Synthetic Biology and Future Production of Biofuels and High-Value Products

11

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Abstract

Synthetic biology aims to build increasingly complex biological systems from standard interchangeable parts. The ideal microorganism for biofuel production may produce a single fermentation product and might possess high substrate utilization and processing capacities. Such microorganisms may also possess fast and deregulated pathways for sugar transport, good tolerance to inhibitors and product, and high metabolic fluxes. The choice to produce such an organism lies between engineering natural function and importing biosynthetic capacity which is affected by current progress in metabolic engineering and synthetic biology. Synthetic biology is bringing together engineers and biologists to design and build novel biomolecular components, networks, and pathways and to use these constructs to rewire and reprogram organisms. Recent findings that plant metabolic pathways can be reconstituted in heterologous hosts and metabolism in crop plants can be engineered to improve the production of biofuels have given new hope for molecular biological approaches in improving food and biofuel production. The de novo engineering of genetic circuits, biological modules, and synthetic pathways is beginning to address these crucial problems and is being used in related practical applications.

Keywords

Electron transport · Photosynthetic process · PSII · Transmembrane complex

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11.1 Introduction

Public concerns over environmental pollution, greenhouse gas emissions, and the shortage of raw oils are increasing, and considerable attention is turning toward alternative, renewable sources of chemical products to reduce both dependency on oil reserves and carbon dioxide emissions into the environment (US Energy Information Administration 2012; Arslan et al. 2012; Kawaguchi et al. 2016; Scheffers et al. 2016; Kumar et al. 2018; Kumar 2020). Analysis by Rogelj et al. (2011) confirms that if the mechanisms needed to enable an early peak in global emissions followed by steep reductions are not put in place, there is a significant risk that the 2 °C target will not be achieved. Long et al. (2015) reported the global food demand of the future by engineering crop photosynthesis and yield potential. Recent reviews on synthetic biology have provided excellent information about the development of synthetic biology (Barber 2009; Khalil and Collins 2010; Erb and Zarzycki 2016; Bhansali and Kumar 2018; Kumar et al. 2019).

The production of numerous sustainable chemicals using engineered microbes has a potential environmental impact with a significant reduction in greenhouse gas emissions (GGEs) while offering the potential of advanced products with improved properties (Wu et al. 2015; Lynch 2016).

Environmental applications of synthetic biology include microbes that sense, report, and degrade toxic chemicals (Hillson et al. 2007; Chen et al. 2014). Besides, it has the capability to produce a variety of chemical products ranging from therapeutics to plastics and biofuels (Fortman et al. 2008; Lee et al. 2012; Beller et al. 2015; Sitepu et al. 2014; Yu et al. 2015; Bhansali and Kumar 2018; French 2019).

Biofuels are environmentally friendly and sustainable sources. Their production including bioethanol, biobutanol, and biodiesel has gained considerable interest (Jiang et al. 2019). Bioethanol was regarded as one of the most promising biofuels, particularly as a carbon-neutral liquid transportation fuel (Jiang et al. 2019). Artificial microbial consortia are specifically constructed to broaden the scope of feedstocks, enhance the productivity of target bio-products, etc. (Jiang et al. 2019). Next-generation biofuels and green chemicals will be produced from lignocellulosic materials, such as agricultural residues, woody energy crops, and municipal solid waste, which are abundant and inexpensive (Carroll and Somerville 2009; Green 2011; Kumar 2020).

The natural fermentation produces alcohols such as ethanol and propanol, lacking the energy density of petroleum fuels (Mackenzie 2013). According to Connor and Atsumi (2010), some of the next-generation biofuels depend on highly precise modification and can produce energy-dense hydrocarbon by introduction of "foreign genes and pathways into central metabolism" of well-studied model organisms such as yeasts and bacteria (Mackenzie 2013).

Engineering of biological systems has emerged as one of the most exciting recent technologies (Nielsen and Keasling 2011; Kumar 2014; Farr et al. 2014; Guo et al. 2016; Gall et al. 2017). The complex oleochemicals that cannot be obtained from

natural sources because of low abundance can be produced by introducing novel synthetic biochemical pathways into platform chassis (Marella et al. 2018).

Jang et al. (2012) reviewed systems metabolic engineering which allows systematic changes of metabolic pathways toward desired goals including enhancement of product concentration, yield, and productivity. Guo et al. (2016) reviewed the development of metabolic engineering and synthetic biology and microbial production of fatty alcohols from renewable feedstock in both *Escherichia coli* and *Saccharomyces cerevisiae*. The boundaries and overlap between metabolic engineering and synthetic biology are often blurry as practitioners often work in both fields, which also share common tools (Couto et al. 2018).

The integration of protein engineering, systems biology, and synthetic biology into metabolic engineering has extended strain engineering from local modification to system-wide optimization. Powerful omics technologies, such as genomics, transcriptomics, proteomics, and fluxomics, have been combined for in-depth understanding of glycerol metabolism and regulation of microorganism at the system level (Wang et al. 2003; Liao et al. 2011; Beckers et al. 2016; Salazar et al. 2009; Kumar 2015; Kumar et al. 2018, 2019).

11.2 Sugar Is the Next Oil

Plant metabolic pathways can be reconstituted in heterologous hosts, and metabolism in crop plants can be engineered to improve the production of biofuels. According to Sanford et al. (2016), the theme of "sugar is the next oil" connects chemical, biological, and thermochemical conversions of renewable feedstocks to products which are drop-in replacements for petroleum-derived chemicals, bio-polymers (Wang et al. 2015; Dai and Nielsen 2015), or are new to market chemicals/materials.

11.3 Bugs to Synthetic Biofuels

Lee et al. (2008) proposed the term bugs to synthetic biofuels. Gaida et al. (2016) reported for the first time the production of n-butanol directly from crystalline cellulose using a single engineered organism—*Clostridium cellulolyticum*, a bacterium. According to Becker and Wittmann (2016), *E. coli* has also entered the precious market of high-value molecules and is becoming a flexible, efficient production platform for various therapeutics, pre-biotics, nutraceuticals, and pigments. This is enabled by systems metabolic engineering concepts that integrate systems biology and synthetic biology into the design and engineering of powerful *E. coli* cell factories.

An artificial *Escherichia coli* binary culture was constructed for the direct conversion of hemicellulose into ethanol. Short-chain alcohols can also be produced in *E. coli* from 2-keto acids, common intermediates in amino acid biosynthetic pathways. By expressing genes in *E. coli*, six short-chain alcohols including

1-propanol, 1-butanol, isobutanol, 2-methyl-1-butanol, 3-methyl-1-butanol, and 2-phenylethanol were produced by non-fermentative pathways (Atsumi et al. 2008a, b; Liao et al. 2016).

11.3.1 Xylose Utilization

Efficient xylose utilization is one of the most important prerequisites for developing an economic microbial conversion process of terrestrial lignocellulosic biomass into biofuels and biochemical (Kwak and Jin 2017). Kwak and Jin (2017) reported a robust ethanol-producing yeast *Saccharomyces cerevisiae* has been engineered with heterologous xylose assimilation pathways. A two-step oxidoreductase pathway consisting of NAD(P)H-linked xylose reductase and NAD^+-linked xylitol dehydrogenase and a one-step isomerase pathway using xylose isomerase have been employed to enable xylose assimilation in engineered *S. cerevisiae* (Alper and Stephanopoulos 2009) (Fig. 11.1).

11.3.2 Xylose Fermenting

Native *Saccharomyces cerevisiae* (Scer) does not consume xylose but can be engineered for xylose consumption with a minimal set of assimilation enzymes, including xylose reductase (Xyl1) and xylitol dehydrogenase (Xyl2) from the xylose-fermenting *Pichia stipitis* (Psti) (Jeffries 2006; Van Vleet and Jeffries 2009). However, xylose fermentation remains slow and inefficient in Scer, especially under anaerobic conditions when NADH cannot be recycled for NAD^+-dependent Xyl2 (Jeffries 2006). Therefore, improving xylose utilization in industrially relevant yeasts is essential for producing economically viable biofuels from cellulosic material (Wohlbach et al. 2011). Yeasts engineered to ferment xylose do so slowly and cannot utilize xylose until glucose is completely consumed (Fig. 11.1). Ha et al. (2011) engineered yeasts to coferment mixtures of xylose and cellobiose (see also Diao et al. 2013).

The development of xylose-utilizing strains of *Saccharomyces cerevisiae* has improved the prospects of lignocellulosic biorefinery, enabling the creation of full-scale second-generation bioethanol production plants worldwide (Diao et al. 2013; Jansen et al. 2017). Tran et al. (2018) successfully developed a high-performance xylose-fermenting strain of *S. cerevisiae*, XUSE, through CRISPR–Cas9-mediated rational engineering and evolutionary engineering. According to Tran et al. (2018), for further engineering, XUSE could serve as a promising platform strain for lignocellulosic biorefinery (see also Estrela and Cate 2016).

11.4 Biosynthetic Pathways of Biofuels

Different pathways of carbon feedstocks are shown by Liao et al. (2016) (Fig. 11.2).

