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Microbial engineering for the production of advanced biofuels

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Advanced biofuels produced by microorganisms have similar properties to petroleum-based fuels, and can 'drop in' to the existing transportation infrastructure. However, producing these biofuels in yields high enough to be useful requires the engineering of the microorganism's metabolism. Such engineering is not based on just one specific feedstock or host organism. Data-driven and synthetic-biology approaches can be used to optimize both the host and pathways to maximize fuel production. Despite some success, challenges still need to be met to move advanced biofuels towards commercialization, and to compete with more conventional fuels.

Concerns about energy security, the global petroleum supply and climate change have increased interest in the production of sustainable and renewable liquid transport fuels[1,2]. The first-generation biofuels, ethanol and biodiesel, are the most widely used of these transport fuels[3]. The use of ethanol is due mainly to its high level of natural production by microbes, through fermentation of cornflour (in the United States) or cane sugar (in Brazil), and not because it is optimal for our existing petroleum-centric transport infrastructure. Ethanol has only 70% of the energy content of gasoline (petrol), it has a high tendency to absorb water from the air, which leads to corrosion in engines and pipes; and its distillation from the fermentation broth is energy intensive[1,4]. Biodiesel is the main biofuel in Europe, and is produced by the transesterification of vegetable oil or animal fats with methanol, but this also has its own limitations: it has only 91% of the energy content of D2 diesel and, because wax can form in the fuel if the temperature is too low, it is difficult to transport with the current distribution infrastructure, so there are geographical limits to its use.

Producing a high volume of first-generation biofuels from food crops creates a link between food and fuel prices. Instead of using starch and sucrose, biofuel production could come from more abundant and underused resources such as lignocellulose, algal biomass and greenhouse gases such as carbon monoxide and carbon dioxide. The non-food energy feedstock lignocellulose is the most abundant biomass on Earth, and consists of about 70% sugars. These sugars, however, require thermal, chemical and biochemical processes before they can be released for microbial fermentation. Algal biomass is a compelling alternative because it can be produced in salt water rather than on arable land, but its collection and dewatering is challenging. Atmospheric CO_2 can be transformed into biofuels through carbon fixation using engineered photosynthetic organisms. Carbon monoxide, from the thermal conversion of lignocellulosic biomass and abundant in steel-mill flu gas, can also be metabolized by microorganisms.

Progress in metabolic engineering, and synthetic and systems biology, have allowed the engineering of microbes to produce advanced biofuels with similar properties to petroleum-based fuels[5,6] (Table 1). When developing an organism or a pathway to produce an advanced fuel, factors including engine type (spark or compression ignition), energy content, combustion quality or ignition delay, cloud point, volatility, lubricity, viscosity, stability, odour, toxicity, water miscibility and cost must be considered[7,8]. The main challenge in using native hosts to convert feedstocks into advanced biofuels is to overcome the endogenous regulation of biofuel-producing pathways to achieve high yields. Reconstruction of advanced biofuel pathways in genetically tractable heterologous hosts, such as *Escherichia coli* and *Saccharomyces cerevisiae*, can work, but this approach presents its own challenges in balancing the enzyme activities and expression to maximize metabolic flux. Less genetically tractable hosts that have high biofuel tolerance or the ability to use non-sugar substrates are interesting alternatives because different feedstocks can be used. In this Review, we discuss the metabolic engineering of the pathways used in the production of advanced biofuels, irrespective of the feedstock and host organism, review data- and synthetic-biology-driven approaches for host and pathway optimization and discuss future directions in the microbial production of advanced biofuels (Fig. 1).

Pathways for alcohol-derived fuels

Short-chain or higher alcohols can be added to gasoline as oxygenates or, in some cases, to replace gasoline altogether. Butanol, for example, has 84% of the energy content of gasoline, limited miscibility with water and is completely miscible with gasoline[9]. In an effort to improve butanol production, species of the natural host *Clostridium* have been engineered to use feedstocks such as glucose[10], liquefied cornflour[11], glycerol (a by-product in the production of biodiesel from fats)[12] and even syngas (a mixture of hydrogen and carbon monoxide)[13]. However, butanol yields in this strict anaerobe seem to be limited by its slow growth rate and intolerance to butanol above 13 g l^{-1}, as well as our limited understanding of its physiology and genetics, and the paucity of synthetic-biology tools to engineer it[9,14,15].

Some of *Clostridium's* limitations can be overcome by the introduction of its butanol pathway into organisms that grow faster, can tolerate high concentrations of butanol or can metabolize alternative feedstocks. *E. coli* has a high growth rate[16]; *S. cerevisiae* has a high tolerance to ethanol and potentially to butanol[17]; *Pseudomonas putida* can overcome toxicity using efflux pumps; *Bacillus subtilis* can change its cell-wall composition in response to solvent toxicity[18]; *Lactobacillus brevis* digests C_5 and C_6 substrates and has a high tolerance to butanol[19]; and the cyanobacterium *Synechococcus elongatus* is able to produce butanol from CO_2 by photosynthesis[20].

Regardless of the host organism, high-titre production of butanol is limited by the reversibility and cofactor specificity of the enzymes

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in the *Clostridium* pathway (Fig. 2). However, this barrier to high butanol production was broken in *E. coli* by using a synthetic butanol pathway that combines enzymes from four different organisms chosen for their biochemical characteristics. Rather than using *Clostridium*'s reversible, flavin-dependent, oxygen-sensitive butyryl-CoA dehydrogenase complex, the synthetic pathway used biosynthetic NADH-dependent *trans*-enoyl-CoA reductase[21]. Increases in NADH, acetyl-CoA and ATP pools by deleting competing pathways and stripping the butanol from the reactor as it is produced have produced 30 $g\,l^{-1}$ of butanol in fermenters[22]. The reversibility of the first step in the *Clostridium* butanol pathway has also been addressed. To favour the production of acetoacetyl-CoA (Fig. 2), instead of thiolase, acetyl-CoA decarboxylase driven by ATP and using CO_2 was used to generate malonyl-CoA and coupled to acetoacetyl-CoA synthase to produce acetoacetyl-CoA releasing CO_2 (ref. 20).

