Synthetic Biology for Biofuels in *Saccharomyces cerevisiae*

8

Wei Suong Teo, Yu Chyuan Heng, Binbin Chen, Hui Ling Lee, Niying Chua, and Matthew Wook Chang

Contents

1 Introduction 134
1.1 Metabolic Engineering 134
1.2 Synthetic Biology Advances Metabolic Engineering 135
2 Short-Chain Alcohols 136
2.1 Isobutanol 136
2.2 n-Butanol 139
2.3 Other Short-Chain Alcohols 140
3 Fatty Acid Derivatives 141
3.1 Fatty Alcohols 141
3.2 Alkanes and Alkenes 143
3.3 Fatty Acid Esters 144
4 Isoprenoids 145
5 Research Needs 150
References 150

W.S. Teo • B. Chen • H.L. Lee • N. Chua • M.W. Chang (✉)
Department of Biochemistry, Yong Loo Lin School of Medicine, and NUS Synthetic Biology for Clinical and Technological Innovation (SynCTI), Life Sciences Institute, National University of Singapore, Singapore, Singapore

School of Life Sciences and Chemical Technology, Ngee Ann Polytechnic, Singapore, Singapore
e-mail: tws3@np.edu.sg; bchc@nus.edu.sg; bchlhl@nus.edu.sg; bchchn@nus.edu.sg; bchcmw@nus.edu.sg

Y.C. Heng
Department of Biochemistry, Yong Loo Lin School of Medicine, and NUS Synthetic Biology for Clinical and Technological Innovation (SynCTI), Life Sciences Institute, National University of Singapore, Singapore, Singapore

Institute of Chemical and Engineering Sciences, Agency for Science, Technology and Research (A*STAR), Singapore, Singapore
e-mail: a0133094@u.nus.edu

S.Y. Lee (ed.), *Consequences of Microbial Interactions with Hydrocarbons, Oils, and Lipids: Production of Fuels and Chemicals*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50436-0_386

Abstract

The yeast *Saccharomyces cerevisiae* has crucial features to facilitate successful genetic engineering and industrial scale fermentations for producing drop-in biofuels. Short-chain alcohols, fatty acid derivatives, and isoprenoids are potential drop-in biofuels where their biosynthesis can help mitigate climate change while ensuring sustainability of energy supply. Here, we review the drop-in biofuel molecules that have been produced in engineered *S. cerevisiae*. Efforts to diversify and optimize biofuel production using synthetic biology and metabolic engineering approaches are discussed. Much improvement will be required to achieve commercial viability.

1 Introduction

The production of biofuels from microbes contributes to ensuring energy security and protecting the environment from destructive drilling for fossil fuels. However, the conventional biofuel, bioethanol is incompatible with current transportation infrastructure and can only be used as blends with gasoline. As a result, the development of drop-in biofuels that can be directly used in fuel distribution systems to power present-day engines holds great importance.

S. cerevisiae has important characteristics as a host organism for biofuel production. This includes the abundance of knowledge about its genetics, physiology, biochemistry, and methods for its genetic manipulation, which will facilitate its engineering. In addition, the ability of *S. cerevisiae* for robust growth in large-scale fermentations at low pH and its resistance to phage attack are crucial for scaling up the bioprocesses. Differing from bioethanol which is its native product, production of drop-in biofuels in *S. cerevisiae* is made possible with metabolic engineering strategies aided by synthetic biology tools.

1.1 Metabolic Engineering

Metabolic engineering emerged early in the 1990s and was first defined as *the directed modulation of metabolic pathways using methods of recombinant technology for the purpose of overproducing fuels and chemical and pharmaceutical products* (Bailey 1991). At its core, metabolic engineering aims to design, construct, and optimize metabolic pathways from a systemic point of view. Strategies such as overexpression of rate-limiting enzymes, deletion of competing pathways, cofactor balancing, and tolerance enhancement are central to metabolic engineering. However, initial implementation of such metabolic engineering strategies was often labor-intensive and restricted to the reconstruction of native metabolic pathways within the host organisms due to a lack of effective tools for engineering.