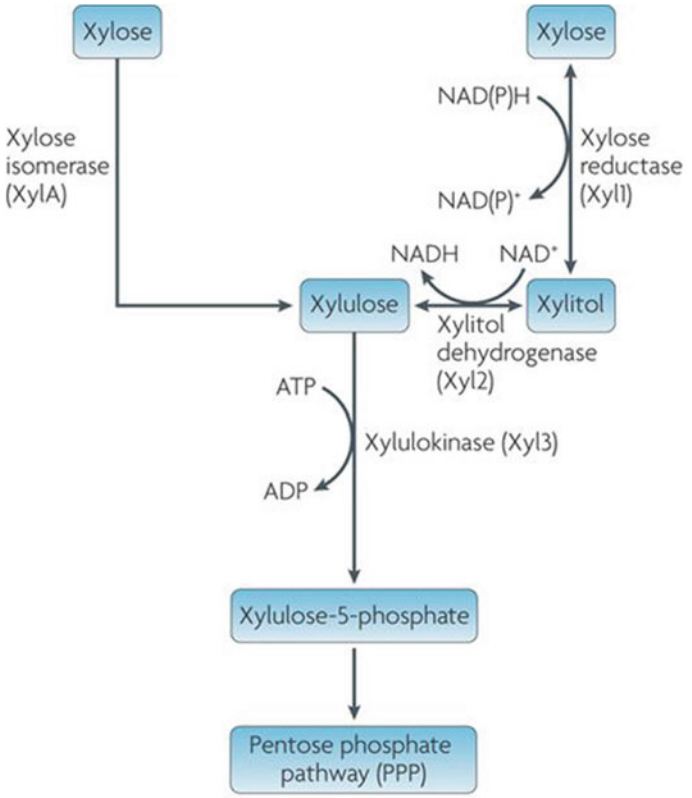

Fig. 11.1 Two routes to xylose assimilation. When xylose enters *Saccharomyces cerevisiae*, it can be incorporated into the pentose phosphate pathway through either the three-enzyme pathway containing a xylitol intermediate or a two-step process that uses a fungal or bacterial xylose isomerase gene. The two-step process bypasses the need for the reducing power that is incorporated in NAD- and NADP-reducing partners and has been shown to improve ethanol production. Xylulose 5-phosphate is formed by both pathways and can enter into central carbon metabolism through the transketolase and transaldolase reactions. (Source: Alper, H. & Stephanopoulos, G. (2009). Engineering for biofuels: exploiting innate microbial capacity or importing biosynthetic potential. *Nature Reviews. Microbiology 7*: 715–723. Retrieved from https://doi.org/10.1038/nrmicro2186. Reproduced with license number 46456408400514)

Different pathways can be assembled to produce molecules not currently used as fuels, but with likely suitable properties, including fatty alcohols (Steen et al. 2010; Feng et al. 2014), methyl ketones (Goh et al. 2012, 2014), γ-hydroxy and dicarboxylic acids (Clomburg et al. 2015), and other fatty acid-derived products.

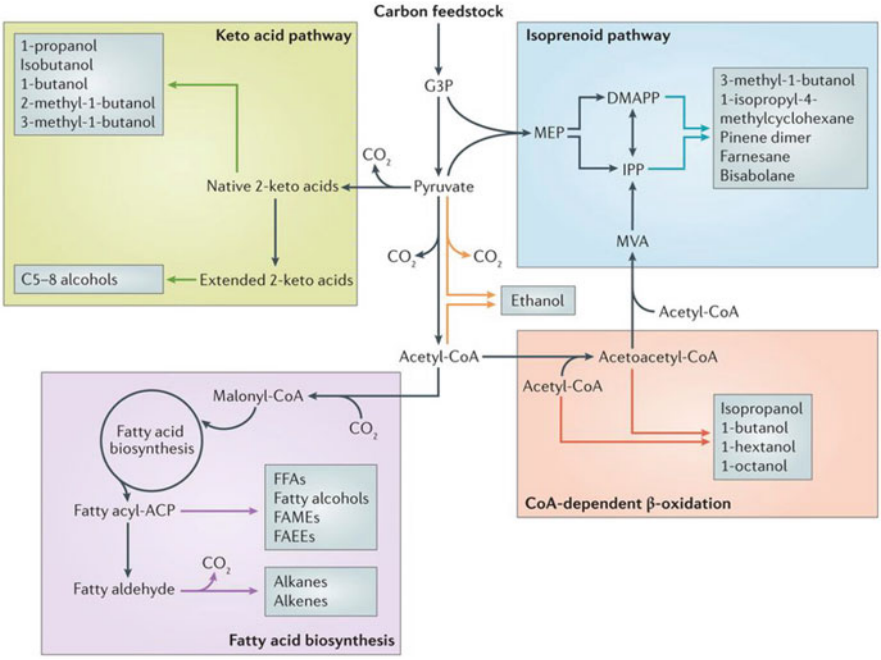

Fig. 11.2 Biosynthetic pathways of biofuels. Ethanol is produced from either pyruvate or acetyl-CoA (orange arrows), with acetaldehyde as a common intermediate. The keto acid pathway (green arrows) can be used to produce both branched and straight-chain alcohols. It uses parts of amino acid biosynthesis pathways for keto acid chain elongation. This is followed by decarboxylation and reduction of the keto acid, analogous to the conversion of pyruvate to ethanol. Fatty acid synthesis (purple arrows) extends acyl-acyl carrier proteins (ACPs) in a cyclical manner, using malonyl-CoA as a precursor. Fatty acyl-ACPs may be converted into free fatty acids (FFAs) with acyl-ACP thioesterase. FFAs can be esterified to esters, such as fatty acid methyl esters (FAMEs) or fatty acid ethyl esters (FAEEs), reduced to fatty alcohols, or reduced to fatty aldehydes followed by decarbonylation to alkanes and alkenes. The CoA-dependent pathway (red arrows) uses reverse β-oxidation chemistry for the production of higher alcohols or decarboxylation of the precursor acetoacetyl-CoA for the production of isopropanol. Isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), the universal precursors of isoprenoid biofuel biosynthesis (blue arrows), may be produced either through the mevalonate (MVA) or methylerythritol 4-phosphate (MEP) pathway. *G3P* glyceraldehyde-3-phosphate. Metabolic engineering for the production of biofuels has been reviewed by Kumar (2010), Kumar (2013), and Kumar (2015). (Source: Liao, J. C., Mi, L., Pontrelli, S., & Luo, S. (2016). Fuelling the future: microbial engineering for the production of sustainable biofuels. *Nature Publishing Group*, Nature Review. Microbiology *14*(5): 288–304. https://doi.org/10.1038/nrmicro.2016.32. Reproduced under license number 4645730007098)

11.5 Metabolic Engineering

Martien and Amador-Noguez (2017) suggested the major goals of metabolic engineering for microbial biofuel production are (1) to direct metabolic flux toward maximum biofuel generation, (2) to enable the use of economical feedstock such as lignocellulose, and (3) to improve stress tolerance to inhibitors produced during pre-processing or biofuel production (Fig. 11.3). Metabolic engineering is a process of optimizing native metabolic pathways and regulatory networks or assembling heterologous metabolic pathways for the production of targeted molecules using molecular, genetic, and combinatorial approaches (Zhu and Jackson 2015). A common strategy of metabolic engineering is to increase the endogenous supply of

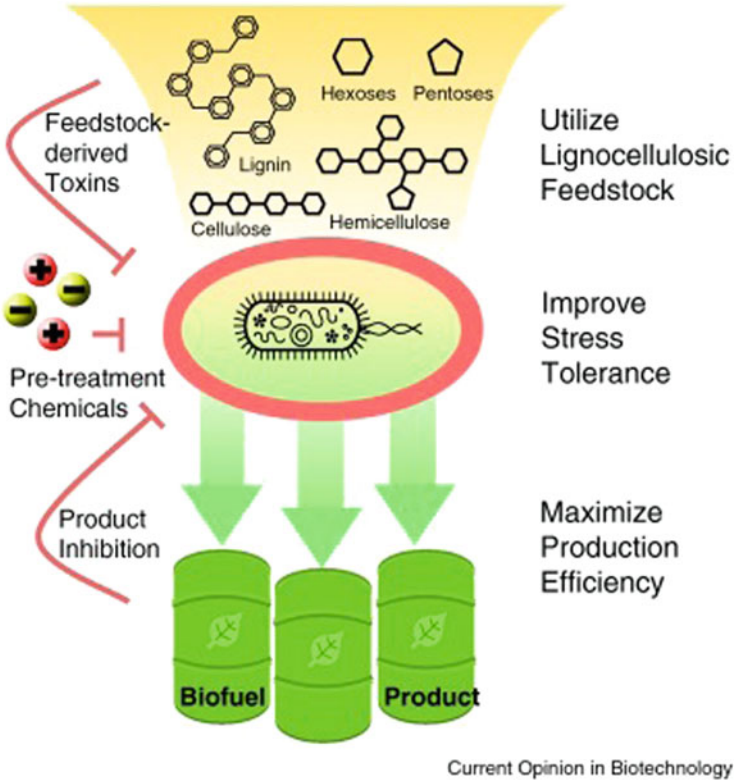

Fig. 11.3 The major goals of metabolic engineering for microbial biofuel production are (1) to direct metabolic flux toward maximum biofuel generation, (2) to enable the use of economical feedstock such as lignocellulose, and (3) to improve stress tolerance to inhibitors produced during pre-processing or biofuel production. The studies featured in this review apply knowledge gained from metabolomics-based methods to achieve these goals. (Source: Martien J.I., and Amador-Noguez D. (2017). Recent applications of metabolomics to advance microbial biofuel production. *Current Opinion in Biotechnology* 43: 118–126. https://doi.org/10.1016/j.copbio.2016.11.006. Reproduced with permission Licence number 4666750205840)

precursor metabolites to improve pathway productivity (Leonard et al. 2010). Several excellent reviews on systems metabolic engineering and synthetic biology have highlighted the motivation and need for pathway balancing (Lee et al. 2008; Völler and Budisa 2017).

