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Microbial production of advanced biofuels

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Abstract:
Concerns over climate change have necessitated a rethinking of our transportation infrastructure. One possible alternative to carbon-polluting fossil fuels are biofuels produced from a renewable carbon source using engineered microorganisms. Two biofuels, ethanol and biodiesel, have been made inroads to displacing petroleum-based fuels, but their penetration has been limited by the amounts that can be used in conventional engines and by cost. Advanced biofuels that mimic petroleum-based fuels are not limited by the amounts that can be used in existing transportation infrastructure, but have had limited penetration due to costs. In this review, we will discuss the advances in engineering microbial metabolism to produce advanced biofuels and prospects for reducing their costs.

Introduction

The US consumes approximately 14 million barrels of oil equivalents every day in transportation fuels or roughly 28 quadrillion BTUs of energy (Department of Energy 2020). In 2019, fossil resources supplied approximately 81% of total energy and 95% of the transportation fuels used (Department of Energy 2020), with renewable resources supplying 11% of total energy and only 5% of transportation fuels. By 2050, energy use is projected to increase by 50%, with renewable resources accounting for only 25% of total use.

In the United States, gasoline is the predominant fuel consumed (approximately 9.5 million barrels per day), diesel fuel is next (at approximately 4 million barrels per day), and jet fuel is last (at approximately 2 million barrels per day). Worldwide, gasoline accounts for 39% of energy used by the transportation sector, with diesel fuel accounting for 36% and jet fuel 12%. Gasoline is a complex mixture of hydrocarbons composed primarily of branched-chain alkanes and aromatics ranging from 4 to 12 carbons in length (Sawyer 1993). Diesel fuel is a mixture of generally linear hydrocarbons ranging from 9 to 23 carbons in length, with an average length of 16 carbons. The types of hydrocarbons in gasoline and diesel have a strong impact on the properties of the fuel. For example, branching and unsaturation leads to greater octane numbers in gasoline (Ghosh et al. 2006) and lower cetane numbers in diesel (Ghosh and Jaffe 2006). Conversely, n-alkanes have higher cetane numbers and lower octane values. While significant branching is detrimental to the diesel cetane number, branching is needed to prevent gelling of linear hydrocarbons at low temperatures.

Similar to gasoline and diesel fuel, jet fuel is a mixture of hydrocarbons. Most jet fuels are based on kerosene and are designed to a specific performance criterion. In the US, standards for fuel for civilian aircraft are set by ASTM International (ASTM 2020), whereas the Department of Defense sets the standards for fuel for military aircraft. Jet A or A-1 are used in most parts of the world except the far north where Jet B is used and in Russia where Jet TS-1 is used.

Worldwide, transportation contributed about one quarter of the total greenhouse gas emissions (Environmental Protection Agency 2020). There are two primary ways to decarbonize transportation: electrification with renewable sources of electricity, and fuels made from renewable resources, namely biofuels. While electrification of the passenger and truck fleet is happening slowly, electrification of air travel is likely to lag significantly, if it ever happens. The development of renewable biofuels and bioproducts (to reduce the price of biofuels) that reduce our reliance on petroleum is critical to energy, environmental, and economic security (Kircher 2015).

The two major biofuels that have been commercialized are ethanol from a variety of sources and biodiesel made from hydrogenated plant oils. Their uses have been limited due to a lack of infrastructure, limitations in the blend wall (e.g., amount of ethanol that can be blended into gasoline, currently 10% in the United States) and the number of flex-fuel automobiles (e.g., that can use more than 10% ethanol), quality of the fuel (e.g., diesel made from vegetable oil), and cost of the fuel. The penetration of biofuels could be deeper if 1) biofuels had similar properties to the fuels currently made from petroleum and 2) if they were significantly less expensive.

One of the only ways to significantly reduce price and the carbon footprint of biofuels is to use renewable lignocellulosic biomass from non-food crops. It has been estimated that there are approximately one billion dry tons of lignocellulosic biomass available annually in the US (Langholtz et al. 2016). If that biomass were converted to biofuels, those biofuels could replace approximately one-third of the petroleum-derived fuels. There have been many important advances in improving bioenergy crops and extracting intermediates from them that can be converted into biofuels (Baral et al. 2019; Lin and Eudes 2020). The other way to increase the penetration of biofuels is to produce biofuels that mimic petroleum-based fuels so they can directly replace petroleum fuels and therefore will not be subject to blend limits. In this article, we review the progress on engineering microorganisms to produce advanced biofuels that will directly replace petroleum-based fuels.

Advanced biofuels and their production pathways

As mentioned above, petroleum-based fuels are a mixture of linear, branched and aromatic hydrocarbons. There are several hydrocarbon-producing pathways in living systems that are capable of producing molecules similar to those found in gasoline, diesel, and jet fuel: isoprenoid, fatty acid, and polyketide, to name a few. In addition to these hydrocarbons, higher alcohols (hydrocarbon chains longer than ethanol) are suitable replacements for gasoline and can be used to synthesize diesel and jet fuel (Brooks et al. 2016) (Figure 1). We review these pathways and the engineering of these pathways to produce advanced biofuels.