An alternative route for the production of alcohols, including butanol, is the Ehrlich, or 2-keto-acid, pathway. This pathway decarboxylates keto acids, the immediate amino-acid precursors, into aldehydes and reduces them to alcohols. The Ehrlich pathway is endogenous to some yeast species, in which the by-products of fermentation are fusel alcohols. Amino-acid-based alcohols, such as *n*-propanol from isoleucine, isobutanol from valine and *n*-butanol from norvaline, can be produced using this pathway[23]. Of these, isobutanol is the closest to industrial use, and although it has a similar energy content to *n*-butanol, its branching gives it improved properties, such as a better octane number (a measure of a fuel's resistance to knocking in spark ignition engines).

Introducing a promiscuous 2-keto-acid decarboxylase and an alcohol dehydrogenase into *E. coli* can produce isobutanol in high yields through the 2-keto-acid pathway[4]. Overexpression of 2-ketoisovalerate biosynthetic genes, deletion of multiple pathways competing for pyruvate consumption and the replacement of the endogenous acetolactate synthase enzyme with one from *B. subtilis* — which has a higher specificity for pyruvate — resulted in 22 $g\,l^{-1}$ of isobutanol. The 2-keto-acid pathway has also been engineered in *Corynebacterium glutamicum*, a bacterium known for its high amino-acid production[24]; *S. cerevisiae*, in which the endogenous 2-ketoisovalerate biosynthetic pathway was upregulated and localized to the cytosol[25]; *Clostridium acetobutylicum*, a cellulolytic bacterium that produces isobutanol directly from crystalline cellulose through consolidated bioprocessing[26]; *S. elongatus*, a photosynthetic bacterium[27]; and *E. coli* engineered to metabolize protein-rich substrates such as the potentially abundant feedstock algal hydrolysate[28]. Isobutanol has also been produced using CO_2 obtained from electrochemically produced formate as the energy source for *Cupriavidus necator* (also known as *Ralstonia eutropha*)[29].

Pathways for isoprenoid-derived fuels

A class of compounds widely used as flavours and pharmaceuticals[30], isoprenoids have the potential to serve as advanced biofuels because of the branches and rings found in their hydrocarbon chain[7,8]. The branching means there are more tertiary carbon atoms to stabilize pressure-induced radicals, reducing premature ignition and increasing the octane number. The isoprenoid-based alcohol isopentanol is a potential

Table 1 | Liquid transportation fuel properties and potential biofuels

Fuel type	Major components	Properties	Potential advanced biofuels
Gasoline	C_4–C_{12} hydrocarbons Linear, branched, cyclic aromatics	Octane number (87–91) Energy content	Butanol, isobutanol, short-chain alcohols, short branched-chain alkanes
Diesel	C_9–C_{23} hydrocarbons Linear, branched, cyclic aromatics	Cetane number (40–60) Good cold properties	Fatty alcohols, alkanes, linear or cyclic isoprenoids
Jet fuel	C_8–C_{16} hydrocarbons Linear, branched, cyclic aromatics	Heat density Very low freezing temperature	Branched alkanes, linear or cyclic isoprenoids

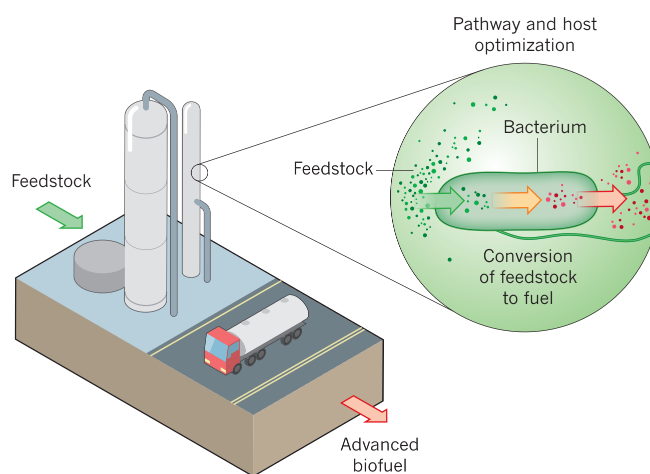

Figure 1 | The production of advanced biofuels. Engineering of bacteria or yeast species through data- or synthetic-biology-driven techniques optimizes the production of advanced biofuels, such as butanol, farnesane and bisabolane from feedstocks (ideally non-food resources such as lignocellulosic biomass, greenhouse gases and algal biomass).

substitute for gasoline. Although branching lowers the cetane number (a measure of combustion quality in compression engines) in diesels, having a few branches in an otherwise straight-chain alkane disrupts stacking and lowers the freezing point. The C_{15} isoprenoids farnesane and bisabolane have cetane numbers (of 58 and 52, respectively) that fall within the expected range for diesel (40–60). Jet-fuel replacements require high-energy content, which can be achieved by the presence of constrained ring structures, such as those found in pinene dimers[31].

Isoprenoid-based biofuels are produced from two universal C_5 precursors, isopentenyl diphosphate and dimethylallyl diphosphate[32], using either the mevalonate or deoxyxylulose-5-phosphate pathways (Fig. 3). Plants are natural sources of isoprenoids, but they do not produce large enough quantities to support biofuel production. Algae, such as *Botryococcus braunii*, produce large quantities, but they grow slowly and produce large amounts of fatty acids, rendering an extract akin to a biocrude, which would require cracking to form biofuels[33,34]. To increase the quantities produced, the deoxyxylulose-5-phosphate and mevalonate pathways (Fig. 3) can be deregulated or overexpressed in their native *E. coli* and *S. cerevisiae*, respectively, and introduced heterologously into these microorganisms[35–40]. Engineering of the mevalonate pathway resulted in 27 $g\,l^{-1}$ of the C_{15} amorphadiene in *E. coli*[37] and 40 $g\,l^{-1}$ in *S. cerevisiae*[38].