Maximizing microbial biofuel production from plant biomass (i.e., lignocellulosic biomass or plant dry matter) requires reprogramming metabolism to ensure a seamless supply of carbon, energy (e.g., ATP), and reducing power (e.g., NAD(P)H) toward engineered biofuel pathways (Martien and Amador-Noguez 2017). Nature exploits a very limited set of just 20 canonical alpha-L-amino acids (cAAs) for the ribosomal translation of peptides and proteins. Reprogramming this process enables the incorporation of additional ncAAs capable of delivering a variety of novel chemical and biophysical properties into target proteins or protein-based complex structures (Agostini et al. 2017). Significant progress has been achieved in understanding and engineering the de novo lipid biosynthesis in *Y. lipolytica* (Zhu and Jackson 2015).

Jones et al. (2015) reviewed metabolic pathway balancing and its role in the production of biofuels and chemicals (Fig. 11.4).

Chae et al. (2017) reviewed recent advances in systems metabolic engineering which analyzes various omics data together, rather than just a single type of omics. The multiomics approach can be used to elucidate various phenomena in a metabolically engineered strain and to identify further engineering targets.

They further resorted to chemical hydrogenation of bisabolene into the final product bisabolane with the ultimate goal of complete microbial production of bisabolane. This will require the reduction of terpenes in vivo using designer reductases and, potentially, balancing cellular reducing equivalents (Peralta-Yahya et al. 2011).

Bisabolane as a biosynthetic alternative to D2 diesel fuel. Peralta-Yahya et al. (2011) identified a novel biosynthetic alternative to D2 diesel fuel, bisabolane, and engineered microbial platforms for the production of its immediate precursor, bisabolene (Fig. 11.5). Peralta-Yahya and Keasling 2010 hypothesized that for a fully reduced monocyclic sesquiterpene, bisabolane may serve as a biosynthetic alternative to diesel (Figs. 11.5 and 11.6). D2 diesel, the fuel for compression ignition engines, is a mixture of linear, branched, and cyclic alkanes with an average carbon length of 16 (Fortman et al. 2008). Bisabolane has a carbon length (C15) close to the average carbon length of diesel (C16). To our knowledge, there are no reports of bisabolane as a biosynthetic alternative to D2 diesel. Source: Peralta-Yahya, P. P., Ouellet, M., Chan, R., Mukhopadhyay, A., Keasling, J. D., & Lee, T. S. (2011). Identification and microbial production of a terpene-based advanced biofuel. *Nature Communications 2*: 483–488. https://doi.org/10.1038/ncomms1494.

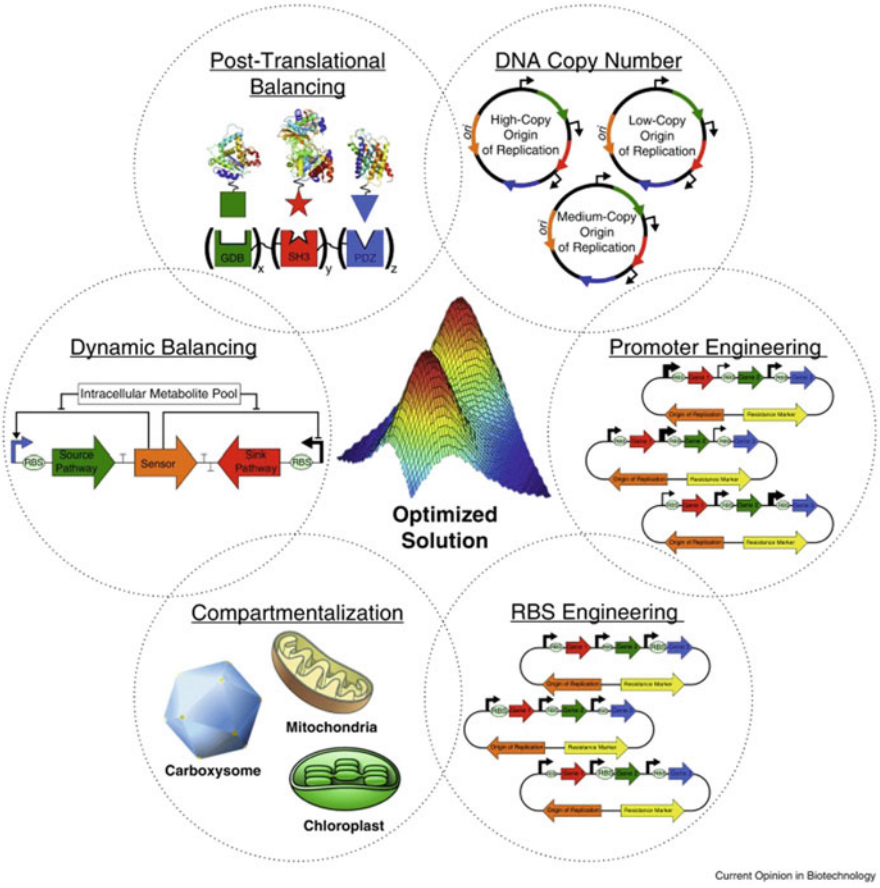

Fig. 11.4 Six major approaches to optimize metabolic pathways in common laboratory organisms such as *E. coli* and *S. cerevisiae*. The left- and right-hand sides of the figure represent modern and classical approaches, respectively. Modern techniques can be summarized as dynamic metabolite monitoring and balancing through critical intermediate chemicals, spatial organization of enzymes by using synthetic scaffolds or fusion proteins, and organelle-level compartmentalization of both metabolites and pathway enzymes to take advantage of elevated concentrations of substrates and enzymes. On the other hand, classical techniques include utilizing plasmid copy number or chromosomal integration modularity by combinational approach; gene expression level control through promoter engineering, including synthetic hybrid promoters (e.g., regulation through toxic chemicals or specific precursors); and lastly, ribosome binding site engineering for each different pathway gene to optimize and normalize their translational efficiencies. (Source: Jones, J.A., Ö. Duhan Toparlak and Mattheos AG Koffas (2015). Metabolic pathway balancing and its role in the production of biofuels and chemicals. *Current Opinion in Biotechnology*, *33*, 52–59. https://doi.org/10.1016/j.copbio.2014.11.013. Reproduced with permission no 4671031226483)

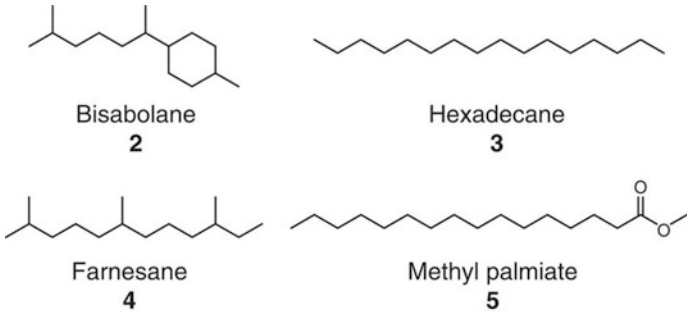

Fig. 11.5 Chemical structures of fuels. Bisabolane (**2**); hexadecane (**3**), a representative molecule for diesel; farnesane (**4**); and methyl palmitate (**5**), a representative molecule for fatty acid methyl esters. (Source: Peralta-Yahya, P. P., Ouellet, M., Chan, R., Mukhopadhyay, A., Keasling, J. D., & Lee, T. S. (2011). Identification and microbial production of a terpene-based advanced biofuel. *Nature Communications 2*: 483–488. https://doi.org/10.1038/ncomms1494. This is an open-access article distributed under the terms of the Creative Commons CC-BY license, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited)

11.5.1 Lycopene

Ma et al. (2019) established a heterologous lycopene pathway in strain YZL141 (Fig. 11.2) by genomic integration of genes encoding GGPP synthase (CrtE), phytoene synthase (CrtB), and phytoene desaturase (CrtI) from different sources. Ma et al.'s (2019) findings are the first, describing lipid-metabolic engineer to promote lycopene overproduction in a non-oleaginous organism (Figs. 11.7 and 11.8).

Using systematic traditional engineering methods, Ma et al. (2019) established high-yield heterologous lycopene biosynthesis in *S. cerevisiae*. Their results confirmed the successful development of an oleaginous biorefinery platform in *S. cerevisiae* that enabled the efficient overproduction of the intracellular lipophilic natural product lycopene.

Efforts to increase terpenoid production in *E. coli* previously focused on (1) overexpression of pathway enzymes and (2) optimizing the expression of enzymes by codon bias (Leonard et al. 2010; Lindberg et al. 2009; Dueber et al. 2009; Tyo et al. 2009). Thus, in addition to metabolic engineering, the molecular reprogramming of key metabolic nodes such as prenyltransferase (GGPPS) and terpenoid synthase (LPS) through protein engineering is required to achieve substantial overproduction of a desired terpenoid product (Keeling and Bohlmann 2006; Tholl 2006; Keeling and Bohlmann 2006; Christianson 2008; Leonard et al. 2010; Peralta-Yahya and Keasling 2010; Kumar 2013).