1.2 Synthetic Biology Advances Metabolic Engineering

While the goal of metabolic engineering to realize the commercially viable microbial production of biochemicals is clearly stated, the means for this goal to be achieved are less well defined, and this is exactly where synthetic biology comes into play. Synthetic biology is an emerging scientific discipline that aims to apply engineering principles of modularity, characterization and standardization to the design, and construction of novel biological systems for the investigation of fundamental biological questions as well as the generation of novel applications. Although still regarded as a relatively young discipline, this field is fast developing – evident by the expanding number of works published and the collection of synthetic biology toolsets currently available.

The realization of high-throughput DNA synthesis not only enabled the advancement of synthetic biology, it also improved how metabolic engineering is carried out. With the availability of genome databases of various organisms and enhanced sequence-to-function knowledge, novel synthetic metabolic pathways can now be constructed through integration of heterologous enzymes and pathways into host organisms to either improve existing functions or create novel functions such as non-native substrate utilization and product formation abilities. Such efforts will be challenging without the availability of a myriad of synthetic biology toolsets that assist at different levels of engineering. Firstly, novel biological part assembly and genome editing tools facilitate combinatorial assembly of DNA elements and genomic modification, speeding up pathway, strain, and library construction. Secondly, well-characterized modular genetic regulatory parts including promoters, terminators, riboswitches, and biosensors allow dynamic temporal regulation of pathway gene expression, permitting fine-tuning of metabolic fluxes to boost product formation. Thirdly, spatial engineering strategies such as pathway compartmentalization, synthetic scaffolding, and protein fusion force components of multistep enzymatic pathways into closer physical proximity, improving pathway efficiency through reduction of intermediate loss and transit time. And lastly, genetic circuit- or biosensor-guided directed evolution accelerates strain optimization, permitting the isolation of genetic variants with phenotypes of improved product formation or improved tolerance toward toxic intermediates or end products (Fig. 1). For the latest reviews on the application of synthetic biology tools for yeast metabolic engineering, readers are referred to (Jensen and Keasling 2014; Williams et al. 2016; Zhang et al. 2015).

Clearly, the emergence of synthetic biology has advanced metabolic engineering by transforming and accelerating the ways how modulation and optimization of metabolic pathways and production strains could be done. In the following parts of this review, the scope will be focused on synthetic biology and metabolic engineering efforts to engineer *S. cerevisiae* for production of drop-in biofuels. These drop-in biofuels include short-chain alcohols, fatty acid derivatives, and isoprenoids.

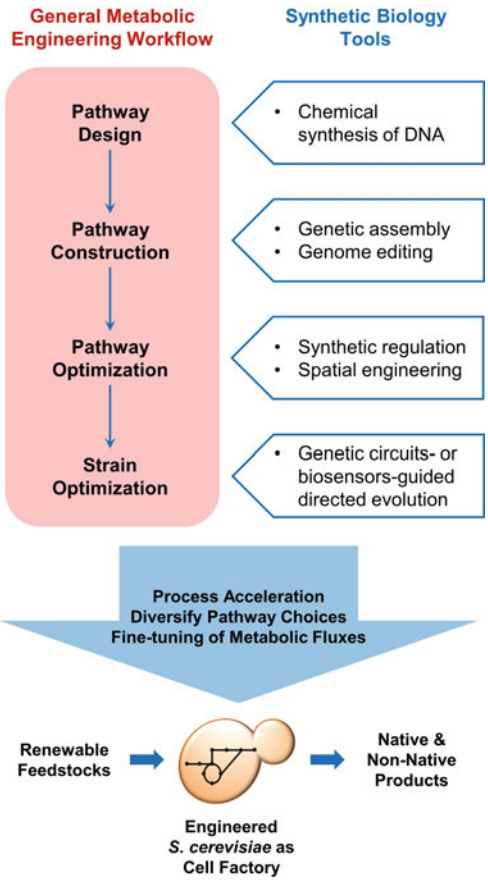

Fig. 1 Incorporation of synthetic biology tools into metabolic engineering workflow and their advantages

2 Short-Chain Alcohols

Ethanol is the most well-known and commonly used short-chain alcohol biofuel. New short-chain alcohol biofuel molecules, mainly butanol isomers, have been identified as viable petrol substitutes due to their high octane number and low hygroscopy (Generoso et al. 2015). The scope of this section will focus on drop-in short-chain alcohol biofuels produced using engineered *S. cerevisiae* (Fig. 2 and Table 1).