Of the isoprenoid-based biofuels, farnesane is the closest to commercialization[41,42]. The renewable products company Amyris based in Emeryville, California, uses the industrial yeast strain *S. cerevisiae* PE-2 to produce farnesene[43]. This is then chemically hydrogenated to farnesane, which is being evaluated as a high-performance advanced biofuel[41]. Bisabolane is another potential biofuel produced through a hybrid process, using microbial catalysis for bisabolene overproduction then chemical catalysis for reduction into bisabolane[44]. To produce bisabolene in *E. coli*, five plant isoprenoid synthases were screened for efficient conversion of farnesyl diphosphate into bisabolene, and the protein expression of the best one was improved using codon optimization for *E. coli*. The levels of the farnesyl diphosphate precursor were increased by codon-optimizing heterologous mevalonate pathway genes and by introducing a second promoter to upregulate transcription of bottleneck genes. Bisabolene was also produced using *S. cerevisiae*, and production by both organisms resulted in more than 900 $mg\,l^{-1}$ (ref. 44). The discovery of bisabolene synthase as a flux-controlling enzyme, prompted the identification of the crystal structure of the most efficient bisabolene synthase[45] — from *Abies grandis* — to aid in its engineering for increased microbial bisabolene production.

Isopentenol and pinene can be chemically converted into isopentanol

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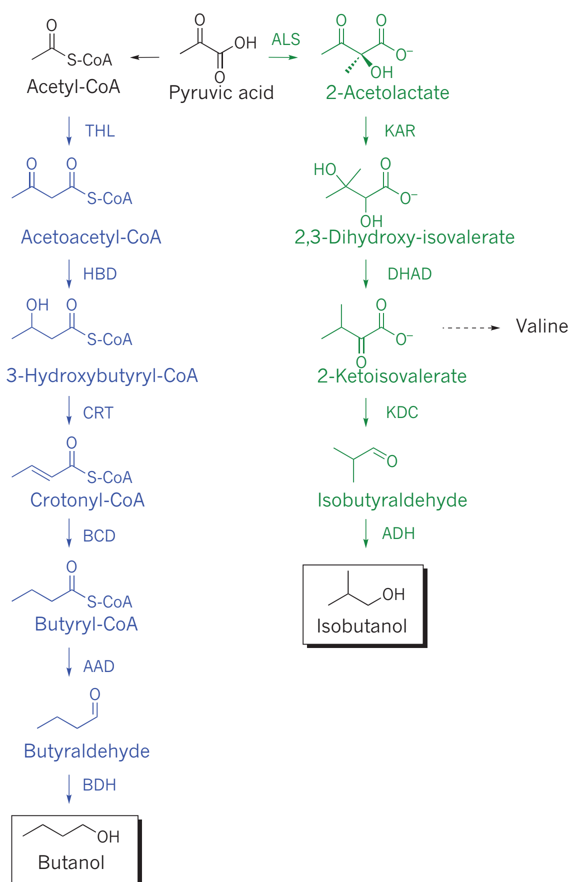

Figure 2 | Metabolic pathways used for the production of alcohol-based biofuels. In blue, *Clostridium's* butanol pathway converts acetyl-CoA into butanol. AAD, butyrladehyde dehydrogenase; BCD, butyryl-CoA dehydrogenase; BDH, butanol dehydrogenase; CRT, crotonase; HBD, 3-hydroxybutyryl-CoA dehydrogenase; THL, thiolase. In green, the 2-keto acid pathway produces isobutanol from pyruvic acid. ADH, alcohol dehydrogenase; ALS, acetolactate synthase; DHAD, dihydroxy-acid dehydratase; KAR, ketol-acid reductoisomerase; KDC, keto-acid decarboxylase.

and pinene dimers[31]. Isopentenol was attained through the dephosphorylation of isopentenyl diphosphate using a phosphatase identified by screening a library of *B. subtilis* complementary DNA[46]. Pinene production in *E. coli* was achieved by re-routing isopentenyl diphosphate and dimethylallyl diphosphate using a geranyl diphosphate synthase and a pinene synthase (Fig. 3). The pinene-specific enzymes were introduced into an *E. coli* strain able to metabolize both xylose and cellulose in a co-culture to produce pinene at 1.5 mg l^{-1} from ionic-liquid-treated switchgrass as the sole carbon source[47].

Pathways for fatty-acid-derived fuels

Fatty acids in the form of phosphoglycerides and triglycerides are the main components of cell membranes, vegetable oils and animal fats, and their hydrophobic acyl chains can be used to produce biofuels. Technology to exploit fatty-acid metabolism is producing an expanded repertoire of fatty-acid-derived chemicals, many of which seem to be suitable alternatives to diesel.

Fatty acids are biosynthesized naturally by a large, multienzyme system called fatty-acid synthase (FAS), using malonyl-CoA as a building block (Fig. 4). Fatty acyl chains are elongated on acyl carrier proteins (ACPs) through repeated cycles of decarboxylative condensation, β-keto reduction, dehydration and enol reduction. Long-chain fatty acids can be released from the ACP by thioesterase-catalysed hydrolysis. The overexpression of a truncated, cytosolic thioesterase from bacteria[48] or plants[49,50] in *E. coli* produces fatty acids in the range of C_8 to C_{18}, depending on the thioesterase expressed. Hydrolysis of acyl-ACP is thought to relieve the feedback inhibition of FAS by the acyl-ACP[51], allowing efficient turnover of FAS and significant overproduction of fatty acids. In combination with the deletion of genes in the fatty-acid degradation pathway (β-oxidation), free fatty acids have been produced at high titres (1.2 g l^{-1}) (ref. 48), providing a large precursor pool for further conversion to advanced diesel fuel.