There are two main precursors which are isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). There are two pathways to generate isoprenoids: the mevalonic acid pathway (MVA, for some bacteria, plants, and higher eukaryotes) and the 2-C-methyl-d-erythritol 4-phosphate/1-deoxy-d-

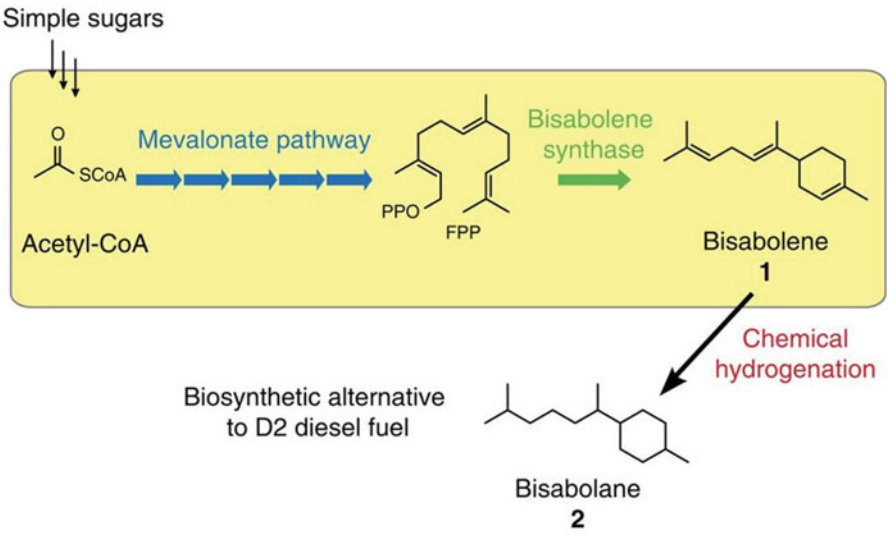

Fig. 11.6 Bisabolane from chemical hydrogenation of microbially produced bisabolene. The engineered microbe (yellow box) converts simple sugars into acetyl-CoA via primary metabolism. A combination of metabolic engineering of the heterologous mevalonate pathway to convert acetyl-CoA into FPP and enzyme screening to identify a terpene synthase to convert FPP into bisabolene (1) is used to produce bisabolene at high titers. Chemical hydrogenation of biosynthetic bisabolene leads to bisabolane (2), a biosynthetic alternative to D2 diesel. (Source: Peralta-Yahya, P.P., Ouellet, M., Chan, R., Mukhopadhyay, A., Keasling, J.D. and Lee, T.S. (2011). Identification and microbial production of a terpene-based advanced biofuel. *Nature Communications 2:* 483–488. https://doi.org/10.1038/ncomms1494. This is an open-access article distributed under the terms of the Creative Commons CC-BY license, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited)

xylulose5-phosphate pathway (DXP, for plants and most of the bacterial strains). The end products of both pathways are the precursors of all terpenoids, some with pharmaceutical relevance such as taxol, artemisinin, and lycopene (Figs. 11.7 and 11.8).

The fully reduced form of the linear terpene farnesene is being pursued as an alternative biosynthetic diesel in the market (Renniger and McPhee 2008).

Generally, butanol was synthesized through traditional acetone–butanol–ethanol (ABE) fermentation process by solventogenic *Clostridium* sp. (Jin et al. 2011; Campos-Fernández et al. 2012; Zheng et al. 2015; Trindade and Santos 2017; Sun et al. 2018; Shanmugam et al. 2018). However, according to Jiang et al. (2018), most *Clostridia* could not directly utilize polysaccharides, such as lignocellulose due to the inexpression of polysaccharide-degrading enzymes. Even though metabolic engineering has provided different alternatives such as improved solvent tolerance and non-acetone-forming strains, systems biology-guided strain engineering and synthetic biology can lead to sustained industrial viability (Birgen et al. 2019).

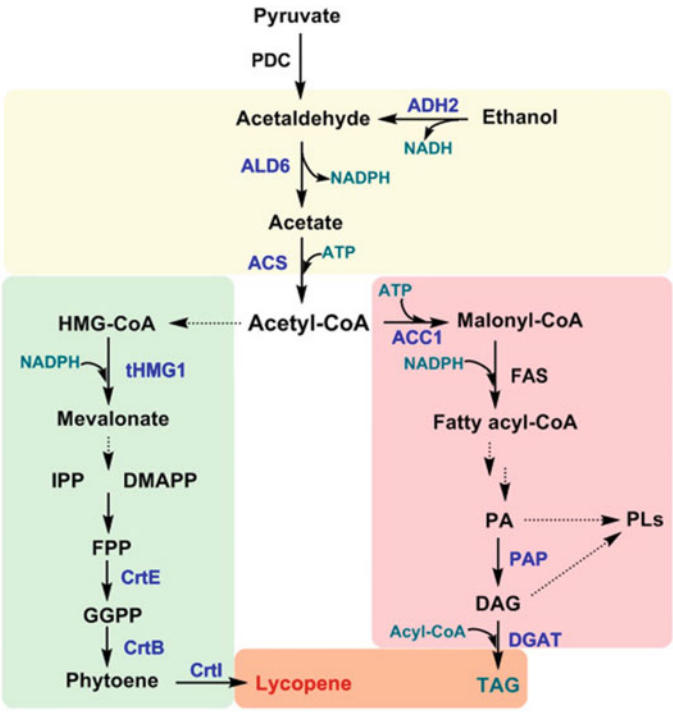

Fig. 11.7 Simplified schematic representation of key fluxes in lycopene biosynthesis coupled with TAG metabolism in *S. cerevisiae*. The acetyl-CoA-producing pathway is highlighted in a yellow rectangle. Reactions associated with TAG synthesis are highlighted in a red rectangle. Lycopene-biosynthetic flux is highlighted in a green rectangle. *PDC* pyruvate decarboxylase, *ADH2* alcohol dehydrogenase, *ALD6* acetaldehyde dehydrogenase, *ACS* acetyl-CoA synthetase, *tHMG1* truncated 3-hydroxy-3-methylglutaryl-CoA reductase, *CrtE* geranylgeranyl diphosphate synthase, *CrtB* phytoene synthase, *CrtI* phytoene desaturase, *ACC1* acetyl-CoA carboxylase, *FAS* fatty acyl-CoA synthetases, *PAP* phosphatidate phosphatase, *DGAT* acyl-CoA: diacylglycerol acyltransferase, *HMG-CoA* 3-hydroxy-3-methyl-glutaryl-CoA, *IPP* isopentenyl diphosphate, *DMAPP* dimethylallyl diphosphate, *FPP* farnesyl diphosphate, *GGPP* geranylgeranyl diphosphate, *PA* phosphatidic acid, *PLs* phospholipids, *DAG* diacylglycerol, *TAG* triacylglycerol. (Source: Ma, T., Shi, B., Ye, Z., Li, X., Liu, M., Chen, Y. & Nielsen, J. (2019). Lipid engineering combined with systematic metabolic engineering of *Saccharomyces cerevisiae* for high-yield production of lycopene. *Metabolic Engineering 52*: 134–142. https://doi.org/10.1016/j.ymben.2018.11.009. Reproduced under license number 4651230668162)

Isobutanol which is a promising second-generation biofuel candidate is already formed as a by-product in fermentations with the yeast *Saccharomyces cerevisiae*, although only in very small amounts (Hammer and Avalos 2017; Wess et al. 2019). Wess et al. (2019) reported that overexpressing a cytosolic isobutanol synthesis pathway and by blocking non-essential isobutanol competing pathways, they could achieve the highest yield ever obtained with *S. cerevisiae* in shake flask cultures.

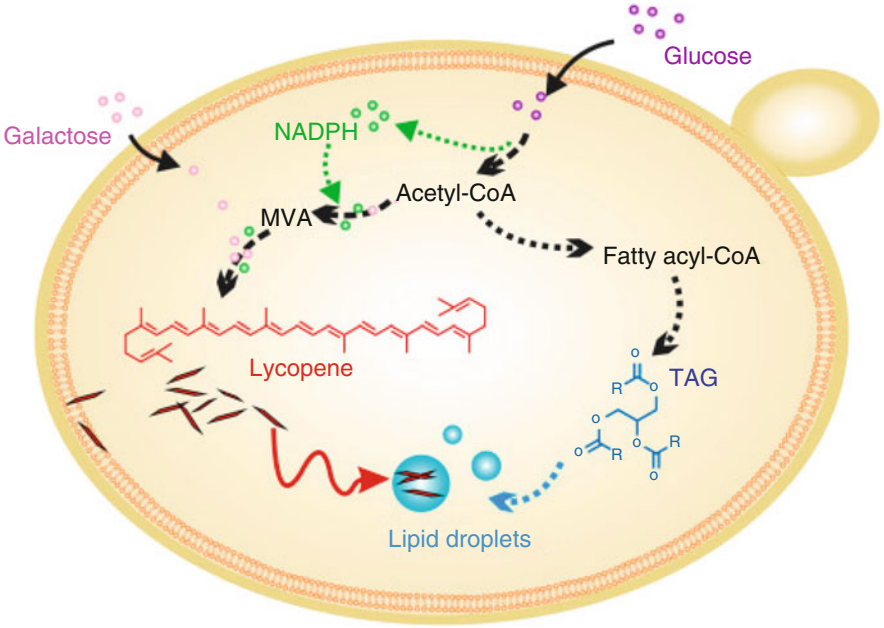

Fig. 11.8 Lycopene biosynthesis in *S. cerevisiae*. *S. cerevisiae* takes up glucose from the extracellular environment, and glucose metabolism results in acetyl-CoA accumulation and the release of NADPH. For lycopene production, acetyl-CoA is used in the endogenous MVA pathway and heterologous carotenoid pathway. Lycopene is distributed in lipid structures (e.g., phospholipid membranes and LDs). For TAG production, acetyl-CoA is used for endogenous fatty acid biosynthesis. TAGs are incorporated into LDs to store energy and dissolve lycopene crystals. Purple spheres represent glucose particles, pink spheres represent galactose, green spheres represent NADPH, and blue spheres represent LDs. Dotted lines represent multiple reactions. (Source: Ma, T., Shi, B., Ye, Z., Li, X., Liu, M., Chen, Y., and Nielsen, J. (2019). Lipid engineering combined with systematic metabolic engineering of *Saccharomyces cerevisiae* for high-yield production of lycopene. *Metabolic Engineering 52*: 134–142. https://doi.org/10.1016/j.ymben.2018.11.009. Reproduced under license number 4651230668162 from RightsLink)

11.5.2 Production of Fatty Acid- and Polyketide-Derived Biofuels

Recently, with the development of metabolic engineering and synthetic biology, microbial production of fatty alcohols from renewable feedstock has been achieved successfully in *E. coli*. Metabolic pathways used for the production of fatty acid- and polyketide-derived biofuels have been presented by Peralta-Yahya et al. (2012) (Fig. 11.9).