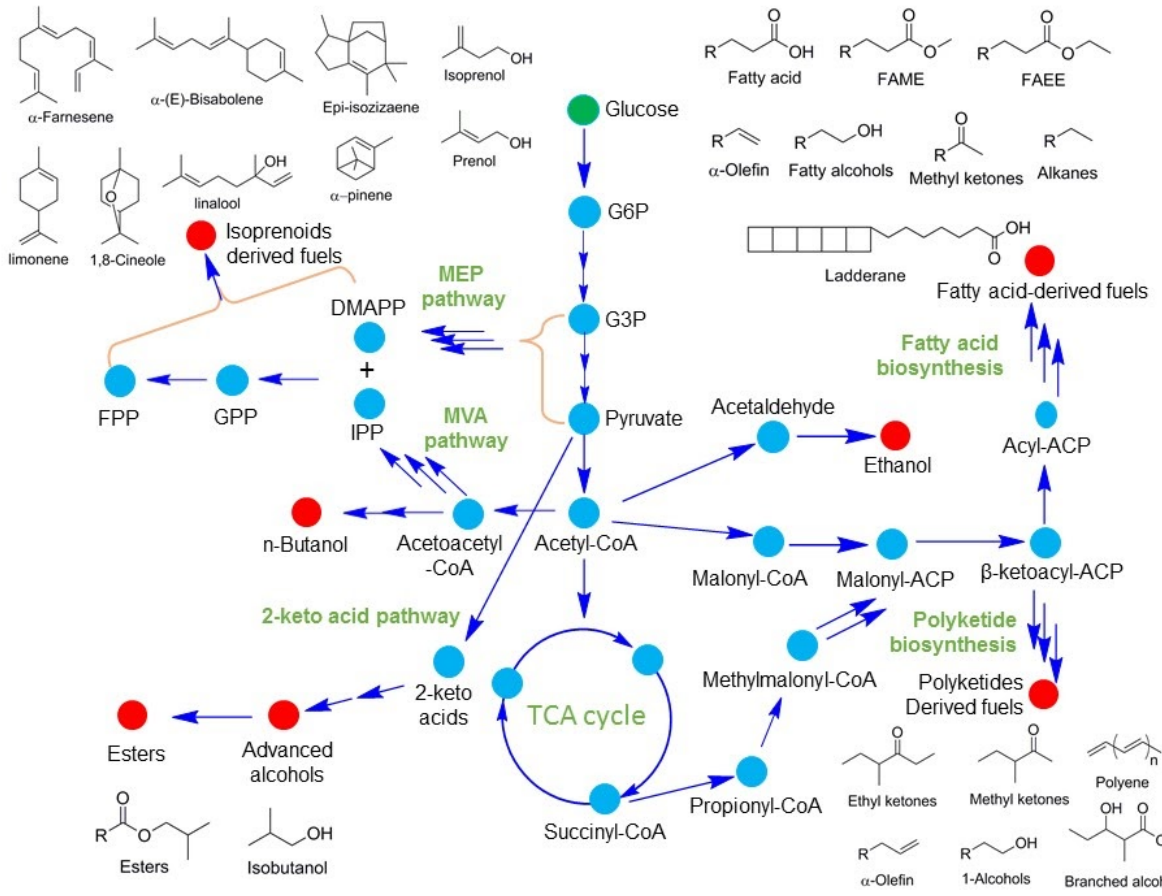

Figure 1. Metabolic pathways for advanced biofuels. Blue circles represent key metabolites, and red circles represent the type of biofuels.

Isoprenoids. Many isoprenoids are excellent biofuel candidates because they are branched and/or cyclic, which prevents gelling at low temperatures and improves octane (George, Alonso-Gutierrez, et al. 2015). Isoprenoids with fewer than 20 carbons are usually considered as fuel targets or precursors to biofuels. Isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) are the two five-carbon (C5) building blocks to isoprenoids, and they are synthesized mostly via two routes: the 2-methyl-d-erythritol-4-phosphate (MEP) pathway and the mevalonate (MVA) pathway (M. Li et al. 2020). Prenyltransferases condense IPP and prenyl diphosphates to produce prenyl diphosphates with an additional five carbons: GPP (C10), FPP (C15), and GGPP (C20) (Rodríguez-Concepción 2014; Oldfield and Lin 2012). Elongated prenyl diphosphates are converted to specific terpenes by terpene synthases or dephosphorylated to alcohols by phosphatases. The cyclization mechanism differs from one terpene synthase to another, but they share common mechanistic aspects such as the formation of a carbocation by diphosphate group

removal (Gao et al. 2012). Both *E. coli* and *S. cerevisiae* have been engineered to produce isoprenoid-based biofuels by co-expression of specific terpene synthases, most of which have been derived from plants. Farnesene and bisabolene are two early examples of isoprenoid biodiesel precursors (Wang et al. 2011; Zhu et al. 2014; Meadows et al. 2016; Peralta-Yahya et al. 2011). Recently, industrial production of farnesene has been reported at titers of 130 g/L by introducing novel pathways to increase cytosolic acetyl-CoA levels in engineered *S. cerevisiae* (Meadows et al. 2016). Multicyclic sesquiterpenes such as epi-isozizaene and pentalenene are highly energy dense fuel precursors for aviation and missile fuels (Harrison and Harvey 2017) and have been produced in engineered *E. coli* and *S. cerevisiae* (Liu et al. 2018). Monoterpenes such as limonene, pinenes, linalool, and 1,8-cineole are precursors to jet fuel and have been produced in *E. coli* and fungi by co-expression of C10-specific prenyl transferase and terpene synthases (Zebec et al. 2016; Zhuang et al. 2019).

Isoprenoid-derived C5 alcohols are being considered for commercial scale production due to their favorable properties as gasoline replacements and as precursors to jet fuel and recent titer improvements (George, Thompson, et al. 2015a; Kang et al. 2019). C5 alcohol production was first demonstrated in *E. coli* by overexpressing *nudF* from *Bacillus subtilis* (Withers et al. 2007). An *E. coli* ortholog (*nudB*) increased isoprenol production further (Chou and Keasling 2012), and extensive engineering efforts improved its yield (close to 70% of theoretical yield) and titer significantly (Zheng et al. 2013; George et al. 2014; George, Thompson, et al. 2015a). To address the toxicity of accumulating IPP, an “IPP-bypass” pathway was developed by leveraging substrate promiscuity of a phosphomevalonate decarboxylase to bypass one phosphorylation step in the MVA pathway (Kang et al. 2016; Aram Kang et al. 2017), and recently, the highest reported titer (10.8 g/L) of isoprenol was achieved via fed-batch fermentation using this pathway (Kang et al. 2019).

Fatty acids. Long, linear hydrocarbons, which are excellent diesel and jet fuel components, can be readily produced from fatty acids (Ruiz et al. 2006; Lennen and Pfleger 2012). In nature, fatty acids form the core of the phospholipid membrane that surrounds most cells (Walther and Farese 2012). Fatty acids have been used traditionally as precursors to biodiesel, produced by trans-esterification of fats with methanol (fatty acid methyl ester (FAME)) or ethanol (fatty acid ethyl ester (FAEE)). The fatty acid synthase (FAS) is a multienzyme system composed of an iterative decarboxylative condensation enzyme, acyl carrier protein (ACP), a series of reducing and dehydrating enzymes, and a thioesterase (TesA) (Janßen and Steinbüchel 2014). Fatty acid biosynthesis is tightly regulated in the host as it is an essential function, and a significant perturbation of this pathway results in altered membrane composition and

severe growth defects (Lennen et al. 2011; Lennen and Pfleger 2013; Budin et al. 2018). There have been various engineering approaches to improve fatty acid biosynthesis for biofuels production (Marella et al. 2018). Two early approaches focused on diverting the pool of acetyl-CoA towards fatty acid biosynthesis or decreasing the degradation of fatty acids. For example, overexpression of acetyl-CoA carboxylase (ACC) led to increased malonyl-CoA levels and improved fatty acid production (Chen et al. 2014; Qiao et al. 2015). Genes for acetate production were knocked out or knocked down in *E. coli* to reduce the loss of acetyl-CoA (Wu et al. 2017). The deletion of β-oxidation genes in the fatty-acid degradation pathway also improved fatty acid production (Steen et al. 2010). As *S. cerevisiae* does not produce cytosolic acetyl-CoA naturally, the cytosolic acetyl-CoA level was increased by overexpression of heterologous ATP:citrate lyase (ACL) (Zhou et al. 2016) and circumventing the native pyruvate dehydrogenase reaction (Krivoruchko et al. 2015; de Jong et al. 2014). As fatty acid biosynthesis demands significant NADPH, improving NADPH supply by engineering the pentose phosphate pathway and/or engineering metabolism to relieve cellular redox imbalances have become important approaches to improve fatty acid biosynthesis (Qiao et al. 2017; Xu, Qiao, et al. 2017). Chain-length control is one of the more recent engineering directions in fatty acids biofuel research as it is directly related to the fuel properties, and several FAS engineering strategies have been used to produce short and medium chain fatty acids (Xu et al. 2016; Zhu et al. 2017).