Because of the ionic nature of their carboxyl group, fatty acids cannot be used directly as biofuel, but they can be readily converted into non-ionic, hydrophobic molecules, such as fatty alcohols, fatty-acid alkyl esters, alkenes and alkanes. Fatty alcohols, a potential biofuel[7], have been biosynthesized at up to 60 mg l^{-1} by the expression of an acyl-CoA synthase to activate fatty acids to acyl-CoAs, followed by reduction by an acyl-CoA reductase through an aldehyde intermediate[48,52]. An acyl-ACP reductase that produces fatty aldehydes and alcohols directly when expressed in *E. coli*[53] has now been discovered, eliminating the need to go through the fatty-acid intermediate.

Methyl ketones in the C_{11} to C_{15} range have favourable cetane numbers and have been produced in engineered *E. coli* at 380 mg l^{-1} (ref. 54). In this pathway, the first three steps of β-oxidation are used to convert fatty acids into β-ketoacyl-CoAs, which are later hydrolysed by a thioesterase to form β-keto-fatty acids, followed by decarboxylation to yield methyl ketones. In addition, fatty-acid methyl and ethyl esters (FAMEs and FAEEs) — the main constituents of biodiesel used in trucks, buses and trains — have been biosynthesized at 1.5 g l^{-1} (ref. 55) by the activation of fatty acids to acyl-CoA, followed by ethanol esterification using a wax-ester synthase[48,56]. Alternatively, fatty acids can be methylated by *S*-adenosylmethionine to produce FAMEs[57] in one step using a fatty-acid methyltransferase. However, high-titre production of FAMEs from this pathway may be challenging because *S*-adenosylmethionine must be regenerated efficiently.

Alkanes are the main molecules in diesel fuel. Although some cyanobacteria naturally produce low levels of long-chain alkanes[53], the discovery of a biosynthetic pathway has allowed their heterologous production[58]. This has created an opportunity to produce long-chain alkanes directly from renewable materials in a single fermentation step and without the need for further chemical modification, such as hydrogenation. Alkane biosynthesis requires acyl-ACP reductase to catalyse the reduction of acyl-ACPs to form fatty aldehydes, and aldehyde decarbonylase to catalyse the decarbonylation of the aldehydes to formic acid and alkanes (or alkenes if the starting acyl groups are unsaturated). At least two other pathways exist for the biosynthesis of terminal alkenes. The first uses a cytochrome P450 enzyme that catalyses a decarboxylative oxidation to convert fatty acids to terminal alkenes[59]. The second involves a polyketide synthase, and produces terminal alkenes through a sulphonation-assisted decarboxylation[60]. A detailed description of the enzymatic mechanism has yet to be reported, and the pathway needs to be optimized to improve efficiency and yield. Finally, the production of long-chain (C_{24}–C_{31}) alkenes by a head-to-head condensation of two fatty acids followed by several reduction steps is also possible[61,62]. However, these long-chain hydrocarbons are waxes and would require further chemical processing, such as hydrocracking or reforming, before being used as fuels.

The building block of FAS-catalysed fatty-acid biosynthesis, malonyl-CoA, is converted from acetyl-CoA and bicarbonate by a multisubunit acetyl-CoA carboxylase. In prokaryotes and eukaryotes, this enzyme is tightly regulated at the transcriptional, translational[63] and post-translational[64] levels, leading to low cellular concentrations of malonyl-CoA. Furthermore, carboxylation of acetyl-CoA to malonyl-CoA consumes one ATP, and the incorporated CO_2 is returned to the solvent on decarboxylative Claisen condensation catalysed by 3-keto-acyl-ACP synthase. Biochemical Claisen condensations, such as those involved in the first step of butanol and polyhydroxyalkanoate biosynthesis, do not involve a decarboxylative mechanism. The consumption of energy and the regulation associated with acetyl-CoA carboxylase might be avoided

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by synthesizing alkyl chains by running β-oxidation, or similar reactions, in reverse[65,66]. Reversing the β-oxidation cycle has been used to produce a diversity of C_4–C_{12} fatty species[67] by overexpressing several fatty-acid degradation enzymes in combination with an unconventional approach of global deregulation and strategic pathway knockouts. The results suggested high levels of butanol and fatty acids could be produced (roughly 7 g l^{-1} of fatty acids and about 14 g l^{-1} of *n*-butanol); however, which of the many deregulated enzymes are responsible for the products and how much product was derived from acetyl-CoA compared with malonyl-CoA remains unclear. If this approach is as useful as it seems, it could pave the way for anaerobic production of higher alkyl-chain products.

Pathways for polyketide-derived fuels

The isoprenoid and fatty-acid biosynthetic pathways produce a variety of regularly branched, cyclic and linear hydrocarbons, but the degree