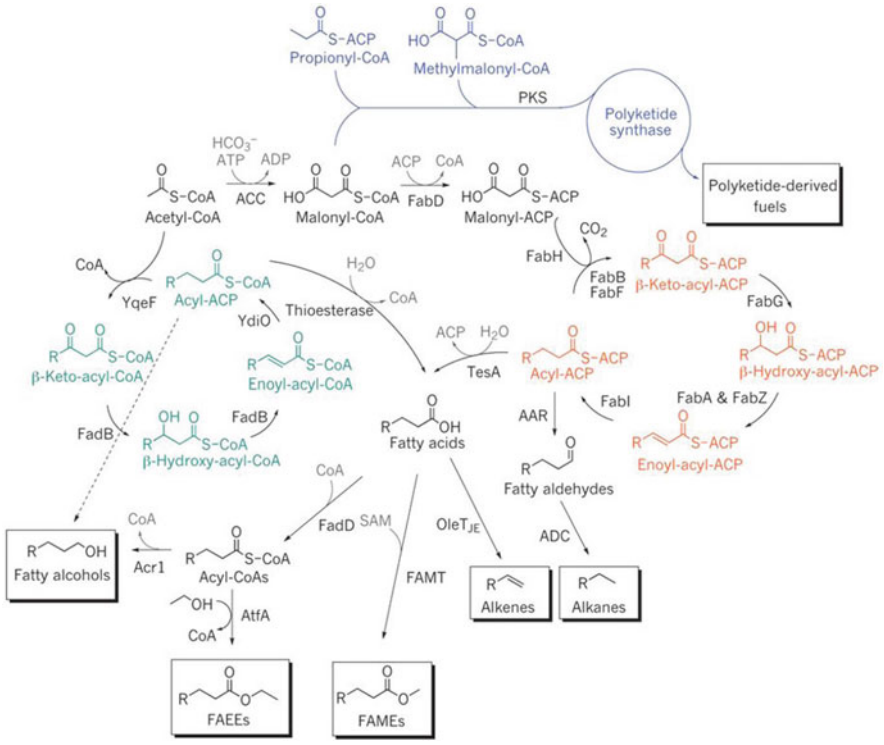

Fig. 11.9 Metabolic pathways used for the production of fatty acid- and polyketide-derived biofuels. The fatty acid biosynthetic cycle is in red, the reversal of the β-oxidation cycle is in green, and polyketide synthase is in blue. *AAR* acyl-ACP reductase, *ACC* acetyl-CoA carboxylase, *Acr1* acyl-CoA 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 and 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. (Source: Peralta-Yahya P.P. et al.(2012). Microbial engineering for the production of advanced biofuels. https://doi.org/10.1038/nature488320-328. Reproduced with license no. 4643340791481)

11.5.3 Synthetic Enzymatic Pathways for the Production of High-Yield Hydrogen

Natural and genetically modified microorganisms cannot produce hydrogen with a yield of more than 4 H_2 per glucose, that is, the Thauer limit (Thauer et al. 2008; Zhang 2011, 2015) (Fig. 11.10), although a theoretical yield is 12 H_2 per glucose. Nature cannot evolve such high-yield hydrogen generation pathways due to two reasons. First, the theoretical yield of hydrogen production is an endothermic reaction so that it cannot co-generate ATP. Second, if a small fraction of reduced

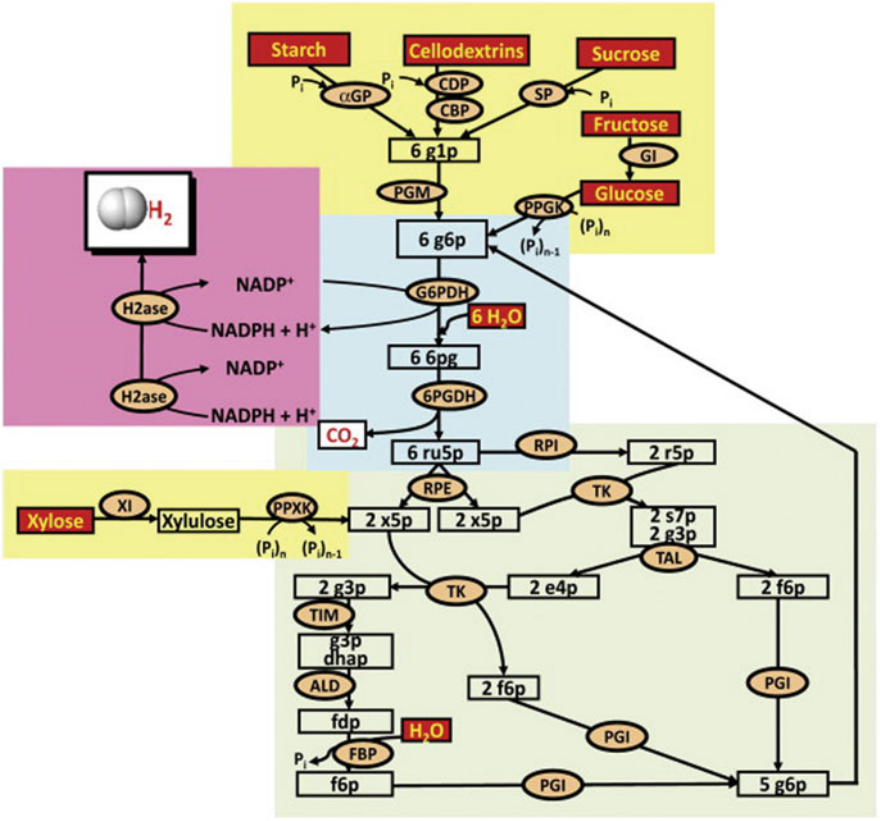

Fig. 11.10 Scheme of in vitro synthetic enzymatic pathways for the production of high-yield hydrogen from a variety of carbohydrates—starch, cellodextrin, sucrose, glucose, fructose, and xylose as well as water. The pathways are compiled and modified from References: Martín del Campo et al. 2013; Myung et al. 2014; Rollin et al. 2016; Ye et al. 2009; Zhang et al. 2007). The enzymes are αGP, alpha-glucan phosphorylase; CDP, cellodextrin phosphorylase; CBP, cellobiose phosphorylase; SP, sucrose phosphorylase; GI, glucose isomerase; XI, xylose isomerase; PPGK, polyphosphate glucokinase; PPXK, polyphosphate xylulokinase; PGM, phosphoglucomutase; G6PDH, glucose-6-phosphate dehydrogenase; 6PGDH, 6-phosphogluconate dehydrogenase; RPI, ribose 5-phosphate isomerase; RPE, ribulose-5-phosphate 3-epimerase; TK, transketolase; TAL, transaldolase; TIM, triose phosphate isomerase; ALD, (fructose-bisphosphate) aldolase; FBP, fructose bisphosphatase; PGI, phosphoglucose isomerase; and H_2ase. P_i and $(P_i)_n$ are inorganic phosphate and polyphosphate with a degree of polymerization of n. The metabolites are g1p, glucose-1-phosphate; g6p, glucose-6-phosphate; ru5p, ribulose 5-phosphate; x5p, xylulose 5-phosphate; r5p, ribose 5-phosphate; s7p, sedoheptulose 7-phosphate; g3p, glyceraldehyde 3-phosphate; e4p, erythrose 4-phosphate; dhap, dihydroxyacetone phosphate; fdp, fructose-1,6-diphosphate; and f6p, fructose 6-phosphate. (Source: Zhang, Y. P. (2015). Production of biofuels and biochemicals by in vitro synthetic biosystems: Opportunities and challenges. *Biotechnology Advances 33*(7): 1467–1483. https://doi.org/10.1016/j.biotechadv.2014.10.009. Reproduced with license number 4652950482642)

NAD(P)H was used to generate ATP via oxidative phosphorylation (Swartz 2013), the presence of oxygen would inhibit oxygen-sensitive hydrogenase activity greatly.

Woodward and his coworkers (Woodward et al. 2000) produced nearly 12 H_2 from the costly glucose 6-phosphate (G-6-P). This pathway comprised three modules: (1) two NADPH generation from G-6-P mediated by two dehydrogenases, (2) hydrogen generation from NADPH mediated by hydrogenase, and (3) regeneration of G-6-P from ribulose 5-phosphate. However, costly substrate G-6-P prevents its potential application so that Woodward did not file a patent for this in vitro synthetic pathway.