2.1 Isobutanol

S. cerevisiae naturally produces small amounts of isobutanol via the Ehrlich pathway from the catabolism of valine (Hazelwood et al. 2008). Isobutanol can also be produced from pyruvate in a biosynthetic pathway, where two molecules of pyruvate are

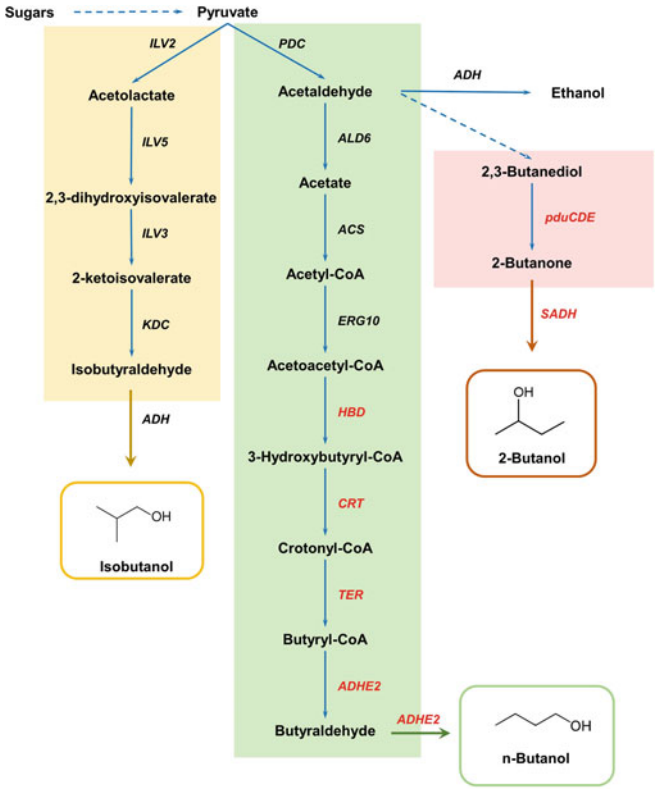

Fig. 2 Biosynthetic pathways of short-chain alcohol biofuels in engineered *S. cerevisiae*. Heterologous genes are colored in *red*. *Dotted lines* indicate multistep reactions. *ILV2* acetolactate synthase, *ILV5* acetohydroxyacid reductoisomerase, *ILV3* dihydroxyacid dehydratase, *KDC* 2-ketoacid decarboxylase, *ADH* alcohol dehydrogenase, *PDC* pyruvate decarboxylase, *ALD6* aldehyde dehydrogenase, *ACS* acetyl-CoA synthetase, *ERG10* acetyl-CoA acetyltransferase, *HBD* 3-hydroxybutyryl-CoA dehydrogenase, *CRT* crotonase, *TER* trans-2-enoyl-CoA reductase, *ADHE2* butyraldehyde dehydrogenase, *pduCDE* diol dehydratase, *SADH* secondary alcohol dehydrogenase

condensed into acetolactate (ALAC) by acetolactate synthase (ILV2). ALAC is then reduced to 2,3-dihydroxyisovalerate (DIV) by acetohydroxyacid reductoisomerase (ILV5). This is followed by conversion of DIV to 2-ketoisovalerate (KIV) by dihydroxyacid dehydratase (ILV3). KIV is decarboxylated by 2-ketoacid decarboxylase (KDC) to isobutyraldehyde, which is then reduced to isobutanol by alcohol dehydrogenase (ADH).

In the first report to engineer yeast for isobutanol production, the biosynthetic enzymes ILV2, ILV3, and ILV5 and the branched-chain amino-acid aminotransferase BAT2 were overexpressed (Chen et al. 2011). Observing that the isobutanol biosynthesis pathway occurred partly in the mitochondria and partly in the cytosol, researchers hypothesized that locating all the enzymes within the same compartment