The types of fatty acid-derived biofuels that have been proposed are numerous. Early work mainly focused on free fatty acids and their esters. Alkanes were synthesized using acyl-ACP reductase and aldehyde decarbonylase (Schirmer et al. 2010; Cao et al. 2016; M.-K. Kang et al. 2017), and terminal alkenes were synthesized using a fatty acid decarboxylase (OleT) or cyanobacterial elongase-decarboxylase (Ols) (Rude et al. 2011; Mendez-Perez et al. 2011; Chen et al. 2015). Fatty alcohols were produced either from fatty acyl-CoA by fatty acyl-CoA reductase or from fatty acids by various reductases (Xu et al. 2016; Youngquist et al. 2013; Akhtar et al. 2013; Cao et al. 2016). Methyl ketones were produced via modification of the β-oxidation pathway and spontaneous decarboxylation of β-keto acids (Goh et al. 2012; Goh et al. 2014). More complex and higher energy products such as ladderanes, which contains linearly concatenated cyclobutane rings (Javidpour et al. 2016), have been proposed as rocket fuels, but the biosynthetic pathway is not known, making their overproduction impossible at this time. Cyclopropane fatty acids and branched fatty acids also have good cold weather properties and can be produced by various systems (Machida et al. 2016; Yu et al. 2014; Czerwiec et al. 2019).

Advanced alcohols. Microbial production of higher alcohols (hydrocarbon chains longer than ethanol) was discovered and explored in Clostridium via the acetyl-CoA dependent fermentation pathway, e.g., acetone-butanol-ethanol (ABE) fermentation (Jones and Woods 1986). Clostridium has been extensively studied for production of higher alcohols (and acetone), but several inevitable drawbacks such as slow growth and spore formation prevent it from being a major workhorse for higher alcohol production (Rabinovitch-Deere et al. 2013). Among advanced alcohols, isobutanol and n-butanol were the most studied fuel targets due to their high energy content and properties similar to gasoline (Harvey and Meylemans 2011). Due to challenges in engineering Clostridium to produce n-butanol, its production has been engineered into *E. coli* and a variety of other hosts. Using NADH accumulation as a driving force, Shen and coworkers were able to boost n-butanol production to 30 g/L (Shen et al. 2011). Heterologous expression of an NADH-dependent CoA-reductase drove the high titer production of n-butanol in an *E. coli* strain that had all other fermentative NADH-consuming pathways deleted. This work also revealed the nature of bottlenecks that affected n-butanol production (Bond-Watts et al. 2011; Bai et al. 2019).

Another important set of higher alcohols are the fusel alcohols derived by catabolism of branched amino acids using the Ehrlich pathway, which is naturally found in yeast. To enhance the productivity of many higher alcohols, this pathway has been engineered into *E. coli* by introduction of a promiscuous 2-keto acid decarboxylase and an alcohol dehydrogenase, enabling high yield production of various higher alcohols (Atsumi et al. 2008). Later it was engineered into cyanobacteria, *Corynebacterium glutamicum*, and other bacterial hosts (Hazelwood et al. 2008; Atsumi et al. 2008; Atsumi et al. 2009; Vogt et al. 2016). Among these alcohols, isobutanol has been engineered to near commercial titers and yields. To expand the portfolio of products derived from alcohol, esters have been produced via esterification of the alcohols with various acyl-CoAs by co-expressing an alcohol O-acyltransferase (Rodriguez et al. 2014; Layton and Trinh 2014). These esters are broadly used as flavors, fragrances, and solvents, and maybe one day as biofuels.

Polyketides. The polyketide biosynthetic pathway is one of the most versatile pathways for production of hydrocarbons with diverse structures, but it has been mostly studied for the complex products they naturally produce, namely therapeutics and pest control agents (Yuzawa et al. 2012; Yuzawa, Keasling, et al. 2017; Yuzawa, Backman, et al. 2018; Yuzawa et al. 2016). As the choice of domains and modules in modular polyketide synthases (PKSs) can provide control of the product structure in a designed manner (Cai and Zhang 2018), short chain hydrocarbons (e.g., fuel targets) can be produced in a predictable manner by rationally recombining domains found

within natural PKSs (Yuzawa et al. 2016). For example, some PKS modules known to release terminal olefins and alcohols (Gehret et al. 2011) can be incorporated into chimeric, multi-modular PKSs to demonstrate production of compounds such as 1-butene, 1-hexene, 1-pentanol and 1-hexanol, which can be oligomerized into jet and diesel fuels (Harvey and Meylemans 2014). An iterative type I polyketide pathway was used to produce an alkene with multiple double bonds that can be chemically hydrogenated to an alkane (Liu et al. 2015). As mentioned above, methyl branching is an important structural feature for gasoline and to prevent gelling of diesel and jet fuel at cold temperatures. Branches in polyketides can be achieved, either through incorporation of methylmalonyl-CoA as a substrate or through methylation using S-adenosyl methionine (SAM) (Poust et al. 2015; Wagner et al. 2016). In one example, fatty acids were multi-methylated using an iterative type I PKS from *Mycobacterium tuberculosis* that accepts methylmalonyl-CoA as a substrate (Menendez-Bravo et al. 2014). Short-chain ketones have been reported as gasoline replacements and produced in *E. coli* engineered with a promiscuous β-keto-thiolase, a CoA transferase, and an acetoacetate decarboxylase from different organisms (Lan et al. 2013; Srirangan et al. 2016; Yuzawa, Deng, et al. 2017). Recently, *Streptomyces albus* was engineered to produce short-chain ketones in excess of 1 g/L from plant biomass hydrolysates (Yuzawa, Mirsiaghi, et al. 2018). Even though there are a few examples of high titer polyketide production and PKSs can be engineered to make biofuels with nearly ideal properties, the reported titers and yields of potential biofuel precursors from engineered PKSs are significantly lower than those from fatty acid or isoprenoid pathways, which is a huge challenge in engineering PKSs to produce biofuels in an economically viable manner (Zargar et al. 2017). Nevertheless, these synthases show great potential to produce tailor-made fuels.

Microbial chassis and carbon source

To produce the desired advanced biofuel, the fuel synthesis pathway must be incorporated into a microbial host. The choice of microbial host is generally dictated by several factors, including the source of carbon (e.g., cellulose, lignin, methane, carbon dioxide, etc.), the toxicity of the substrate (or anything in it) or the fuel itself, and the processing conditions needed to transform the substrate and/or produce the fuel. Heterologous pathways to produce advanced biofuels have been translated to a variety of hosts, which has expanded the use of different substrates and cultivation conditions. Isobutanol production has been engineered into a wide variety of hosts beyond the foundational studies in *E. coli* and *S. cerevisiae*. The isobutanol pathway has been engineered into *Corynebacterium glutamicum*, an industrial actinobacterium that has higher tolerance to isobutanol and is a native high titer producer of branched

chain amino acids, precursors to isobutanol ((Smith et al. 2010; Blombach et al. 2011). These engineered *C. glutamicum* strains produced up to 4.9 g/L from glucose. The isobutanol pathway has also been deployed into clostridia that use cellulosomes to hydrolyze cellulose. A mesophile, *Clostridium cellulolyticum*, was engineered to produce isobutanol at 660 mg/L and a thermophile, *Clostridium thermocellum*, produced 5.4 g/L of isobutanol, representing a 45% yield from cellulose (Higashide et al. 2011; Lin et al. 2015).