11.5.4 Synthetic Biology Tools and Methodologies

Synthetic biology today encompasses an increasing number of tools and methodologies to facilitate strain construction and optimization. Synthesizing, sequencing, and introducing DNA sequences into living cells are cheaper and easier than ever (DiCarlo et al. 2013). Codon optimization, directed evolution (Korman et al. 2013), screening enzyme libraries, and incorporating non-natural amino acids (Cirino et al. 2003) all provide ways of improving or generating novel enzymatic activities (see also Jagadevan et al. 2018) (Fig. 11.11).

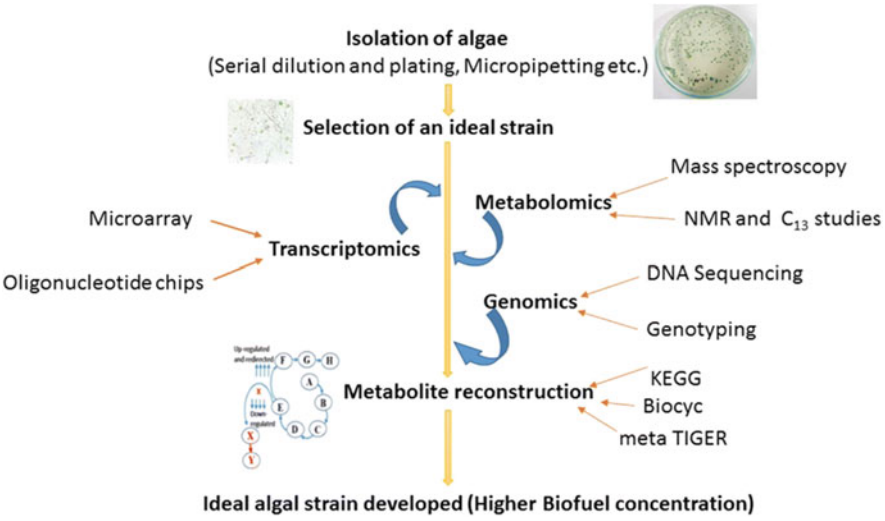

Fig. 11.11 Pictorial representation of the overall process toward biofuel production in microalgae using synthetic biology approach (i.e., isolation, selection of an ideal strain, redirecting the metabolism to maximize synthesis of the targeted biofuel). (Source: Jagadevan, S., Banerjee, A., Banerjee, C., Guria, C., Tiwari, R., & Baweja, M. (2018). Biotechnology for Biofuels Recent developments in synthetic biology and metabolic engineering in microalgae towards biofuel production. *Biotechnology for Biofuels 11*: 1–21. https://doi.org/10.1186/s13068-018-1181-1. Used under creative commons license)

The major challenge of the modern era is the transition to a bio-based economy. Biofuels are a key part of this landscape, but challenges to efficiently and cost-effectively produce biofuels still remain (Tyner 2012; Taheripour et al. 2012).

The standard of skill and expertise in synthetic biology and metabolic engineering has made significant strides over the past 25 years, and now the production of numerous chemical products with a range of market applications is available (Lynch 2016). Tatsis and O'Connor (2016) demonstrated with examples how the metabolic pathways of plants can be successfully harnessed using several metabolic engineering approaches. According to O'Connor (2015), one approach to harness plant metabolic pathways is to reconstitute the biosynthetic genes into a heterologous organism.

Hybrid processes: Hybrid processes combine the biochemical and chemical processes to enhance competitiveness of bio-based products (Beerthuis et al. 2015) such as polymers, and bioplastics will grow their market share by synergizing and collaborating with the chemical process industry (Babu et al. 2013). Creating the necessary process flow sheets, assessing cost sensitivities, and identifying bottlenecks upfront by the use of modeling, simulation, and techno-economic analysis will aid in a successful scale-up (Earhart et al. 2012; Claypool and Ramon 2013; Claypool et al. 2014; Harrison et al. 2015).

Reducing cell wall digestibility: Lignin concentration also increases with the maturation of plants and is associated with reduced cell wall digestibility (Jung and Deetz 1993). Cell wall lignification creates an access barrier to potentially digestible wall material by microorganisms if cells have not been physically ruptured. Traditional breeding has focused on increasing total dry matter digestibility rather than cell wall digestibility, which has resulted in minimal reductions in cell wall lignification (see Kumar et al. 2018). While major reductions in lignin concentration have been associated with poor plant fitness, smaller reductions in lignin provided measurable improvements in digestibility without significantly impacting agronomic fitness (Jung et al. 2012; see also Kumar et al. 2018).

The engineering of proteins along with pathways is the key strategy in achieving microbial biosynthesis and overproduction of pharmaceutical, chemical products, and biofuels.

11.5.5 Exploiting Diversity and Synthetic Biology for the Production of Algal Biofuels

Engineering of algal metabolism has an important role in the improvement of growth and biomass accumulation (Angermayr et al. 2009; US DOE 2010; Georgianna and Stephen 2012; Case and Atsumi 2016; Meyer et al. 2016; Shih et al. 2014). Manipulating the primary carbon-fixing enzyme Rubisco could also increase efficiency. The cultivation of algae in industrial photobioreactors or agricultural ponds aims to harvest as much solar energy as possible (Figs. 11.13 and 11.14) Efforts to improve photosynthetic efficiency have not been specific to algae; as a strategy, it has been proposed for increasing the yield of land plants to keep pace with increasing

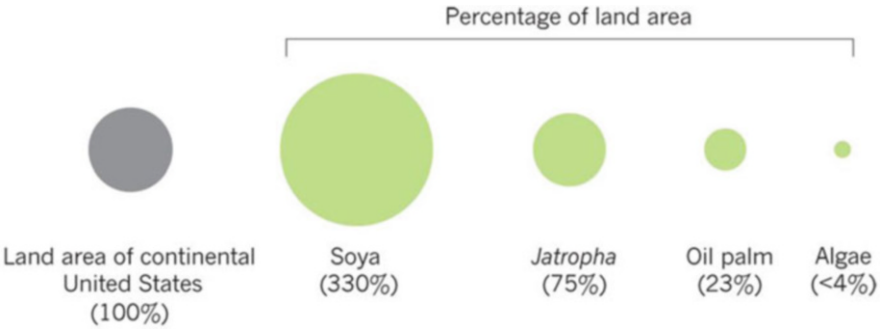

Fig. 11.12 Comparison of oleaginous crops. The United States consumes 25% of the world's petroleum. The land area needed to replace all domestic and imported petroleum used in the United States is shown as a percentage relative to the land area of the United States. The area required for algae is estimated to be significantly less than for any other biomass source (Dismukes et al. 2008). (Source: Georgianna, D. R. & Stephen, P. (2012). Exploiting diversity and synthetic biology for the production of algal biofuels, Nature *488*: 330–335. https://doi.org/10.1038/nature11479. Reproduced under license number 4646381493445 from RightsLink)

food demand where usable cropland is limited (US DOE 2010). Jagadevan et al. (2018) reviewed the upcoming field of microalgae employed as a model system for synthetic biology applications and highlighted the importance of genome-scale reconstruction models and kinetic models, to maximize the metabolic output by understanding the intricacies of algal growth (see also Georgianna and Stephen 2012) (Figs. 11.12, 11.13, and 11.14).

11.5.6 Biofuel from Protein Sources

According to Huo et al. (2011), biofuels are currently produced from carbohydrates and lipids in the feedstock. They suggested the use of proteins to synthesize fuels. Huo et al. (2011) applied metabolic engineering to generate *Escherichia coli* that can deaminate protein hydrolysates, enabling the cells to convert proteins to C4 and C5 alcohols at 56% of the theoretical yield (Huo et al. 2011) (Fig. 11.15).

Liu et al. (2017) reviewed the production of organic acids, especially carboxylic acids, as renewable sources of chemical products to substitute fossil fuels. They have been applied in a wide range of industries, including pharmaceutical, food, cosmetic, polymer, detergent, and textile (Becker and Wittmann 2016; Huo et al. 2011). The more economical and sustainable production of organic acids can be expected with the combination of these modern engineering techniques (Liu et al. 2017; Giessen and Silver 2017).

