeds

ICE (Inventory of Composable Elements) is a physical as well as a freely open-source web-based digital repository platform for microbial strains, sequences, parts, and plant seeds[32]. ICE supports community standards including the Synthetic Biology Open Language (SBOL) data-exchange format[33] as well as the SBOL Visual[34] representation of annotated DNA sequences. Multiple repositories across the world can exchange information in a granular access-controlled manner using ICE's "Web of Registries" functionality. The JBEI Public Registry (https://public-registry.jbei.org) stores the strains, sequences, parts, and seeds associated with JBEI publications. The journal *ACS Synthetic Biology* has announced an initiative in which it had deployed its own ICE repository for authors to store and share all of the strains, sequences, parts, and seeds associated with their published manuscripts[35].

4.2 DeviceEditor/j5: Biological computer-aided design and DNA assembly automation

DeviceEditor is a web-based biological computer-aided-design canvas that enables users to visually design combinatorial DNA constructs[36] (see Figure 7). DeviceEditor depicts part types (*e.g.*, promoter, terminator) using SBOL Visual[34] standard glyphs. j5 automates the design and cost-optimization of modern scar-less combinatorial DNA assembly methods[37]. j5 receives design information from DeviceEditor, and interfaces with PR-PR (a cross-platform laboratory automation system)[6, 38] to enable the downstream execution of the designed DNA assembly protocols on laboratory automation devices including liquid handling robotics and microfluidic platforms[39-41]. A U.S. Patent for j5 has been issued[42], and j5 has been exclusively licensed for commercial-use and distribution to TeselaGen (a JBEI startup company founded on j5 technology). Through the JBEI Public j5 web-server (https://j5.jbei.org) there are more than 1,800 academic, non-profit, and government registered users of DeviceEditor and j5 at more than 460 institutions worldwide.

4.3 DIVA: DNA design, implementation, and validation automation

DIVA (Design, Implementation, and Validation Automation) is a web-based collaborative biological design and fabrication platform that increases research efficiency and productivity through enabling a division of labor (separation of design from fabrication tasks) and design aggregation to scales benefitting from laboratory automation devices. A small team of technical staff, leveraging laboratory automation devices, build and verify all designs submitted for construction. Over 3,000 constructs have been designed within DIVA at JBEI across all 4 of its divisions, and over 1,100 of these have been submitted for construction. The DIVA platform has been deployed at the JGI, and has been licensed for non-commercial use to the University of Edinburgh and the National University of Singapore.

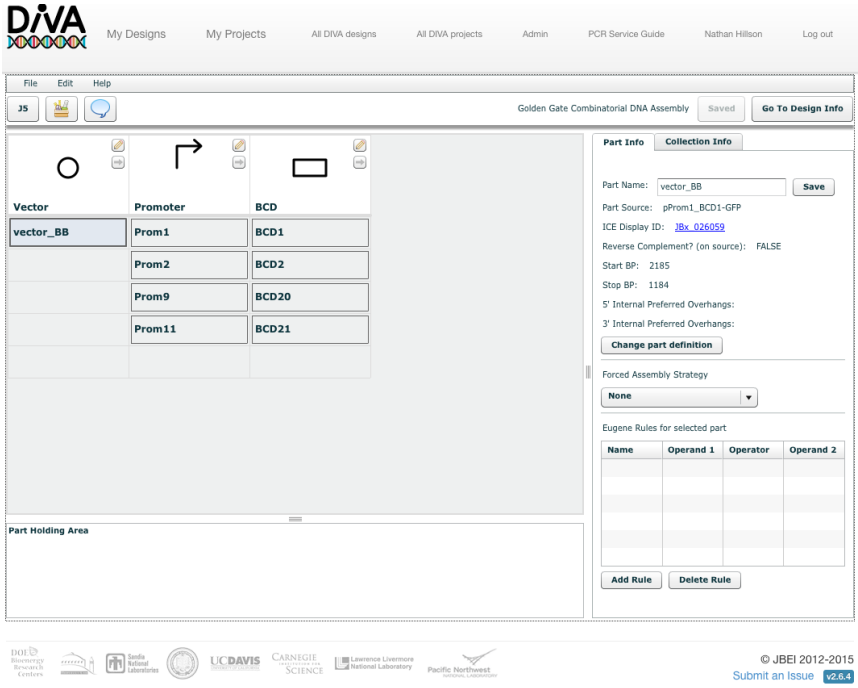

Figure 7. DeviceEditor user interface. Shown at left is a 16-variant combinatorial design (4 promoters combined with 4 bicistronic-devices)[6], with columns left to right corresponding to DNA 5' to 3', and rows top to bottom corresponding to different part variants. SBOL Visual glyph column headers indicate the type of parts contained within a column. At right is information for the selected "vector_BB" part, indicating its location within its source sequence (with a link to its entry within ICE), along with its forced assembly strategy and the Eugene rules specified for it.