Table 1 Short-chain alcohol biofuels produced by engineered *S. cerevisiae*

Biofuel	Titer (mg/L)	Carbon source (g/L)	References
Isobutanol	154	Glucose (40)	Chen et al. (2011)
	63	Glucose (20)	Matsuda et al. (2012)
	143	Glucose (20)	Kondo et al. (2012)
	151	Glucose (40)	Lee et al. (2012)
	630	Glucose (40)	Brat et al. (2012)
	58	Glucose (20), glycine (15)	Branduardi et al. (2013)
	635	Glucose (100)	Avalos et al. (2013)
	1620	Glucose (100)	Matsuda et al. (2013)
	377	Glucose (100)	Park et al. (2014)
	224	Glucose (20)	Ida et al. (2015)
	600	Galactose (38), glucose (2)	Yuan and Ching (2015)
	331	Glucose (20)	Park et al. (2016)
n-Butanol	2.5	Galactose (20)	Steen et al. (2008)
	16.3	Glucose (20)	Krivoruchko et al. (2013)
	92	Glucose (20), glycine (15)	Branduardi et al. (2013)
	120	Glucose (20)	Lian et al. (2014)
	243	Glucose (20)	Si et al. (2014)
	20	Glucose (20)	Lian and Zhao (2015)
	130	Glucose (20)	Schadeweg and Boles (2016)
	1050	Glucose (20)	Shi et al. (2016)
2-Butanol	4	Glucose (20)	Ghiaci et al. (2014)
3-Methyl-1-butanol	130	Glucose (100)	Avalos et al. (2013)
	766	Glucose (100)	Park et al. (2014)
2-Methyl-1-butanol	118	Glucose (100)	Avalos et al. (2013)

would increase the production of isobutanol. This proved true as relocating ILV2, ILV5, and ILV3 out of the mitochondrial matrix into the cytosol improved isobutanol production (Brat et al. 2012; Matsuda et al. 2012). Relocation of the metabolic pathway into the mitochondria by relocating KDC and ADH also increased isobutanol production (Avalos et al. 2013; Yuan and Ching 2015).

Efforts to further optimize isobutanol production through the elimination of competing pathways were carried out. These include (i) channeling pyruvate toward isobutanol production instead of acetyl-CoA biosynthesis through deletion of LPD1, a subunit of pyruvate dehydrogenase (Matsuda et al. 2013); (ii) reducing ethanol flux via pyruvate by deletion of PDC1, a major pyruvate decarboxylase (Kondo et al. 2012); (iii) blocking the isobutyrate, pantothenate, or isoleucine biosynthetic pathways through deletion of aldehyde dehydrogenase (ALD6), ketopantoate hydroxymethyltransferase (ECM31), or threonine deaminase ILV1, respectively (Ida et al. 2015); and (iv) deleting branched-chain amino acid aminotransaminase (BAT1), involved in valine biosynthesis (Park et al. 2014).

Furthermore, isobutanol production was improved through codon optimization and screening for highly active enzyme candidates (Brat et al. 2012; Lee et al. 2012), as well as expression of a constitutively active Leu3Δ601 transcriptional activator (Park et al. 2014) to increase transcription of endogenous genes in the valine and leucine biosynthetic pathways. As cofactor imbalance limits isobutanol production, overexpression of enzymes responsible for transhydrogenase-like shunts such as pyruvate carboxylase, malate dehydrogenase, and malic enzyme boosted NADPH cofactor availability and improved isobutanol production (Matsuda et al. 2013). Recently, the mitochondrial-localized pathway was optimized by increasing the mitochondrial pool of pyruvate through mitochondrial pyruvate carrier expression and removal of competing pathways by deletion of BAT1, ALD6, and LPD1 (Park et al. 2016).

Despite the aforementioned engineering approaches, isobutanol production in yeast is still unsatisfactory, indicating the existence of rate-limiting steps. Possible reasons for suboptimal yields were explored using a cytosolic, cofactor-balanced isobutanol pathway. Massive accumulation of pathway intermediates and ALAC degradation products was observed, which is consistent with the hypothesis that the enzyme ILV3, which possesses iron–sulfur clusters, could be a key bottleneck (Milne et al. 2016).

2.2 n-Butanol

The initial pathway used to produce n-butanol in yeast was first derived from *Clostridium spp.* (Steen et al. 2008). In this pathway, two molecules of acetyl-CoA are condensed to acetoacetyl-CoA, which is converted to 3-hydroxybutyryl-CoA and then into crotonyl-CoA. Crotonyl-CoA is reduced to butyryl-CoA, followed by butyraldehyde, and finally, n-butanol. In the first demonstrated n-butanol production in *S. cerevisiae*, enzyme homologs from different organisms were screened for highly active clostridial n-butanol biosynthetic pathway enzymes (Steen et al. 2008).