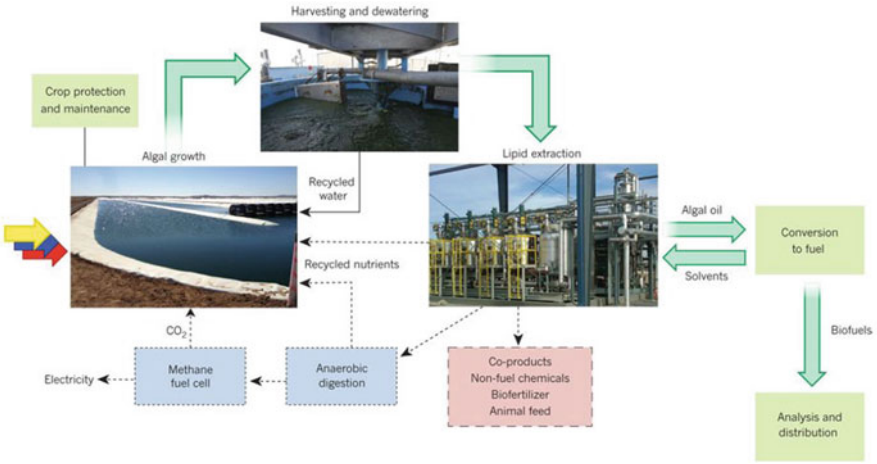

Fig. 11.13 Algal biofuel production: Light, water, and nutrients (yellow, blue, and red arrows) are required for algal growth in ponds. Some of the processes involved in algal biofuel production are common to most systems (green arrows). After fuel molecule extraction, there are alternative uses for algal biomass (dashed arrows); many of these can produce co-products that are beneficial for economic and life cycle analysis considerations. (Images courtesy of Sapphire Energy, San Diego, California). (Source: Georgianna, D. R., & Stephen, P. (2012). Exploiting diversity and synthetic biology for the production of algal biofuels, Nature *488*: 330–335. https://doi.org/10.1038/nature11479. Reproduced under license number 4646381493445 from RightsLink)

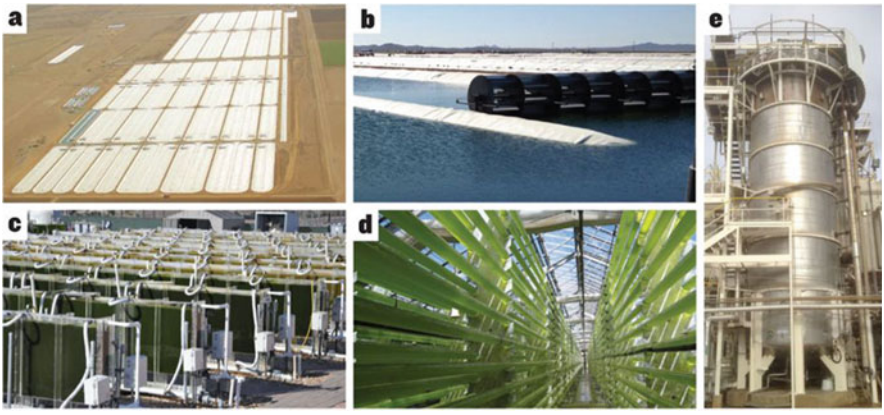

Fig. 11.14 Algae cultivation methods: (**a**) Algal ponds of 0.5 ha and 1 ha are part of the first commercial-scale algal biofuel facility in the United States at Sapphire Energy's Integrated Algal BioRefinery. They cover an area of 400 m wide by 1600 m long at a location near Columbus, New Mexico. (**b**) A single one-million-liter paddle-wheel-driven pond from the Columbus facility. (**c**) A pilot-scale flat panel photobioreactor developed at the Laboratory for Algae Research and Biotechnology at Arizona State University in Mesa (image courtesy of Q. Hu). (**d**) A commercial-scale tubular photobioreactor designed and constructed by IGV and operated by Salata in Germany (image courtesy of C. Grewe). (**e**) An industrial-scale fermentation tank for heterotrophic cultivation of microalgae at Martek Biosciences, part of DSM in Heerlen, the Netherlands (image courtesy of D. Dong). (Source: Georgianna, D. R. and Stephen, P. (2012). Exploiting diversity and synthetic biology for the production of algal biofuels. *Nature 488*: 330–335. https://doi.org/10.1038/nature11479. Reproduced under license number 4646381493445 from RightsLink)

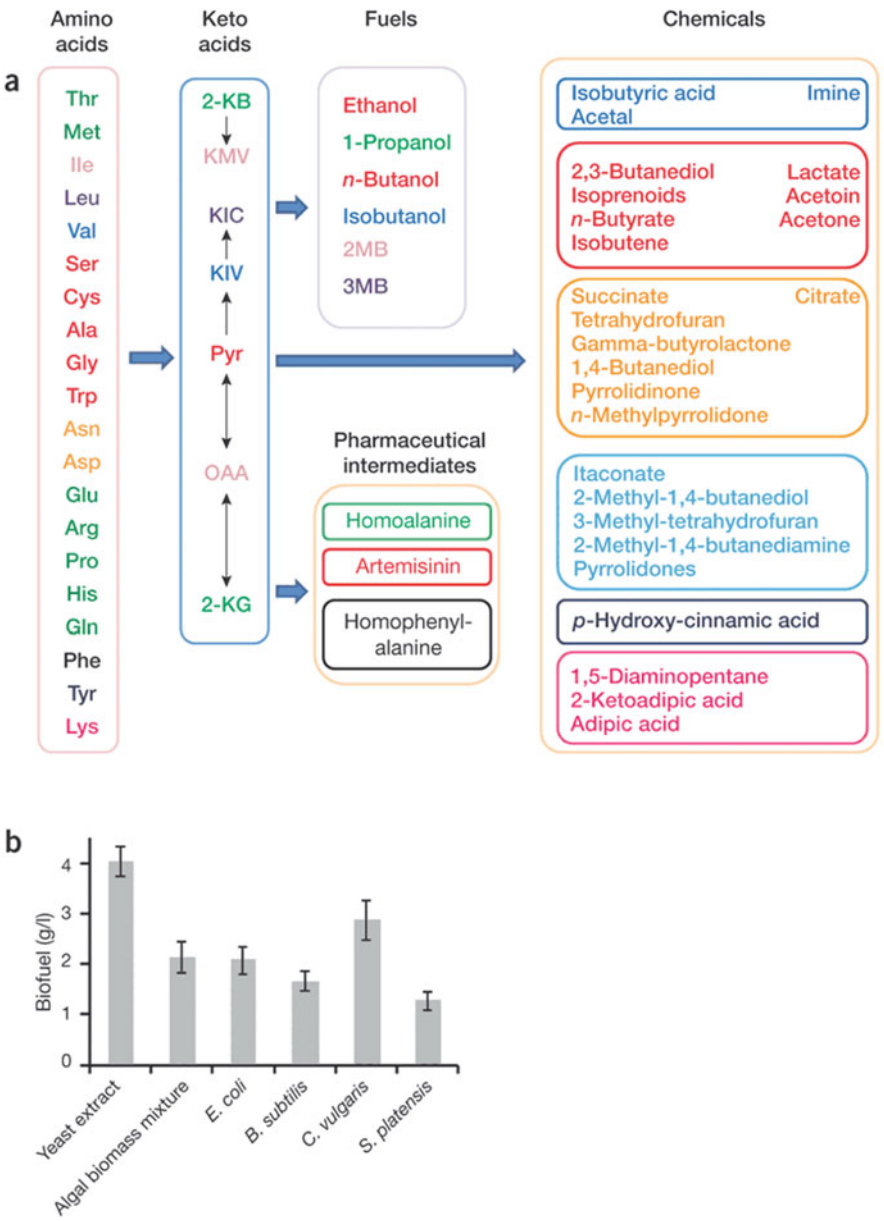

Fig. 11.15 Biofuel production and biorefining scheme from algal or bacterial protein sources: (**a**) The proposed protein-based biorefinery scheme. Amino acids are deaminated to various keto acids, which are then used to produce fuels, chemicals, and pharmaceutical intermediates. The colors link products and intermediates to the amino acids from which they are derived. (**b**) Biofuel (EtOH, iBOH, 2 MB, 3 MB) produced from the engineered *E. coli* strain YH83 grown in flasks using algal or bacterial cell hydrolysates. Small laboratory-scale reactors (1 L or 30 L) were used to grow bacterial and algal cells individually. The algal biomass mixture includes *C. vulgaris*, *P. purpureum*, *S. platensis*, and *S. elongatus*. All protein sources were adjusted to contain 21.6 g/L peptides and amino acids. Error bars indicate s.d. ($n = 3$). *OAA* oxaloacetate, *2-KB*

11.5.7 Metabolic Engineering in Methanotrophic Bacteria

Methane is 38-fold more effective at promoting global warming than carbon dioxide on a molar basis over a span of 20 years (Howarth 2015). Thus, harnessing methane is one of the most important near-term goals for biochemical engineering (Lee and Kim 2015). Methane as natural gas or biogas is the least expensive source of carbon for (bio)chemical synthesis (Kalyuzhnaya et al. 2015).

Methanotrophs are bacteria that grow on methane as their sole carbon and energy source. Methanotrophic bacteria and microbes converting methane into value-added products are both promising approaches for taking advantage of methane as a future bio-feedstock. There is resurgent interest in mitigating methane in the atmosphere as a greenhouse gas (Shindell et al. 2012) and in part its abundance, its low cost, and its potential to create liquid value-added products (Conrado and Gonzalez 2014). The activation of methane by a single species, *Methanosarcina acetivorans*, creates possibilities for metabolic engineering for anaerobic methane conversion to other products (Santos et al. 2011; Fei et al. 2014; see review Kalyuzhnaya et al. 2015; Soo et al. 2016; Mcanulty et al. 2017). It might also be possible to engineer strains that grow directly on cellulosic biomass, or other abundant and inexpensive substrates, such as methane or CO_2 (Espaux et al. 2015).

Despite these optimistic signs, a significant number of gaps in the fundamental knowledge of methanotrophy need to be filled to allow the potential of these systems to be fully reached (Kalyuzhnaya et al. 2015).

11.5.8 Engineered Microbial Biofuel Production and Recovery Under Supercritical Carbon Dioxide

Supercritical carbon dioxide ($scCO_2$) has been used for the depolymerization of lignocellulosic biomass to release fermentable sugars (Luterbacher et al. 2010). Brock et al. (2019) proposed a high-pressure fermentation strategy, coupled with in situ extraction using the abundant and renewable solvent supercritical carbon dioxide ($scCO_2$), which is also known for its broad microbial lethality to avoid end-product toxicity, culture contamination, and energy-efficient product recovery. They reported the domestication and engineering of a $scCO_2$-tolerant strain of *Bacillus megaterium*, to produce branched alcohols that have potential use as biofuels (Brock et al. 2019).