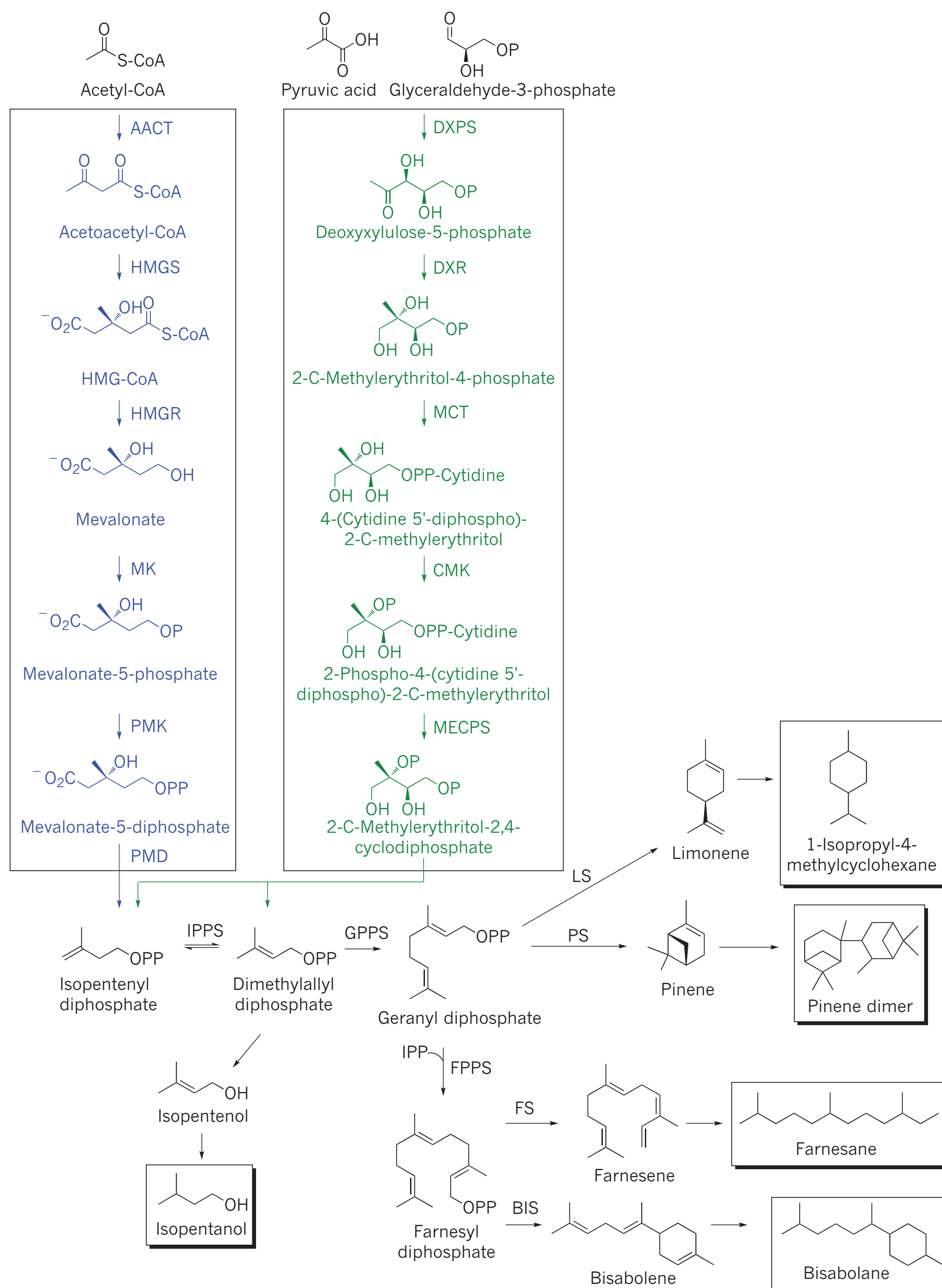

Figure 3 | Metabolic pathways used for the production of isoprenoid-based biofuels. In blue is the mevalonate pathway and in green is the deoxyxylulose-5-phosphate (DXP) pathway. AACT, acetyl-CoA transferase; BIS, bisabolene synthase; CMK, 4-(cytidine-5′-diphospho)-2-C-methylerythritol kinase; DXPS, DXP synthase; DXR, DXP reductoisomerase; FPPS, farnesyl diphosphate synthase; FS, farnesene synthase; GPPS, geranyl diphosphate synthase; HMGR, HMG-CoA reductase; HMGS, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase; IPP, isopentenyl diphosphate; IPPS, IPP isomerase; LS, limonene synthase; MCT, 2-C-methylerythritol-4-phosphate cytidyltransferase; MECPS, 2-C-methylerythritol-2,4-cyclodiphosphate synthase; MK, mevalonate kinase; PMD, phosphomevalonate decarboxylase; PMK, phosphomevalonate kinase; PS, pinene synthase.

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to which the hydrocarbons can be tailored is limited. The polyketide biosynthetic pathway is the most versatile for producing hydrocarbons with diverse structures, but it is relatively unexplored for fuels. This diversity means that they have been used as antibiotics, insecticides and antitumour agents[68]. Moreover, reduced polyketides are a good source of hydrocarbons that can produce biofuels as a derivative. They are biosynthesized through the decarboxylative condensation of malonyl-CoA by multidomain polyketide synthases, which share striking similarities with FASs. Polyketide synthases have been studied extensively over the past 30 years, creating an opportunity for engineering them to produce biofuels[69] (Fig. 4).

Modular type I polyketide synthases are the most extensively engineered[68,70], in part because they use different catalytic domains for each round of chain extension, providing the best control over polyketide structures. Each polyketide synthase contains several modules, a set of catalytic domains that are responsible for one round of polyketide chain extension and sub-sequence modification of the synthesized β-ketone. A downstream module can often process non-natural intermediates from the previous module, allowing the engineering of a modular type I polyketide synthase by several means: changing the precursor used for polyketide biosynthesis through the manipulation of the acyltransferase domains[71]; altering the structure of each extension unit by domain mutation, substitution and insertion within each module[72]; and changing the chain length of polyketides by module deletion, substitution or *de novo* design[70].

Optimization of hosts and pathways

High yields and productivities are crucial for the economical production of biofuels. Data-driven and synthetic-biology approaches for host and pathway optimization are showing promise in achieving these goals.