To boost n-butanol production, researchers sought to increase the availability of cytosolic acetyl-CoA. This was done by expressing alcohol dehydrogenase (ADH2), ALD6, acetyl-CoA synthetase (ACS), and acetyl-CoA acetyltransferase (ERG10), in strains lacking malate synthase (MIS1) or citrate synthase (CIT2) (Krivoruchko et al. 2013). n-Butanol production was further improved by redirecting glycolytic flux toward acetyl-CoA through inactivation of alcohol dehydrogenases (ADH1 and ADH4) and glycerol-3-phosphate dehydrogenases (GPD1 and GPD2) which are involved in ethanol formation and needed for glycerol production, respectively, and expression of pyruvate dehydrogenase (PDH), PDH-bypass, and ATP-dependent citrate lyase (ACL) (Lian et al. 2014). Availability of acetyl-CoA was also increased by overexpressing pantothenate kinase (CoaA), adding pantothenate to the medium and expressing an ATP-independent acetylating acetaldehyde dehydrogenase, ADHE (A267T/E568K) (Schadeweg and Boles 2016).

Besides the clostridial pathway, other n-butanol biosynthetic pathways have also been discovered. An endogenous n-butanol pathway dependent on the catabolism of threonine in *S. cerevisiae* was found (Si et al. 2014). The leucine biosynthetic pathway enzymes, KDCs, and ADHs were able to convert glucose via threonine into n-butanol. By overexpressing pathway enzymes and deleting competing pathways (ADH1 and ILV2), n-butanol production was improved. The researchers further showed that a mutant HOM3 allele encoding a feedback-insensitive aspartate kinase enabled deregulation and overproduction of threonine. The localization of the n-butanol pathway to the mitochondria further increased the production level. In addition, a citramalate synthase (CIMA), which enables an alternative route to α-ketobutyrate from pyruvate and acetyl-CoA, was able to act synergistically with the threonine degradation pathway. Overexpression of LEU1, LEU2, and LEU4 which catalyze reactions converting α-ketobutyrate to α-ketovalerate, LEU5, a mitochondrial carrier protein involved in the accumulation of CoA in the mitochondrial matrix, NFS1, a cysteine desulfurase to stabilize LEU1, CIMA, ADH, and KDC, further improved n-butanol production (Shi et al. 2016). Other notable approaches to n-butanol production were attempted including the reversal of the β-oxidation pathway in the cytosol (Lian and Zhao 2015) and the use of a putative glycine degradation pathway (Branduardi et al. 2013).

2.3 Other Short-Chain Alcohols

In order to produce 2-butanol, a heterologous pathway which converts 2,3-butanediol to 2-butanol via 2-butanone was engineered (Ghiaci et al. 2014). Using a TEV-cleavage-based system, all three subunits of a B12-dependent diol dehydratase from *Lactobacillus reuteri* and its two-subunit activating enzyme were expressed in equal amounts. The mature dehydratase enzyme, together with a secondary alcohol dehydrogenase from *Gordonia spp.*, enabled 2-butanol production. Production was boosted by increasing the availability of NADH, which was achieved by deleting GPD1 and GPD2 and growth under anaerobic conditions.

S. cerevisiae naturally produces small amounts of 3-methyl-1-butanol and 2-methyl-1-butanol via the Ehrlich pathway from the catabolism of leucine or isoleucine, respectively (Hazelwood et al. 2008). By engineering yeast to produce isobutanol through relocation of the isobutanol pathway into the mitochondria, the production of 3-methyl-1-butanol and 2-methyl-1-butanol was also increased. This could be attributed to the considerable overlap in the upstream ILV genes (ILV2, ILV3 and ILV5) and identical downstream, Ehrlich degradation pathways of KDC and ADH (Avalos et al. 2013). In another work, leucine biosynthetic genes LEU2 and $LEU4^{D578Y}$, a feedback inhibition-insensitive mutant of LEU4, were additionally overexpressed in a strain engineered for isobutanol production, resulting in increased 3-methyl-1-butanol production (Park et al. 2014).