Fig. 11.15 (continued) 2-ketobutyrate. (Source: Huo, Y.-X., Cho, K. M., Rivera, J. G. L., Monte, E., Shen, C. R., Yan, Y. & Liao, J. C. (2011). Conversion of proteins into biofuels by engineering nitrogen flux. *Nature Biotechnology 29*(4): 346–351. https://doi.org/10.1038/nbt.1789. Reproduced with permission under license number 4646190098001 from RightsLink)

11.5.9 Solar-to-Chemical and Solar-to-Fuel Technology

Recent researches in solar-to-chemical and solar-to-fuel technology describe the use of solar energy to convert CO_2 to desired chemicals and fuels. The direct conversion of carbon dioxide to chemicals and fuels presents a sustainable solution for reducing greenhouse gas emissions and sustaining our supply of energy (Liao et al. 2016). According to Woo (2017), ultimately solar energy must be used for CO_2 reduction and conversions to provide a sustainable system, and this system is now available in the forms of solar-to-chemical (S2C) and solar-to-fuel (S2F) technologies. The S2C and S2F technology must be developed to capture and convert the essential feedstocks using only three inputs (CO_2, H_2O, and solar energy) to produce the desired value-added chemicals and fuels. Woo (2017) reviewed carbon capture utilization (CCU) for the reduction of greenhouse gas emissions.

Photoautotrophic cyanobacterial platforms have been extensively developed on this principle, producing a diverse range of alcohols, organic acids, and isoprenoids directly from CO_2 (Savakis and Hellingwerf 2015). Recent breakthroughs in the metabolic engineering of cyanobacteria, adoption of the light-harvesting mechanisms from nature, photovoltaics-derived water-splitting technologies have been integrated with microbial biotechnology to produce desired chemicals (Woo 2017).

Photosynthetic organisms (including cyanobacteria) have been engineered to produce value-added chemicals, providing a number of promising S2C and S2F platforms. Thus, hybrid systems comprising an electrochemical in situ hydrogen-evolution reaction at the electrode and the biological CO_2 fixation using autotrophic bacteria have been suggested as an alternative S2C and S2F platform.

11.5.10 Implementing CRISPR–Cas Technologies for Obtaining High-Value Products

Several approaches of rebalancing or rewiring of the metabolic network and the use of dynamic metabolic control strategies to conditionally reduce essential competitive fluxes have yielded better results. Liu et al. (2013) reviewed recent advances that allow more precise regulation of gene expression in plants, including synthetic promoters, transcriptional activators, and repressors.

The use of newer gene silencing technologies, including CRISPR interference, makes transcriptional tuning an attractive platform for any desired microbe (Lynch 2016). Success in using CRISPR–Cas9 for gene targeting in laboratory *S. cerevisiae* strains was first demonstrated in 2013 (DiCarlo et al. 2013) Estrela and Cate (2016) reviewed the use of CRISPR–Cas9 technology for energy biotechnology in *S. cerevisiae.* They further reported that recently, other bacteria have been successfully edited, such as *Streptomyces* (Cobb et al. 2015; Huang et al. 2015; Tong et al. 2015), *Lactobacillus reuteri* (Oh and van Pijkeren 2014), *Taumatella citrea* (Jiang et al. 2015), *Streptococcus pneumoniae*, and *E. coli* (Jiang et al. 2015).

In metabolic engineering, of photosynthetic, cyanobacteria can use CO_2 as a building block to synthesize carbon-based chemicals. In recent years, clustered regularly interspaced short palindromic repeats (CRISPR)-dependent approaches have rapidly gained popularity for engineering cyanobacteria. Behler et al. (2018) reviewed CRISPR-based tools for the metabolic engineering of cyanobacteria. Rather than utilizing CRISPR-based genome editing, CRISPR interference (CRISPRi) offers an alternative, viable approach for cyanobacterial engineering which relies on an enzymatically inactive dead Cas9 (dCas9) (Yao et al. 2016). Increased understanding of various CRISPR mechanisms and systems will undoubtedly inspire more advanced approaches for the engineering of biological hosts such as cyanobacteria (Behler et al. 2018).

Yeasts are widely used host organisms in biotechnology to produce fine chemicals, industrial biocatalysts, biopharmaceuticals, food additives, and renewable biofuels (Kim et al. 2015). Within 5 years, the CRISPR–Cas system has emerged as the dominating tool for genome engineering while also changing the speed and efficiency of metabolic engineering in conventional (*Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*) and non-conventional (*Candida albicans*, *Yarrowia lipolytica*, *Pichia pastoris* syn. *Komagataella phaffii*, *Kluyveromyces lactis*, and *C. glabrata*) yeasts (Raschmanová et al. 2018).

11.6 Discussion

Metabolic pathway optimization is generally a very challenging endeavor because of the complex regulation that cells have evolved to maintain homeostasis and robustness (Nielsen and Keasling 2016: Wang et al. 2017). In vitro synthetic biosystems provide several other biomanufacturing advantages, such as easy product separation, open process control, fast reaction rate, broad reaction condition, tolerance to toxic substrates, etc. According to Lynch (2016), many challenges still remain; these recent efforts further support the potential of this discipline in making a significant impact in the production of high-volume industrial products, with the potential to displace petroleum with more sustainable alternatives. According to Woo (2017), synthetic biology-inspired metabolic engineering of next-generation microbes will be established to accommodate more efficient S2C and S2F platforms.

Hence, rather than trying to understand how synthetic biology is shaped by commercial forces, it might be better to understand sciences like synthetic biology as co-emerging with new market regimes and forms. Energy-rich parts of the world look to the Global South. As many observers have pointed out, biofuel crops compete with food crops and through deforestation reduce biodiversity more generally (Chakravorty et al. 2009; Shaik and Kumar 2014; Kumar et al. 2018).

According to Mackenzie (2013), in synthetic biology, this conflict between food and fuel is mentioned as something that must be avoided in the development of advanced biofuels by using microbes to produce fuel without relying too heavily on feedstocks or other inputs that compete with agriculture.

11.7 Conclusion

Sustainable large-scale production of biofuels will require the integration of knowledge across many disciplines. In the short term, the major research opportunities for plant biologists seem to be in identifying promising species, knowing paths of biofuel production, and altering genes to produce more or insert missing links or synthesize required protein into organisms. Large parts of next-generation biofuels exist in partial realizations: metabolic models, research projects, pilot plants, and various other technologies in testing. As the industrial reality of synthetic biology, next-generation biofuels can also prompt us to consider synthetic biology less from the perspective of epistemic value and more from the perspective of the mode of existence of technical objects.

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I also acknowledge with thanks the different figures with legends reproduced with permissions from the following publications: Figure 11.1. Alper, H. & Stephanopoulos, G. (2009). Engineering for biofuels: exploiting innate microbial capacity or importing biosynthetic potential, Nature Reviews. Microbiology *7*: 715–723. Retrieved from https://doi.org/10.1038/nrmicro2186. Reproduced with license number 46456408400514. Figure 11.2**.** Liao, J. C., Mi, L., Pontrelli, S. & Luo, S. (2016). Fuelling the future: microbial engineering for the production of sustainable biofuels. *Nature Publishing Group*, Nature Review. Microbiology *14*(5): 288–304. https://doi.org/10.1038/nrmicro.2016.32. Reproduced under license number 4645730007098. Figure 11.3. Martien J.I. and Amador-Noguez D. (2017). Recent applications of metabolomics to advance microbial biofuel production. *Current Opinion in Biotechnology*, 43, 118–126. https://doi.org/10.1016/j.copbio.2016.11.006. Reproduced with permission license number 4666750205840. Figure 11.4. Jones, J.A., Toparlak, Duhan, Ö., and Koffas, Mattheos AG (2015). Metabolic pathway balancing and its role in the production of biofuels and chemicals. *Current Opinion in Biotechnology 33*, 52–59. https://doi.org/10.1016/j.copbio.2014.11.013. Reproduced with permission no 4671031226483. Figures 11.5 and 11.6. Peralta-Yahya, P. P., Ouellet, M., Chan, R., Mukhopadhyay, A., Keasling, J. D., & Lee, T. S. (2011). Identification and microbial production of a terpene-based advanced biofuel. *Nature Communications 2*(May): 483–488. https://doi.org/10.1038/ncomms1494. This is an open-access article distributed under the terms of the Creative Commons CC-BY license, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Figures 11.7 and 11.8. Ma, T., Shi, B., Ye, Z., Li, X., Liu, M., Chen, Y. & Nielsen, J. (2019). Lipid engineering combined with systematic metabolic engineering of *Saccharomyces cerevisiae* for high-yield production of lycopene. *Metabolic Engineering 52*(November 2018): 134–142. https://doi.org/10.1016/j.ymben.2018.11.009. Reproduced under license number 4651230668162. Figure 11.9. Peralta-Yahya P. P. et al. (2012). Microbial engineering for the production of advanced biofuels. https://doi.org/10.1038/nature488320–328. Reproduced with license no. 4643340791481. Figure 11.10. Zhang, Y. P. (2015). Production of biofuels and biochemicals by in vitro synthetic biosystems: Opportunities and challenges. *Biotechnology Advances 33*(7): 1467–1483. https://doi.org/10.1016/j.biotechadv.

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