Data-driven approaches have the potential to streamline the engineering of microorganisms for the production of fuels and chemicals, possibly reducing the number of constructs needed to optimize a biofuel-producing organism. Functional-genomics approaches are well suited to identifying the rate-limiting steps in biosynthetic pathways, such as using targeted proteomics to identify two enzymes in the heterologous mevalonate pathway that were poorly expressed[73]. Increasing the enzyme expression levels through codon optimization of the relevant genes, in addition to increasing messenger RNA levels by the introduction of an additional 5′ promoter to the most poorly expressed gene, led to a three-fold improvement in sesquiterpene production[73]. By contrast, computer modelling of an organism's metabolism may be best suited to identifying off-pathway targets to increase biofuel production. For example, a metabolic model suggested that deletion of the NADPH-dependent glutamate dehydrogenase would lead to a ten-fold increase in the production of sesquiterpenes in *S. cerevisiae*[74]. Deleting the predicted gene, which increased the availability of NADPH for an important mevalonate pathway enzyme, nearly doubled sesquiterpene production[74]. Systems-biology approaches look beyond metabolism, identifying molecular mechanisms that could be applied to enhance biofuel production, and have been used to identify efflux pumps that

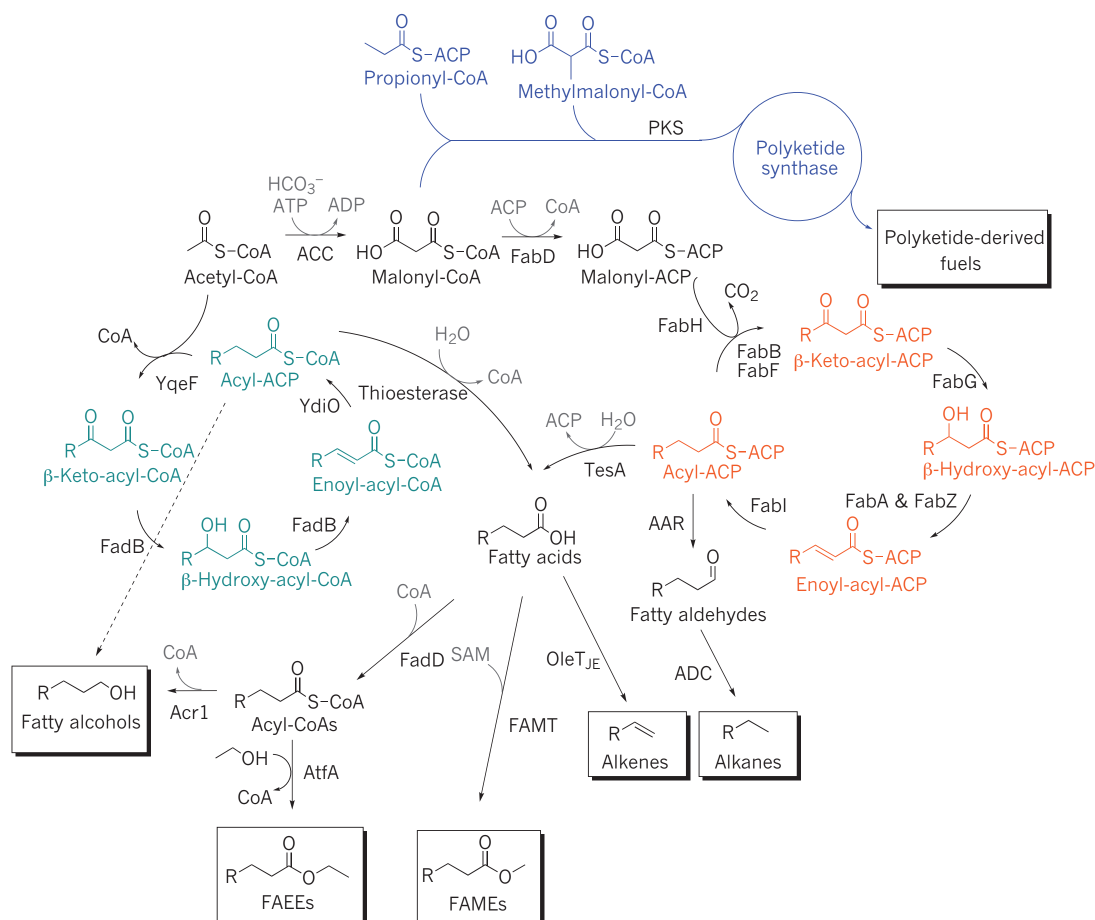

Figure 4 | Metabolic pathways used for the production of fatty-acid- and polyketide-derived biofuels. The fatty-acid biosynthetic cycle is in red, the reversal of the β-oxidation cycle is in green and polyketide synthase is in blue. AAR, acyl-ACP reductase; ACC, acetyl-CoA carboxylase; Acr1, acyl-CoA reductase; ADC, aldehyde decarbonylase; AtfA, wax-ester synthase; FabB, β-keto-acyl-ACP synthase I; FabD, malonyl-CoA:ACP transacylase; FabF, β-keto-acyl-ACP synthase II; FabG, β-keto-acyl-ACP reductase; FabH, β-keto-acyl-ACP synthase III; FabA & FabZ, β-hydroxyacyl-ACP dehydratase; FabI, enoyl-acyl-ACP reductase; FadB, enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase; FadD, acyl-CoA synthase; FAMT, fatty acid methyltransferase; $OleT_{JE}$, *Jeotgalicoccus* sp terminal olefin-forming fatty-acid decarboxylase; PKS, polyketide synthase; TesA, acyl-ACP thioesterase; YdiO, enoyl-CoA reductase; YqeF, thiolase.

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could aid in the production of biofuels that are toxic to the cell. Of the 43 potential biofuel efflux pumps that were identified from sequenced bacterial genomes, the expression of one pump improved *E. coli* survival in the presence of limonene, a potential biofuel precursor, and increased yield[75].

An extensively tested approach is to control flux of the biosynthetic pathways directly at the transcriptional, translational and post-translational levels (Fig. 5). Metabolic pathways can be controlled in several ways. The number of copies of a particular gene can be manipulated by increasing how many are integrated into the genome[76] or by placing the gene in a vector with high or low copy number[77]. The strength of constitutive and inducible promoters can be chosen to match the desired transcription initiation rate[78], and orthogonal, inducible promoters can be used to regulate parts of pathways, or multiple pathways independently[79]. Synthetic terminators can be engineered to control transcription termination efficiencies[80]. The metabolic pathway can be modulated at the translational level by inserting functional RNA segments into intergenic regions of operons to regulate the processing[81] and stability[82] of mRNAs that encode individual pathway enzymes. The strength of ribosome-binding sites can be predicted and designed using computational methods, allowing the control of the protein translation efficiencies[83]. Enzyme stability can be altered through the control of protein degradation rate using peptide tags[84].