3 Fatty Acid Derivatives

Fatty acid derivatives, such as fatty alcohols, alkanes, alkenes, and fatty acid esters, have a wide range of applications such as surfactants, lubricants, detergents, and fuels. With the introduction of novel pathways in yeast, endogenous fatty acids can be converted into useful fatty acid derivatives (Fig. 3 and Table 2).

3.1 Fatty Alcohols

To produce fatty alcohols in *S. cerevisiae*, NADPH-dependent fatty acyl-CoA reductase (FAR) from mouse (*Mus musculus*), mFAR1, which reduces fatty acyl-CoAs in the cell into fatty alcohols, was expressed. Overexpression of fatty acid biosynthetic genes, specifically acetyl-CoA carboxylase (ACC1), fatty acid synthase

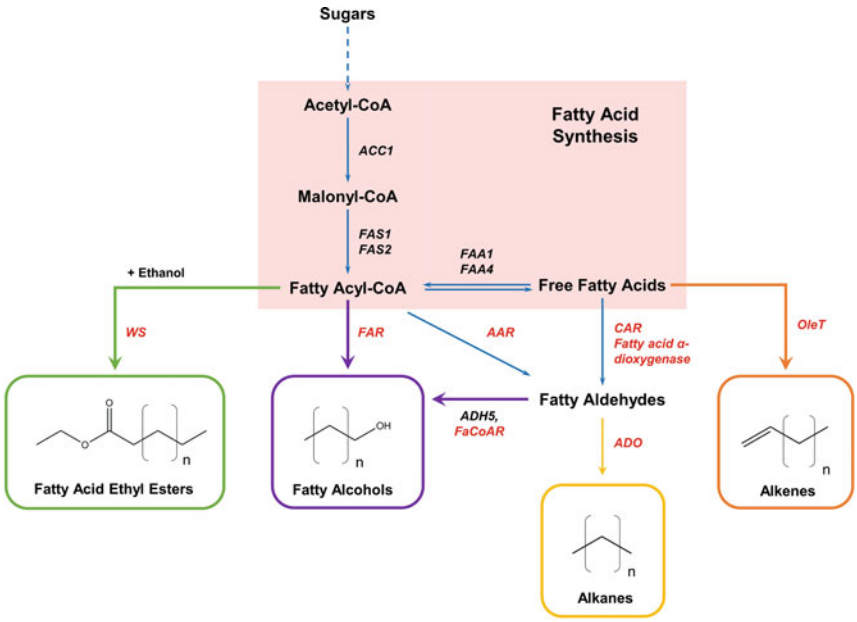

Fig. 3 Biosynthetic pathways of fatty-acid-derived biofuels in engineered *S. cerevisiae*. Heterologous genes are colored in *red*. *Dotted lines* indicate multistep reactions. *ACC1* acetyl-CoA carboxylase, *FAS1* fatty acid synthase 1, *FAS2* fatty acid synthase 2, *FAA1* fatty acyl-CoA synthetase 1, *FAA4* fatty acyl-CoA synthetase 4, *WS* wax ester synthase, *FAR* fatty acyl-CoA reductase, *AAR* fatty acyl-CoA/ACP reductase, *CAR* carboxylic acid reductase, *ADH5* alcohol dehydrogenase 5, *FaCoAR* bi-functional fatty acyl-CoA reductase, *ADO* fatty aldehyde deformylating oxygenase, *OleT* fatty acid decarboxylase