Fatty acid-derived biofuels have also been produced in a range of microbial hosts (Yan and Pfleger 2020). Oleaginous yeasts, primarily *Yarrowia lipolytica*, which naturally produce triacylglycerides under nitrogen limitation, have been engineered to produce a suite of fatty-acid derived molecules, including, fatty acid ethyl esters and medium chain methyl ketones (Hanko et al. 2018; Gao et al. 2018). These molecules may serve as drop-in replacements or blendstocks for diesel engines. These molecules have largely been produced using glucose or glycerol as a substrate; oleaginous yeasts *R. toruloides* and *Lipomyces starkeyii* natively metabolize xylose, but strains of *Y. lipolytica* have been engineered to metabolize xylose by the addition of heterologous pathways (Li and Alper 2019). For bacteria, *Pseudomonas putida* has been engineered to produce medium chain methyl ketones from both glucose and lignin-related aromatics, and methyl ketone production from plant hydrolysates was shown to be enhanced by the presence of biomass-derived amino acids, which are co-metabolized with the sugar and aromatic substrates (Dong et al. 2019). Fuel range hydrocarbons have been also produced indirectly by *P. putida* from lignin-derived compounds through funneling of intermediates from lignin depolymerization to medium-chain length polyhydroxyalkanoates (mcl-PHAs), which are produced through fatty acid metabolism. These mcl-PHAs were subjected to tandem thermal depolymerization of mcl-PHAs and catalytic deoxygenation to produce C_7-C_{12} hydrocarbons (Linger et al. 2014).

As mentioned above, terpenes produced by the isoprenoid pathway are versatile molecules that can be used as drop-in replacements for gasoline, diesel and jet fuel. Examples for all three of these fuels types have been demonstrated in non-model hosts (Table 1). Isopentenol was produced in *C. glutamicum (Sasaki et al. 2019)*. Titers of >1 g/L liter were achieved with glucose as substrate as well as sorghum hydrolysate derived from ionic liquid pretreatment. *C. glutamicum* tolerated higher levels of both isopentenol compared to *E. coli*. Bisabolene has been produced from glucose, xylose and *p*-coumarate in an engineered strain of *R. toruloides (Yaegashi et al. 2017)*. This process has been scaled to a 20-L one-pot process at > 2 g/L using ionic-liquid pretreated sorghum as a feedstock (Sundstrom et al. 2018). And monoterpenes, jet fuel precursors, have also been produced by *R. toruloides* (Zhuang et al. 2019).

Table 1. Advanced biofuel production by non-model hosts

Host	Molecule (titer)	References
Corynebacterium glutamicum	Isobutanol (4.9 g/L) Isopentenol (1.3 g/L)	(Blombach et al. 2011) (Sasaki et al. 2019)
Clostridium cellulolyticum	Isobutanol (660 mg/L)	(Higashide et al. 2011)
Clostridium thermocellum	Isobutanol (5.4 g/L)	(Lin et al. 2015)
Yarrowia lipolytica	Methyl ketones (315 mg/L) Fatty acid ethyl esters (1.2 g/L)	(Hanko et al. 2018; Gao et al. 2018)
Pseudomonas putida	Methyl ketones (1.1 g/L)	(Dong et al. 2018)
Rhodosporidium toruloides	bisabolene (2.2 g/L) 1,8-cineole (35 mg/L)	(Sundstrom et al. 2018) (Zhuang et al. 2019)

Biofuels from CO_2. In addition to production from plant-derived sugars and aromatics, advanced biofuels have been produced from CO_2 using photosynthetic and non-photosynthetic autotrophic bacteria. Isobutanol has been produced in single cell cyanobacteria *Synechocystis* PCC 6803 and *Synechococcus elongatus* PCC 7942 at ~ 1 g/L and >40 mg/L/h (Li et al. 2014; Miao et al. 2018)) (Varman et al. 2013). As with heterotrophic hosts, isobutanol toxicity limited production and UV mutagenesis was employed to identify mutants that improved tolerance to isobutanol in *S. elongatus* (Miao et al. 2018) *Cupriavidus necator*, a well-studied chemoautotroph capable of rapid growth on H_2/CO_2, was engineered to produce isobutanol from H_2/CO_2 and formate. Formate was delivered exogenously and generated from CO_2 in a bioelectrochemical reactor in the presence of *C. necator (H. Li et al. 2012)*. *C. necator* has also been engineered to produce medium chain methyl ketones from both sugars and H_2/CO_2 by the same strategies to impair native fatty acid beta-oxidation (Dong et al. 2018).

Product, intermediate, pathway toxicity. Another factor that greatly impacts the host choice is the toxicity of the final product, intermediates in the production pathway, or the pathway itself. Inhibitory aspects of the starting material or carbon source must also be overcome.

Biofuels are predominantly hydrocarbon based (Beller et al. 2015) and, as such, are growth inhibitory (Mukhopadhyay 2015). For example, the bio-gasoline target isopentenol is toxic to model microbes such as *E. coli* at concentrations > 2% (Foo et al. 2014; Dunlop et al. 2011), as are the terpene-based jet-fuel targets and precursors limonene (Chubukov et al. 2015), pinene (Niu et al. 2018) and cineole (Mendez-Perez et al. 2017). Isobutanol, another prominent biofuel target, is also toxic to many microorganisms that have been engineered for its production (Minty et al. 2011; Chong et al. 2014; Song et al. 2018; Ouellet et al. 2011). The partitioning of these compounds into the cell-membrane is considered to be a prominent aspect dictating their toxicity (Jin et al. 2014) (Zingaro et al. 2013; Chen et al. 2013).

Most starting materials, such as non-food plant biomass or agricultural waste, require pretreatment to depolymerize and release the metabolizable components. A well-reviewed topic (Pienkos and Zhang 2009; Ostadjoo et al. 2018; Mukhopadhyay et al. 2012), pretreatment often results in the generation of toxic byproducts. Examples are furfural and HMF that arise from the desiccation of sugars during acid pretreatment of plant biomass. In other cases, residual reagents used in the pretreatment may also be toxic to downstream enzymes and microbes. For instance, ionic liquids, a highly efficient set of reagents for the depolymerization and deconstruction of a range of feedstocks, can be inhibitory to both downstream enzymes (Park et al. 2012) and microbes, even at residual levels (Ouellet et al. 2011; Yu et al. 2016).

For these challenges two main approaches have been used and have their own strengths and weaknesses. One is the use of a model or highly genetically tractable microbial host, and engineering into it the desired phenotypes, namely resistance to the toxicity. The other is to use microbial strains that have native capability to tolerate the inhibitory compound. In addition to strain improvements, removal of final products or clean-up of the starting material is a powerful process strategy to address these toxicities (Freeman et al. 1993; Li et al. 2016) - this approach is described below, under Engineering for Scale-up.

Engineering model hosts. Both targeted and combinatorial approaches have been used to engineer microbial systems to counter these toxicities. In a targeted approach, specific genes known to enable tolerance are upregulated, downregulated, or deleted. Predominantly, chaperones, cell wall components, transporters and regulators are commonly discovered targets, and continue to be examined to engineer robust hosts (Thorwall et al. 2020; Gong et al. 2017). The microbial cell wall also plays a critical role in physiological response to many key stresses as well as core functions such as respiration. Perturbation of the cell membrane can disrupt the efficiency of the electron

transfer chain (Budin et al. 2018). The importance of cell wall engineering for biofuel production has also been recently reviewed (Sandoval and Papoutsakis 2016). Recent examples for other targeted studies are the systematic evaluation of chaperones to aid in butanol production (Xu et al. 2019). Transporters have specifically been an attractive target due to their ability to export the final product, and have been developed for hemiterpenes (e.g. isopentenol, (Basler et al. 2018)), monoterpenes (e.g. limonene, (Dunlop et al. 2011)), isobutanol (He et al. 2019), fatty acids (Lennen and Pfleger 2013) and other hydrocarbons (e.g. hexene (Mingardon et al. 2015) and decane (Chen et al. 2013)). However, genes of other functional categories have also been discovered using broader approaches. One example is a CRISPR-based study that investigated 31 loci and revealed the role of housekeeping genes such as *recA* and genes of unknown function (*yjjZ*) in enhancing biofuel production (Otoupal and Chatterjee 2018).