These controls are effective in the engineered pathways, which are optimized on the basis of an initial set of conditions. However, the conditions may change when used in bioreactors and the control system cannot respond, potentially leading to suboptimal production of biofuels[85]. To address this, researchers have developed a dynamic sensor-regulator system (DSRS) that can adjust the metabolic pathway according to the metabolic status of the host and regulate the production of FAEE in engineered *E. coli*[55]. In this strategy, a biosensor that detects the cellular concentration of an intermediate acyl-CoA was used, and its cognate regulators — promoters responsive to the sensor — were engineered to control the expression of genes involved in FAEE biosynthesis. The DSRS regulated both the production and the consumption of pathway intermediates dynamically, according to the cellular concentration of acyl-CoA. As a result, the DSRS not only increased FAEE yield by threefold, but also reduced the cellular concentration of toxic intermediates and improved the genetic stability of FAEE-producing strains. Similar sensor-regulator systems that exert control at the translational level[86] can be developed to dynamically regulate other heterologous pathways.

Enzyme activity is a common bottleneck in the engineering of metabolic pathways. Many native enzymes are allosterically regulated by metabolites at the post-translational level; for example, *S. cerevisiae* mevalonate kinase is inhibited by several downstream intermediates, including farnesyl diphosphate. To overproduce farnesyl diphosphate, feedback-inhibition resistant mutants or homologues, such as mevalonate kinase from the archaeon *Methanosarcina mazei*[87], can be used to increase pathway flux. Furthermore, synthetic protein scaffolds could be used to spatially assemble metabolic enzymes and to catalyse multistep reactions synergistically[88]. When this system was applied to the heterologous mevalonate pathway in *E. coli*, mevalonate production was increased 77-fold, even though less protein was produced.

Moving to commercialization

To make the innovations in this Review sustainable, commercialization on a massive scale is needed. Advanced biofuels must be economically competitive with existing products, overcoming the primary economic drivers of feedstock price, and overall process productivity and yield. Although many of the products described have suitable performance characteristics, the potential yield for each product is limited by the theoretical yield of the production pathway[42], and this limit sets a bottom price that a product can achieve for a feedstock. Commercialization requires advancing laboratory-scale processes to yields and productivities that approach the theoretical (generally this will be 85% of what is theoretically possible) and scaling them up to reactors that will be

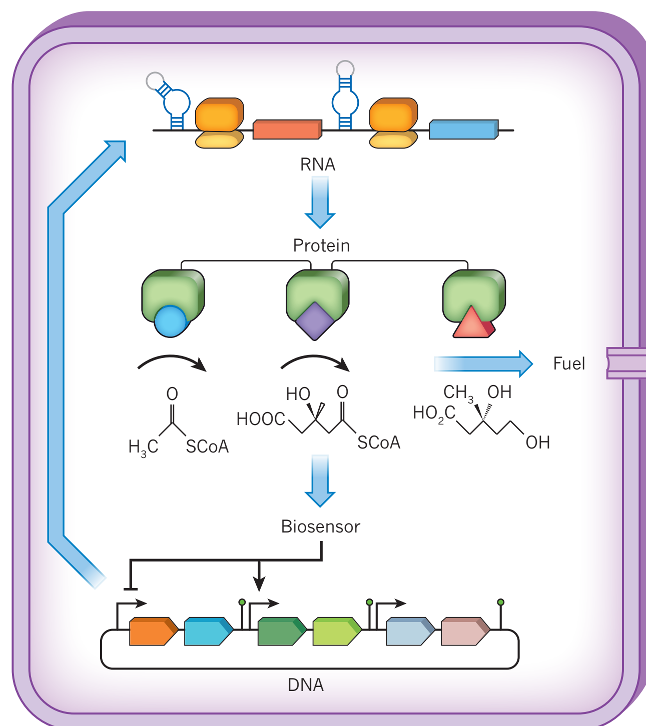

Figure 5 | Synthetic biology tools are used to maximize the production of biofuels from hosts. In DNA, genes involved in the synthesis of fuel can be introduced at the desired numbers into the host genome. Promoter (black arrows) and terminator sequences (green pins) can be engineered to control transcription rates. In the translation process, ribozymes (hairpins) can be inserted into functional RNA segments between the genes and ribosome binding sites can be engineered to control transcription initiation rates. Synthetic protein scaffolds incorporated into the pathway spatially assemble enzymes to efficiently transfer intermediates from one enzyme to the next in the pathway, increasing metabolic flux. Biosensors can detect specific metabolites in the host and regulate either transcription or translation dynamically, and the production of intermediates. Cellular pumps transport the fuel outside the cell (pumps).

more than 600 m^3 (roughly a million times larger than most lab-scale fermentations). The greatest challenges to commercialization are engineering catalysts to reach the yields and productivities required to meet economic targets, and scaling these processes without losing performance. Many of the advanced biofuels in the pipeline are stuck in an expensive purgatory, with only a few technologies for higher alcohols, butanol and isobutanol reaching the later phases of commercialization. These technologies are anaerobic processes that can be used in existing ethanol facilities, and have products that are naturally produced or engineered pathways linked to microbial fermentative growth[89,90]. Despite tremendous investments in isoprenoid and fatty-acid-derived products, commercial success has yet to be demonstrated. As these technologies become more refined, the more they differ with regard to industrial host, process and product trade-offs, and feedstock. Indeed, the closer commercialization becomes, the more crucial is the choice of host.