4.4. jQMM: Algorithms for actionable metabolic engineering insights

The JBEI Quantitative Metabolic Modeling library (jQMM, Birkel *et al.* in preparation) provides an open-source framework for modeling metabolic fluxes (see Figure 8). jQMM brings together Flux Balance Analysis and ^{13}C Metabolic Flux Analysis, using ^{13}C labeling experiment data to constrain comprehensive genome-scale models through two-scale ^{13}C Metabolic Flux Analysis (2S-^{13}C MFA)[1]. The jQMM library has been demonstrated to suggest gene knock-out targets in *S. cerevisiae* that increase fatty acid yield by ~40% (Ghosh *et al*. in review), to investigate the metabolism of glucose-repression (Shymansky *et al*, submitted), and to increase limonene and bisabolene production by ~40% and ~200% respectively[2]. Some jQMM functionality has been run on the KBase platform (https://kbase.us), and future efforts aim to make the full jQMM library available via KBase.

4.5. Experiment Data Depot (EDD): Repository for standardized experiment data

The EDD is a freely open-source web-based repository for actionable proteomics, metabolomics, transcriptomics, flow cytometry, plate reader, and BioLector data (Morell *et al.* in preparation). EDD data visualization capabilities facilitate data quality assessment, and experiment data can be exported (and is also accessible through a RESTful API) in standard data-exchange formats. The JBEI Public EDD (https://public-edd.jbei.org) stores experiment data associated with JBEI publications.

4.6. Arrowland: Multi-omics data visualization

Arrowland is a web-based tool for visualizing overlays of functional genomics data (*e.g.*, transcriptomics, proteomics, metabolomics and fluxomics), and comparisons between different time points or conditions, through an interactive multi-scale map. Arrowland enables users to click on a metabolic reaction, and

predict the flux profile were that reaction to be knocked out. Arrowland is available through the JBEI Public web-server (https://public-arrowland.jbei.org).

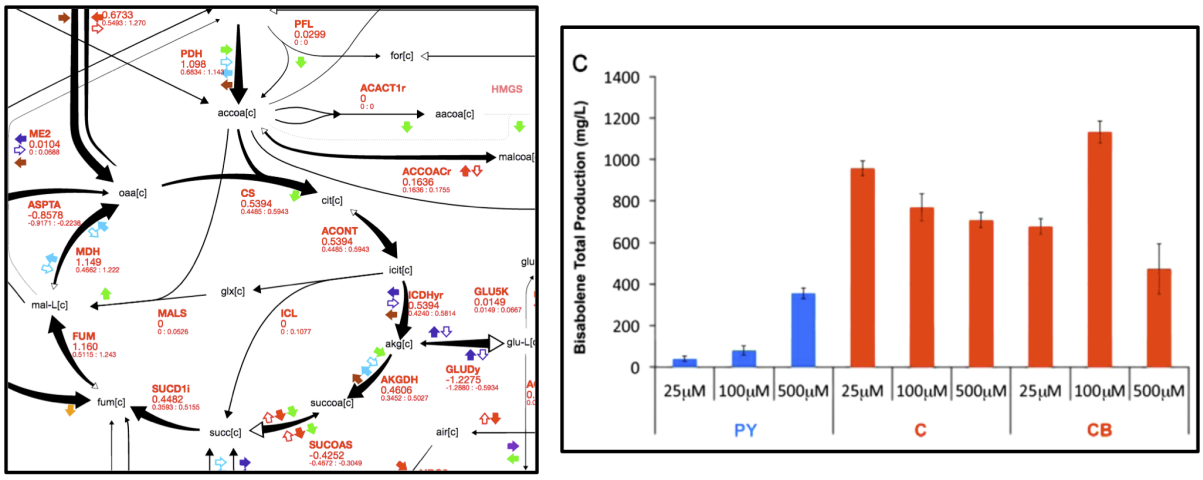

Figure 8. jQMM metabolic flux profiles and increases in bisabolene production. Shown at left are metabolic flux profiles obtained through 2S ^{13}C MFA by constraining genome-scale models with ^{13}C labeling data[1]. Right panel shows the increase (in red) of bisabolene production using plasmid designs obtained through PCAP[2].

5. FUTURE OUTLOOK

As we continue to optimize microbial host chassis to meet the sophisticated demands of carbon-efficient, energy-efficient, and scalable conversion, it will become increasingly necessary to explore additional microbial hosts. An important future objective is to develop robust new hosts that demonstrate conversion capabilities (*e.g.*, co-utilization of mixed carbon sources, heterologous expression of proteins, tolerance to toxic intermediates and final products[43]) under industrially relevant conditions (*e.g.*, low pH). In addition to new microbial hosts, there is a vast expanse of new molecules that are potentially accessible to us. We envision a future in which a single design algorithm, using a molecule of interest as input, successfully combines DNA sequences into a metabolic pathway that efficiently produces the desired molecule. Such hosts, algorithms, and engineered pathways will further enable the process of engineering biological systems for biofuel production as well as for a broad variety of other biological engineering goals.

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