Engineering microbial systems to address substrate toxicity has also relied on 'omics-guided discovery of targets that could be modulated to enhance tolerance. Initial functional genomics studies were valuable in understanding gene targets that could be used to generate more robust hosts. Furan compounds, such as furfural and HMF, are well known side products in the renewable carbon use pipeline and toxic to microorganisms (Glebes et al. 2014; Yang et al. 2018), and they remain a topic of research to enable use of acid pretreated biomass (Kurgan et al. 2019; Jung et al. 2019). Several key studies explored the development of HMF-tolerant strains for production of isobutanol, highlighting the requirement for strains to have multiple non-native capabilities (Song et al. 2017). In the case of ionic liquids (ILs), both IL-tolerant enzymes and microbes have been developed. Examples include the development of an *E. coli* chassis that, via the deletion of the regulator RcdA, could be made tolerant to ethyl methyl imidazolium acetate (EMIM), an IL. This chassis was then used to express both IL-tolerant cellulases, as well as a biojet fuel target, limonene, and was able to convert pretreated cellulose with toxic levels of EMIM directly to the final product (Frederix et al. 2016). More recently, a laboratory evolution experiment led to the discovery of the role of a cytochrome component for IL tolerance, resulting in even superior production of limonene from IL-pretreated biomass (Eng et al. 2018). Another study targeting a novel IL-responsive transporter in *E. lignolyticus* led to the discovery of the transporter and also the corresponding regulator that is now the basis of a new category of inducible promoters using crystal violet (Frederix et al. 2014; Ruegg et al. 2018) Recent studies have also used salt tolerance as an enhancer for both IL tolerance and biofuel production in the presence of such inhibitory reagents and *E. coli* strains adapted for ionic liquid tolerance were used for the production of isoprenol (Wang et al. 2019).

Engineering naturally tolerant hosts. As tools for genetically modifying any microbe of interest are becoming more available, a viable approach to develop robust biofuel production hosts is the use of microbes that are natively tolerant to a common set of inhibitors. Prior to work on biofuels, there has been significant work on solvent tolerance for biodegradation and biocatalysis. Selection of microbial systems for these applications provided some of the first examples of bacterial and fungal strains that are highly tolerant to a range of hydrocarbons and aromatic compounds. Well reviewed elsewhere, key examples are *P. putida* strains that have both cellular export systems and hydrocarbon catabolism systems that lead to this phenotype (Yang et al. 2016; Ramos et al. 2002). Other microbial hosts, such as *C. glutamicum* and *Zymomonas mobilis*, also show innate tolerance to certain key biofuel targets including isopentenol (Sasaki et al. 2019) and ethanol (Yang et al. 2016; Ramos et al. 2002). These tolerances have been further enhanced using evolution or targeted engineering (Stella et al. 2019; Wang et al. 2020; Shui et al. 2015; Fuchino and Bruheim 2020). Gram positive microbes, such as *Rhodococcus opacus*, are natively tolerant to many aromatic compounds and hydrocarbons (Castro et al. 2016). And many fungi such as the oleaginous yeast *R. toruloides* are natively tolerant to a wide range of ionic liquids and other prepreatment reagents (Yaegashi et al. 2017). An interesting caveat in the use of naturally tolerant hosts for final product tolerance is the case of *P. putida*, which contains degradative pathways for many desired final products (W.-J. Li et al. 2020; Thompson et al. 2020). Degradation of the final product is not an economically viable method for dealing with toxicity, and therefore additional studies of these phenotypes are essential before they can be used in the design of microbial production systems.

In the context of both model and non-model hosts, recent progress in examining the toxicity response is dominated by next-generation methods that leverage combinatorial assays, massively parallel sequencing and automation. Among these, two approaches are particularly suitable for tolerance engineering. Adaptive laboratory evolution (ALE) has been used in several examples to evolve tolerance towards a range of inhibitory compounds, substrates and reagents (Sandberg et al. 2019). ALE works by repeatedly subculturing cells, which selects for the fastest growers (Conrad et al. 2011). As such, it relies on the powerful tool of evolution to find genotypes that improve growth in a systematic fashion. ALE was used to develop *E. coli* hosts with higher tolerance to aromatics than native *E. coli* (McCloskey et al. 2018), resulting in recruitment of genes that may not have been chosen in a targeted effort. Another uniquely powerful approach is RB-TnSeq: random barcode transposon-site sequencing (Wetmore et al. 2015). RB-TnSeq conducts mutant fitness profiling in high throughput by incorporating random DNA barcodes into transposons. Barcode sequencing is then used to assay mutant fitness across very sets of conditions. Wetmore et al performed 387 successful

genome-wide mutant fitness assays, representing 130 different microbe-carbon source combinations, and identified 5,196 genes with significant phenotypes across the five bacteria. Since then, RB-TnSeq has been used to discover new catabolism pathways in *P. putida* (Levulinic acid (Rand et al. 2017), I,4-butanediol (W.-J. Li et al. 2020), lysine (Thompson et al. 2019), short-chain alcohols (Thompson et al. 2020) and valeric acid (Thompson et al. 2019)), essential genes in *Synechococcus elongatus* PCC 7942 (Rubin et al. 2015), and the role of mutant phenotypes for 11,779 protein-coding genes that had not been previously annotated with a specific function (Price et al. 2018). Both ALE and RB-TnSeq resulted in identification of genes not easily intuited from prior studies or even other 'omics experiments, and provided valuable targets to design robust microbial chassis. ALE data and fitness data from published studies are available to the public via accessible databases (ALE db (Phaneuf et al. 2019) and fit browser (Price et al. 2018)), making them valuable resources to be used by the research community in general.

Pathway and pathway Intermediates. Pathways can impact the cell in two major ways One is by creating an imbalance for energetics or redox of the cell, such as by overuse of the pool of reducing cofactors or accumulation of a toxic intermediate, and the second is due to the burden of protein production. Eliminating intermediate imbalance and balancing redox is a key part of optimizing pathways (A Kang et al. 2017; Meadows et al. 2016). However, in a few cases the toxicity of these intermediates itself was used to develop the biodesign approach. An example is the accumulation of FPP which was reduced by using a dynamic sensor responsive system (Dahl et al. 2013; Zhang et al. 2012). GIven the specificity of an intermediate to a pathway, general solutions are challenging to devise. However, innovative solutions to this problem could be found in the development of synthetic organelles, such as carboxysomes where reactions that produce toxic byproducts and intermediates can be sequestered (DeLoache et al. 2016). Another way to solve this problem is to tether proteins together (Ajikumar et al. 2010; Mitsuhashi and Abe 2018; Hu et al. 2020; Wang et al. 2011; Dueber et al. 2009) or co-localize proteins (Jäger et al. 2018) to minimize diffusion of intermediates (Dueber et al. 2009). Laboratory evolution can also be used to select for cells that do not accumulate toxic intermediates and have high flux to the final product. For example, ALE was used to adapt *E. coli* strains to tolerate methylglyoxal, a common toxic intermediate (McCloskey et al. 2018).