Yeast's robustness, our extensive fermentation knowledge, the availability of genetic tools, its tolerance to industrial conditions and solvents (to butanol this is more than 20 $g\,l^{-1}$), its low medium pH and its lack of susceptibility to bacteriophage[91,92] make it the preferred organism for the production of butanol and isoprenoid-derived biofuels[91]. However, it is unable to digest the five-carbon sugars present in lignocellulosic biomass, such as xylose and arabinose; and its natural ability to produce ethanol can hinder metabolic engineering efforts to produce advanced biofuels. In addition, there are few synthetic biology tools for pathway optimization, and its lower protein expression levels compared with *E. coli* can limit the flux through biofuel producing pathways.

At least three renewable biofuel enterprises are pursuing

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higher-alcohol production in yeast using slightly different strategies. Gevo based in Englewood, Colorado, has linked the production of isobutanol to anaerobic growth, and selected for strains that approach theoretical yields[89]. This, in combination with stripping the isobutanol from the broth through continuous flash evaporation, has resulted in more than 90% of the theoretical yield and a commercial-scale plant. Butamax in Wilmington, Delaware, has constructed various metabolic pathways that lead to butanol[93]. And Butalco in Fuerigen, Switzerland, has developed the technology to construct strains of yeast that can metabolize five-carbon sugars, and proposes using only endogenous genes to improve isobutanol production[94].

Exploitation of fatty-acid metabolism is being pursued in a variety of host organisms. Fatty-acid-derived compounds, such as FAMEs, fatty alcohols, alkanes and olefins, can be produced from *E. coli* in single-step fermentation from carbohydrate. LS9 in South San Francisco, California, chose *E. coli* to exploit because of its exceptionally high rate of fatty-acid biosynthesis (0.3 g l^{-1} per hour per gram of dry cell weight, based on the 30-minute doubling time and 9.7% lipid content of cell mass), its natural ability to secrete the compounds and consume both five-carbon and six-carbon sugars, the extensive industrial precedent in the commercial production of metabolically engineered small molecules (such as 1,3-propanediol, lysine and phenylalanine) and its ease of engineering. But, *E. coli* does have limitations, such as a preference for neutral pH and susceptibility to bacteriophages. Using cyanobacteria for the production of fatty-acid-derived compounds from CO_2 in photobioreactors is another option. The production of biodiesel and the renewable diesel precursor triacyl glyceride[95] have gravitated towards oleaginous algae; these GRAS (generally recognized as safe, classification by the US Food and Drug Administration) organisms naturally produce high levels of intracellular oil in both photobioreactors and heterotrophic fermentations[96]. However, until these technologies begin producing fuels at competitive prices, the jury is out on whether there will be widespread adoption.

Future directions

The past two years have seen advances in the use of different, inexpensive non-food feedstocks, and the creative engineering of metabolic pathways and enzymes for the production of advanced biofuels. The feasibility of these biofuel production pathways has been shown by the conversion of syngas, CO_2, algal hydrolysate and switchgrass into higher alcohols, fatty acids and isoprenoid-derived biofuels. Two years ago, the question would simply have been: which advanced biofuel can be made from glucose in high yield? But now researchers are also asking which feedstock can cost-effectively produce advanced biofuels in high yield. The challenge lies in producing a high yield of advanced biofuels, requiring the engineering of both the substrate use and advanced biofuel-producing pathways.

Advances in the production of alcohol-derived biofuels came from improving the kinetics of metabolic-pathway enzymes and generating metabolic driving forces to increase the flux through the pathways. Although biochemical characterization of metabolic-pathway enzymes is laborious, determining their biochemical parameters has allowed major breakthroughs in the production of biofuel. For example, *in vitro* reconstitution of the *E. coli* FAS determined the limits of the pathway for fatty-acid production[97]. For isoprenoid-derived biofuels, the effective union between biocatalysis and chemical catalysis has produced advanced biofuels, but the extensive in-pathway regulation by allosteric enzymes that respond to metabolic intermediates still needs to be overcome. Protein engineering could be used to remove or engineer the desired allostery into metabolic-pathway enzymes. Advances in chemical catalysis to allow the inexpensive conversion of biological compounds into fuels will help to make these a competitive alternative to conventional products.

Engineering the fatty-acid pathway has led to the production of several types of biofuel with different physical and combustion properties. Although the fuel properties of fatty alkanes are the most similar to conventional diesel fuel, the increased degree of unsaturation in alkenes may cause the formation of radicals during combustion. By contrast, oxygen-containing fatty methyl ketone and fatty-acid alkyl esters have higher cetane numbers and flashpoints, endowing shorter ignition delay and safer transport, respectively, but they have lower energy content and higher cloud points, which limits their use at very cold temperatures. Combining different types of fatty-acid-derived biofuel may allow fine-tuning for optimal fuel properties. Engineering the substrate specificity of FASs and the downstream biosynthetic pathways to alter length, functionality, and the degree of fatty-acid saturation and branching can further optimize fuel properties. In addition, engineered fuels with new features could be biosynthesized by polyketide synthases[98]. The challenges to this engineering will be designing the functional linkage between each domain and module, the proper folding and phosphopantetheinylation of the designed polyketide synthases[99], and the low catalytic efficiency and limited number of turnovers of engineered polyketide synthases. Advanced design tools and experimental techniques will facilitate the tuning of the polyketide-derived fuel properties by manipulating polyketide synthase.

Both data-driven and synthetic-biology approaches are powerful tools for trouble shooting and optimizing engineered metabolic pathways. Improving the efficiency and yield of biofuels further will require more potent tools, including techniques for stable maintenance of gene copy number and more precise control of mRNA and protein levels, especially in a dynamic manner that would automatically adjust the pathway according to its own metabolic flux. Furthermore, enzyme activities and substrate specificities can be altered by protein engineering[100]. Artificial enzymes with new functions can even be created by incorporating unnatural amino acids and computation-based protein design.

The development of pathways to expand the source of feedstock and to tune the properties of biofuel products, the creation of more metabolic engineering techniques to improve pathway flux and additional synthetic-biology approaches to optimize microbial hosts and boost production should see one or more advanced biofuels make it to market. ■

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