Table 2 Fatty acid derived biofuels produced by engineered *S. cerevisiae*

Biofuel	Titer (mg/L)	Carbon source (g/L)	References
Fatty alcohol	98	Galactose (18), glucose (2)	Runguphan and Keasling (2014)
	98	Galactose (60)	Tang and Chen (2015)
	1111	Glucose (100)	Feng et al. (2015)
	1300	Glucose (30)	Sheng et al. (2016)
	1500	Glucose (~320)	Zhou et al. (2016)
	20.3	Glucose (20)	Jin et al. (2016)
Alkane	0.022 mg/gDCW	Glucose (30)	Buijs et al. (2015)
	0.07	Galactose (18), glucose (2)	Foo et al. (2015)
	0.8	Glucose (30)	Zhou et al. (2016)
Alkene	3.7	Glucose (180)	Chen et al. (2015)
Fatty acid ethyl ester	n.i.	Galactose (20)	Kalscheuer et al. (2004)
	520	Glycerol (20), sodium oleate (1)	Yu et al. (2012)
	8.2	Glucose (20)	Shi et al. (2012)
	5.4	Galactose (18), glucose (2)	Runguphan and Keasling (2014)
	15.8	Glucose (20)	Shi et al. (2014a)
	17.2	Glucose (20)	Valle-Rodríguez et al. (2014)
	25.4	Glucose (114)	Thompson and Trinh (2014)
	48	Glucose (20)	Shi et al. (2014b)
	5100 mg/gCDW	Glucose (20)	de Jong et al. (2014)
	4.4	Glucose (20)	de Jong et al. (2015)
	10.5 mg/gCDW	Glucose (20)	Eriksen et al. (2015)
	n.i.	Glucose (20)	Lian and Zhao (2015)
Fatty acid short- and branched-chain alkyl esters	234	Glucose (20), galactose (20)	Teo et al. (2015)

n.i. not indicated in the publication, *gDCW* g dry cell weight

1 (FAS1), and fatty acid synthase 2 (FAS2), as well as the malic enzyme from the oleaginous fungus *Mortierella alpina* to increase cytosolic NADPH level, led to increased fatty alcohol production (Runguphan and Keasling 2014).

Fatty alcohols were also produced by expressing a FAR from barn owl (*Tyto alba*), TaFAR. Together with ACC1 overexpression, the production of fatty alcohols

was enhanced by expressing a heterologous ATP-dependent citrate lyase, which increases the cytosolic acetyl-CoA supply, and knocking out RPD3, which negatively regulates the INO1 gene involved in phospholipid metabolism (Feng et al. 2015). Fatty alcohol production titers could also be improved by using the same TaFAR enzyme and deleting the DGA1 gene to block the fatty acyl-CoAs-dependent pathway of TAG synthesis (Tang and Chen 2015).

Medium chain fatty alcohols were produced via targeted expression of TaFAR in the peroxisome of *S. cerevisiae*. Compartmentalizing TaFAR into the peroxisomal matrix enabled interception of medium chain fatty acyl-CoAs generated from the β-oxidation pathway and their conversion into medium chain fatty alcohols. Along with the previously mentioned ACC1, overexpression of PEX7 to enhance TaFAR targeting efficiency into the peroxisome further improved fatty alcohol production (Sheng et al. 2016).

Instead of using FARs to convert fatty acyl-CoAs into fatty alcohols, a pathway utilizing free fatty acids (FFAs) was engineered. The expression of *Mycobacterium marinum* carboxylic acid reductase (MmCAR) converts FFAs into fatty aldehydes, which are then reduced to fatty alcohols by overexpressed ADH5. To increase fatty alcohol production, FFA intermediates are accumulated through deletion of the main fatty acyl-CoA synthetase-encoding genes FAA1 and FAA4, which prevent reactivation of FFAs into fatty acyl-CoAs. Further, POX1 encoding the fatty acyl-CoA oxidase was also deleted to prevent FFAs degradation through β-oxidation. Deletion of the aldehyde dehydrogenase-encoding genes HFD1 and ADH6, coupled with expression of a bi-functional fatty acyl-CoA reductase from *Marinobacter aquaeolei VT8* (FaCoAR) which has high activity toward long-chain fatty aldehydes, further increased fatty alcohol production (Zhou et al. 2016). Introduction of a biosynthetic pathway involving cytosolic thioesterase, rice α-dioxygenase, and endogenous aldehyde reductases into a yeast strain lacking FAA1 and FAA4 genes enabled production of odd chain-length fatty alcohols (Jin et al. 2016).