Growth inhibition due to protein expression or localization burden could be observed in some cases, such as with the use of membrane associated proteins (Wagner et al. 2007) (Gubellini et al. 2011). Among other burdens, membrane protein overexpression causes stresses due to titration of the cellular secretory system (Wagner et al. 2007)

(Jensen et al. 2017) (Baumgarten et al. 2017; Baumgarten et al. 2018). However, there have been several useful studies that have developed approaches to mitigate or overcome these stresses. An interesting example is the use of toxicity from the membrane protein expression itself to dynamically regulate its expression (Boyarskiy et al. 2016).

Carbon efficient biofuel production

An important aspect of microbial production of biofuels is the conservation of carbon converted from biomass substrates to fuel products. A challenge for reduced, long chain molecules like fatty acids and terpenes is that the substantial amounts of carbon are lost as CO_2. As an example, conversion of glucose to limonene, a monoterpene proposed as a jet fuel precursor, has a theoretical yield of 0.32 g/g of glucose (Baral et al. 2019). This yield takes into account glucose converted to product as well as glucose converted to CO_2 to provide ATP and NADPH for C-C formation and reduction. Removing the requirement for glucose to provide energy and reducing equivalents raises the theoretical yield to 0.45 g/g for the glucose limonene conversion. Two strategies are required to achieve this increase in carbon conservation: 1) bypassing the glycolytic pathway to avoid pyruvate dehydrogenase-mediated decarboxylation of pyruvate to acetyl-CoA and, 2) compensating for this bypass by introducing external reducing equivalents to generate ATP/NADPH. The phosphoketolase shunt, studied most intensively in bifidobacterial species, produces acetyl phosphate from pentose phosphate intermediates xylulose-5-phosphate or fructose- 6-phosphate (Henard et al. 2015). Acetyl phosphate is converted by acetyl-CoA synthetase to acetyl-CoA.This shunt fulfills the requirement to bypass glycolysis to produce, in theory, three moles of acetyl-CoA from one mole of glucose. This strategy, which has been referred to as non-oxidative glycolysis, has been implemented in *E. coli* by expressing phosphoketolase and impairing the native glycolytic pathway, improving the yield of acetate from glucose from 66% to 83% under anaerobic conditions (Lin et al. 2018). In principle, this non-oxidative glycolysis strategy could be combined with the introduction of a source of external reducing equivalents, such as H_2, to reduced products with improved carbon conservation as described above for limonene (Bogorad et al. 2013). In practice, a complete bypass to produce an acetyl-CoA-derived product has not yet been reported; however, a phosphoketolase shunt has been installed into a farnesene-producing strain of *S. cerevisiae* (Meadows et al. 2016), leading to a 25% improvement in carbon conservation. The ability to express phosphoketolase in a variety of hosts suggest this two step strategy to improve carbon conservation for biofuel production has many avenues for success (Henard et al. 2015). A promising host to implement the two step strategy is *C. necator*, since it has two

native, oxygen tolerant hydrogenases to deliver external reducing equivalents and phosphoketolase expression has been demonstrated in this host (Fleige et al. 2011).

Systematic approaches to improve titers, rates and yields (TRY)

A fundamental obstacle to produce commercially viable biofuels involves obtaining high titers, rates and yields needed to make the fuel commercially viable (Van Dien 2013; Chubukov et al. 2016). Indeed, given the commodity nature of biofuels, at least 90% of theoretical yield, 300 g/L for titer and 5.0 g/L h for productivity are necessary TRY targets for biofuels to be competitive with petroleum-based fuels. While rational engineering approaches, which rely on a deep knowledge of pathway and host metabolism, have proven successful in the past (Kang et al. 2019; Tian et al. 2019; George, Thompson, et al. 2015b), less bespoke and more systematic methods are desirable. These systematic methods have the advantage that they can be applied to any host, pathway or metabolite, and do not require in-depth knowledge of metabolism. This in-depth knowledge of metabolism takes years to obtain, and may not even be available for non-model hosts.

Coupling production to growth. ALE has been successfully used to generate strains with improved growth on suboptimal carbon sources (Strucko et al. 2018), or better tolerance of high temperatures that might be necessary to maintain sterility, or for downstream processing (Caspeta et al. 2014) or ionic liquids used to pretreat biomass (Mohamed et al. 2017). At times, growth rate increases result in associated production increases (Lennen et al. 2019), but this need not necessarily be the case. Making this connection necessary (i.e., growth is necessarily linked to product synthesis) involves a procedure called growth coupling (Shepelin et al. 2018), which typically relies on making cell growth dependent on an intermediate of the desired product. The design of the selection mechanisms that couple growth and production is mostly an art, rather than a science, and relies on bespoke approaches that are not generalizable to many products. Hence, although growth coupled strategies have been experimentally shown for the production of lactic acid (Fong et al. 2005; Zhou et al. 2003), alanine (Zhang et al. 2007), n-butanol (Shen et al. 2011), succinate (Machado et al. 2012), malate (Machado et al. 2012), carotenoids (Reyes et al. 2014), and 1,4-butanediol (Tai et al. 2016), a systematic approach applicable to any product is desirable. Harder et al (Harder et al. 2016) provided experimental proof that itaconic acid can be increased through the use of minimal cut sets, a generalizable computation approach. Indeed, theoretical studies using this approach suggest that growth coupling for most metabolites can be obtained for *E. coli* and *S. cerevisiae (von Kamp and Klamt 2017)* by knocking out enough genes (often on the order of ~10 or more). This surprising

theoretical result awaits experimental verification, which appears to be underway (Banerjee et al. 2020).

Computational approaches to increase TRY. A popular approach has been to leverage Genome Scale metabolic Models (GSMs), which provide an exhaustive description of metabolic reactions encoded in the genome (King et al. 2016; Thiele and Palsson 2010). Using GSMs as a basis, a multitude of computational algorithms have been developed to predict the effect of genetic modifications or pinpoint the genetic modifications that increase production of the desired metabolites (Maia et al. 2016; Zomorrodi et al. 2012; Landon et al. 2019): e.g., OptKnock (Burgard et al. 2003), OptForce (Ranganathan et al. 2010), OptCouple (Jensen et al. 2019), OptORF (Kim and Reed 2010), k-OptForce (Chowdhury et al. 2014), CiED (Fowler et al. 2009), MOMA (Segrè et al. 2002), ROOM (Shlomi et al. 2005), RobustKnock (Tepper and Shlomi 2010), ReacKnock (Xu et al. 2013), FSEOF (Choi et al. 2010), EFMs (Zanghellini et al. 2013), EMILiO (Yang et al. 2011), OptReg (Pharkya and Maranas 2006), OptGene (Patil et al. 2005), RegKnock (Xu 2018), FOCuS (Mutturi 2017), GACOFBA (Salleh et al. 2015), OptStrain (Pharkya et al. 2004), GDLS (Lun et al. 2009), among others. However, only a few of these methods have been experimentally shown to lead to improved TRY. Optknock is among the first of these methods and it attempts to achieve growth coupling as defined above. Optknock has been successfully used to increase lactate production by 25-73% (Fong et al. 2005) and 1,4-butanediol by 300% (Yim et al. 2011) in *E. coli*, to increase anaerobic production of 2,3-butanediol to a titer of 2.3 g/l and a yield 0.113 g/g in *S. cerevisiae (Ng et al. 2012)*, and to increase the respiratory rate in *G. sulfurreducens (Izallalen et al. 2008)*. Cipher of evolutionary design (CiED) has been leveraged to increase flavanone yields by 600% in *E. coli (Fowler et al. 2009)* and increase production of leucocyanidin and catechin by 400% and 200%, respectively, in the same host (in conjunction with Metabolic Optimization of Metabolic Adjustment, MOMA) (Chemler et al. 2010). MOMA has also been combined with OptGene to help increase production of sesquiterpenes by 85% (Asadollahi et al. 2009) and vanillin by 500% (Brochado et al. 2010) in *S. cerevisiae*. Flux variability scanning based on enforced objective flux (FSEOF) has been used to improve lycopene production in *E. coli* by 320% (Choi et al. 2010). FluxDesign combined with (EMA) has provided insights to increase production of lysine in *C. glutamicum* by 200% *(Becker et al. 2011)* and of isobutanol in *B. subtilis* by 230% (S. Li et al. 2012). Optforce has been shown to improve internal malonyl-CoA levels by 400% in *E. coli,* leading to record levels of naringenin (Xu et al. 2011), and 20% yield increase of fatty acids in the same host (Ranganathan et al. 2012). OptGene has been used by itself to increase succinate titer by 3000% and succinate yield by 4300% in *S. cerevisiae (Otero et al. 2013)*. GSMs have also been used in conjunction with ^{13}C Metabolic Flux Analysis (^{13}C MFA) to

identify bottlenecks or imbalances in *S. cerevisiae*, and suggest engineering strategies that improved fatty acid production by 70% (Ghosh et al. 2016) and helped produce fatty alcohols at the level of 1.2 g/L (d'Espaux et al. 2017). These successes underscore the utility of GSM in metabolic engineering, and highlight the need for more experimental tests and comparisons between different approaches.

Machine Learning. Machine learning (ML) has recently emerged as a new approach to improve TRY (Kim et al. 2019; Presnell and Alper 2019; Volk et al. 2020). ML provides predictions by statistically learning patterns in experimental data, rather than concentrating on the underlying biological mechanism. While the application of machine learning to synthetic biology is still nascent, it has shown promise by predicting translation initiation sites (Clauwaert et al. 2019), protein function (Ryu et al. 2019), biosynthetic pathways (Segler et al. 2018), the strength of regulatory elements (Meng et al. 2013), enzyme kinetic parameters (Heckmann et al. 2018), CRISPR guide efficacy (Chuai et al. 2018), optimal growth temperatures (Li et al. 2019) and pathway intermediate concentration (Lee et al. 2013), to name a few. ML has also been applied to systematically improve TRY. A precursor of ML, Principal Component Analysis (PCA), has been used to guide metabolic engineering. by mMapping proteomics to production. These through PCA led to recommendations led to producing a 40% increase of limonene and 200% of bisabolene in *E. coli* by (Alonso-Gutierrez et al. 2015). Another example involves a 200% increase of n-butanol production in *E. coli* by focusing on metabolomics data (Ohtake et al. 2017). Quadratic regressions have been used in combination with Design of Experiments (DOE) models to predict violacein production in *E. coli* and increase its production by 320% *(Xu, Rizzoni, et al. 2017)*. Violacein was also the product of choice to be optimized in *S. cerevisiae* through artificial neural networks, leveraging the data generated through the systematic MiYA YeastFab Assembly strategy, leading to a 240% increase in production (Zhou et al. 2018). Ensemble models have been used to relearn the Michaelis-Menten relationship purely from data for limonene and isopentenol producing *E. coli*, enabling actionable recommendations for their improvement (Costello and Martin 2018). Ensemble models have also been used to improve by 21% dodecanol production in *E. coli*, showing also some limitations of this data-driven approach (Opgenorth et al. 2019). Neural networks and a support vector regressor (SVR) have been used to fine tune the translational control of a limonene producing pathway in *E. coli* through the engineering of RBSs, increasing production by 60% (Jervis et al. 2019). Promoter choice in pathway design has been guided through the use of gaussian processes and ensemble models to improve lycopene production in *E. coli* (HamediRad et al. 2019) and tryptophan synthesis in *S. cerevisiae* (105% improvement in productivity) *(Zhang et al. 2019)*. Given the utility of machine learning in the field, new algorithms that quantify uncertainty

prediction and are specially designed for synthetic biology use cases are being created (Radivojević et al. 2019). In sum, machine learning shows a great potential for a more systematic metabolic engineering (Yadav et al. 2012), especially if combined with automation (Carbonell et al. 2019).

Engineering for scale-up

With the development of advanced robotics and molecular biology techniques, an array of tools have enabled high-throughput metabolic engineering, significantly reducing the time and effort required to achieve high flux to novel biofuel pathways (Dietrich et al. 2010; Choi et al. 2019; Marcellin and Nielsen 2018). Fermentation process development and scale-up have remained comparatively bespoke, creating a significant bottleneck for commercial deployment of advanced biofuel technologies (Crater and Lievense 2018; Wehrs et al. 2019). Due to the massive size of commercial biofuel fermentors - up to 500 m^3 for aerobic processes and 4,000 m^3 for anaerobic processes (Davis et al. 2018; Marcellin and Nielsen 2018) - overcoming scale-up challenges is of paramount importance for successful commercialization. Achieving consistently high yields and titers under production conditions necessitates precise control of process parameters including pH, substrate feed rate, dissolved oxygen, and in situ product removal. Such controls are not accommodated in simple batch cultivation - effective evaluation of new strains therefore requires scale-down cultivation in tightly controlled systems that effectively mimic industrial conditions to ensure process robustness and scalability. While conventional bioreactors remain labor intensive and low-throughput, improved robotics and low-cost sensors have enabled a new generation of bioreactor systems with capabilities nearing those of their full-scale counterparts, allowing automation and statistical design of experiments approaches to interrogate non-intuitive interactions between process variables. Microplate growth systems are now engineered to enable fed-batch operation, pH control, automated sampling, and real-time monitoring of pH, DO, and culture density (Yang et al. 2016; Cruz Bournazou et al. 2017; Gruber et al. 2017). Highly automated, disposable (Chang et al. 1997) bioreactors offer additional functionalities, including precise dissolved oxygen and feed control and continuous monitoring of process offgas (Tai et al. 2015). High-throughput chemostat bioreactors can target phenotypic response to slight variations in process conditions (Wong et al. 2018). When coupled with advanced computational fluid dynamics modeling (Haringa et al. 2017; Bach et al. 2017; Anane et al. 2019), such systems allow increasingly accurate scale-down testing and de-risking of industrial biofuel fermentations, even before the commercial bioreactor design is finalized.

In addition to tight process controls, the production environment features pressures, sheer rates, product titers, and spatial heterogeneities poorly reflected in small-batch cultivation. Microbial contamination and genetic instability also threaten process stability and robustness at scale, necessitating novel control mechanisms (Rugbjerg and Sommer 2019; Brexó and Sant'Ana 2017). While conventional antibiotics and antimicrobials are cost-prohibitive at scale, targeted application of sulfite (Shaw et al. 2016; Wang and Coates 2017; Chang et al. 1997), strain modification for chlorite resistance (Shaw et al. 2016; Wang and Coates 2017; Chang et al. 1997), and genetic modifications encoding affinity for xenobiotic nitrogen and phosphorus sources (Shaw et al. 2016) could potentially enable low-cost bioreactor hygiene control. Genetic stability can be enhanced by decoupling growth and production phases, eliminating the metabolic incentive to increase production of biomass at the expense of biofuel. A number of tools have emerged to facilitate this decoupling, utilizing nutrient limitation or low-cost induction agents suitable for application at scale (Ruegg et al. 2018; Dahl et al. 2013; Lo et al. 2016). High product titers often threaten productivity via toxicity and feedback inhibition - in addition to tolerance engineering approaches, these challenges may be overcome by process designs featuring in situ product removal (Dafoe and Daugulis 2014; Woodley et al. 2008).

In situ product recovery has been a critical component of recent scale-up success stories. Industrial production of farnesene has been facilitated by phase separation of the hydrophobic fuel molecules in extractive fermentation (Tsuruta et al. 2009). For bio-isobutanol, product toxicity was overcome at commercial scale via continuous solvent or gas stripping of product in recirculating anaerobic bioreactors (Xue et al. 2014).

Future Perspectives

Fuel properties and bio-advantaged fuels. Given ongoing improvements in metabolic engineering, host onboarding, and accelerated process development, we can now envision a future in which feedstocks and products are increasingly fungible. While earlier technologies targeted natural metabolites and high flux pathways to generate high yields and titers, reduced development costs could enable production of bio-advantaged fuels - molecules which leverage the exquisite specificity of enzymatic production to achieve favorable properties for production, separation, and optimal combustion. Leveraging the natural advantages of enzymatic synthesis enables deployment of fuel molecules with highly tunable properties unachievable via petrochemical routes - enabling potential improvements in octane/cetane number, melting point, energy density, and sooting tendency (Figure 2). A recent survey of

bioblendstocks for light duty gasoline engines utilized a computational merit function (Farrell et al. 2018) to generate a top 10 list of blendstocks likely to enable fuel efficiency exceeding that of E10 premium gasoline, including seven alcohols, cyclo-pentanone, di-isobutylene, and mixed furan derivatives (Gaspar 2019). These bioblendstocks may generate additional value via synergistic blending with lower cost fuel mixtures. For example, blending unsaturated C5 alcohols into base gasoline fuel reveals that the research octane number of the blended fuel significantly exceeds that of both neat components (Monroe et al. 2019). For diesel and jet fuels, the limited number of biological pathways to highly reduced, long-chain molecules has previously hindered efforts to create such bio-advantaged fuels. Straight-chain molecules generated via the fatty acid synthase pathway tend to suffer from high melting points and poor cold flow, while tuning of isoprenoid-derived fuels is limited to 5-carbon increments. Ongoing development of more flexible metabolic pathways to long-chain, highly reduced molecules, including polycyclic terpenes (Liu et al. 2018), non-canonical terpenes (Ignea et al. 2018), polyketides (Yuzawa, Mirsiaghi, et al. 2018; Curran et al. 2018), and functionalized aromatics (Huccetogullari et al. 2019) is needed. An emerging suite of modeling tools now enables precision targeting of both fuel properties and metabolic pathways to target bio-advantaged molecules, as predicted a priori of strain development by an emerging suite of modeling tools (Das et al. 2018; Whitmore et al. 2016; Saldana et al. 2011). This “fuel properties first” approach was recently demonstrated for catalytic upgrading of volatile fatty acids to hydrocarbons, enabling down-selection of prospective molecules to a branched C14 hydrocarbon that reduced overall sooting tendency by over 10% when blended to 20% volume in a base petroleum diesel (Huo et al. 2019).

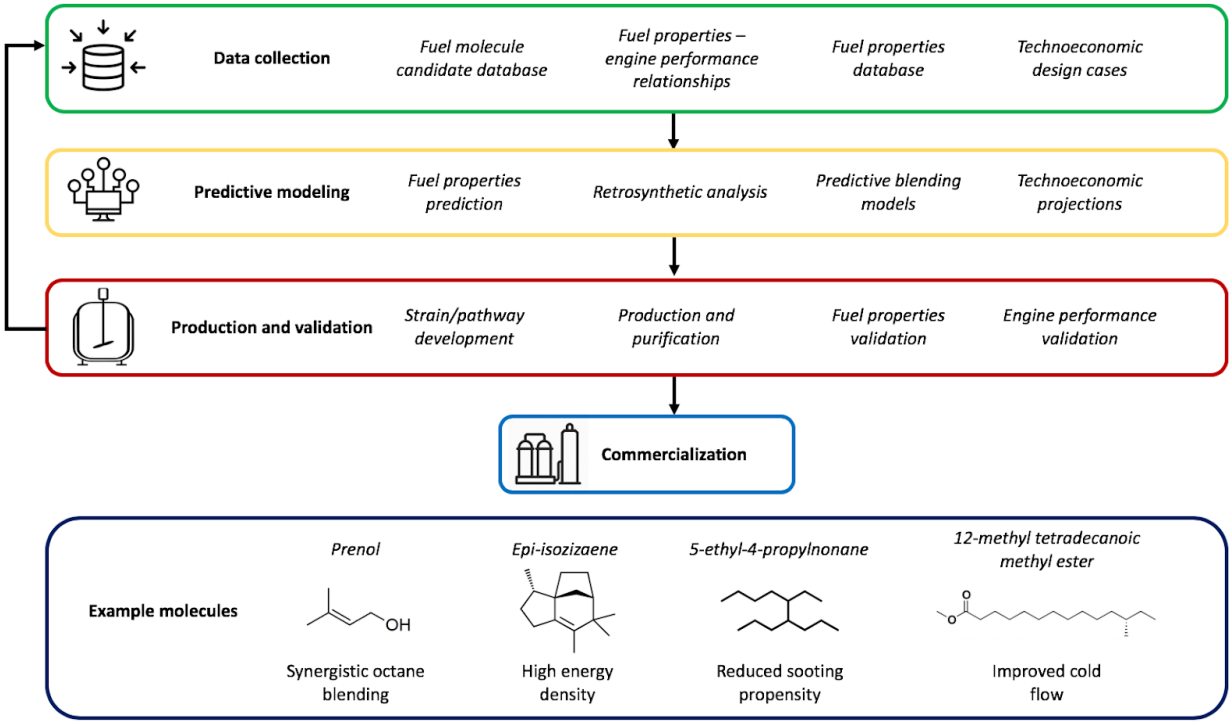

Figure 2. Idealized fuel-properties first approach for identification and screening of bio-advantaged fuels. High throughput computational models are leveraged to down-select candidate molecules for more resource intensive production and validation trials.

Fuel costs and the integrated biorefinery. The high cost of producing biofuels and the low cost of petroleum-based biofuels make biofuels a difficult sell. Besides improvements to engineered microorganisms so that they use all components in cellulosic biomass and lose as little carbon dioxide as possible (as outlined above), other ways to reduce the cost of biofuels is through the production of co-products, either in the plant that is the source of the lignocellulosic biomass for the biofuels (Yang et al. 2020; Lin and Eudes 2020) or by the same engineered microorganism that is producing the biofuel. Ideally, this co-product would somehow benefit biofuel production in addition to reducing the cost. As the volume of fuels needed greatly exceeds any one commodity chemical, it is likely that multiple co-products will be needed.

More and better microbial hosts. Finally, the carbon source for the biofuel and the process for producing the biofuel are highly integrated. Continued development of microbial hosts for biofuel production will be necessary to utilize waste products, not just from agriculture but also from municipalities, potentially including plastics, which will reduce costs. Further reductions in costs will come from producing the fuels under

non-sterile conditions, which will require engineered microorganisms that can withstand extreme conditions that few others can survive. The development of tools and pathways that will function in these hosts will keep microbial synthetic biologists busy for years to come.

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