3.2 Alkanes and Alkenes

To produce alkanes in yeast, *Synechococcus elongatus* fatty acyl-CoA/ACP reductase (SeAAR), which converts fatty acyl-CoAs into fatty aldehydes, and fatty aldehyde deformylating oxygenase (SeADO), which reduces fatty aldehydes into alkanes, were expressed. Elimination of HFD1 and expression of a redox system were found to be essential for alkane biosynthesis (Buijs et al. 2015). In a bid to avoid using fatty acyl-CoAs as substrates for alkane production, an alternative pathway was engineered to convert sugars to alkanes via FFAs, where a fatty acid α-dioxygenase from rice was expressed in *S. cerevisiae* to convert FFAs to fatty aldehydes. Co-expression of a SeADO converted the aldehydes into the desired alkanes. Alkane production was increased by overproducing FFAs through deletion of FAA1 and FAA4 (Foo et al. 2015). Production of alkanes from FFAs was also carried out using MmCAR to catalyze FFAs conversion into fatty aldehydes. HFD1

and POX1 were deleted to boost fatty acid supply, whereas ADH5 deletion led to an increased alkane production and decreased fatty alcohol accumulation. To further increase flux toward alkanes, the expression of SeADO was upregulated, one copy of MmCAR was integrated into the genome, and *Nostoc punctiforme* NpADO was expressed (Zhou et al. 2016).

By introducing a one-step fatty acid decarboxylation pathway into yeast, terminal alkenes were produced (Chen et al. 2015). In an attempt to boost production level, fatty acid decarboxylases (OleT) homologs were screened to select highly active enzymes and the best performing OleT was found to be from *Jeotgalicoccus sp. ATCC 8456* (OleTJE). The engineering of precursor and cofactor availability and OleTJE gene expression tuning further improved production.

3.3 Fatty Acid Esters

Fatty acid ethyl esters (FAEEs) are biodiesels converted industrially from chemical transesterification and esterification processes. Bioconversion of fatty acyl-CoAs into FAEEs requires a fatty acyl-CoA:alcohol transferase (wax ester synthase, WS) that can accept ethanol as the alcohol substrate. FAEEs were first detected in yeast expressing WS from *Acinetobacter calcoaceticus ADP1* devoid of four storage lipids biosynthetic genes, namely, diacylglycerol acyltransferase (DGA1), a phospholipid: diacylglycerol acyltransferase (LRO1), and two acyl-coenzyme A:sterol acyltransferases (ARE1 and ARE2) (Kalscheuer et al. 2004).

To improve production of FAEEs, researchers carried out various engineering strategies. Heterologous WS homologs were compared to determine the best performing FAEE-producing WS where WS from *Marinobacter hydrocarbonoclasticus DSM 8798* (ws2) showed the highest specificity for ethanol as the alcohol substrate (Shi et al. 2012). As ACC1 overexpression resulted in limited FAEE production improvement, ACC1 activity was improved by mutating the enzyme at Ser659 and Ser1157, abolishing posttranslational regulation by protein kinase SNF1 (Shi et al. 2014a). Elimination of nonessential and competing fatty acid utilization pathways through deletion of ARE1, ARE2, DGA1, LRO1, and POX1 genes enabled accumulation of more FAEEs (Valle-Rodríguez et al. 2014). Integration of six copies of ws2 expression constructs into chromosomal delta sites, and further overexpression of acyl-CoA binding protein (ACB1) involved in the transport of acyl-CoA esters and attenuation of the feedback inhibitory effect of acyl-CoAs on enzymes such as fatty acid synthetase, acetyl-CoA carboxylase, and long-chain acyl-CoA synthetase led to increased FAEEs production (Shi et al. 2014b). Another strategy used was the channeling of carbon flux toward acetyl-CoA by overexpressing ADH2, ALD6, and *Salmonella enterica* ACS^{L641P} (de Jong et al. 2014). Engineering the availability of redox cofactor NADPH through expression of bacterial NADP+-dependent glyceraldehyde-3-phosphate dehydrogenase (gapN) (Shi et al. 2014b) and phosphoketolase pathway (de Jong et al. 2014) increased FAEE production. Single copies of ws2, ADH2, ALD6, *S. enterica* ACS^{L641P}, $ACC1^{S1157A,\ S659A}$, and ACB1 were introduced into a *S. cerevisiae* strain lacking

ARE1, ARE2, DGA1, LRO1, and POX1. However, this led to lower FAEE titer than reported in earlier studies, where a single copy of ws2 was believed to be rate limiting (de Jong et al. 2015).

Production of FAEEs was also increased by overexpressing fatty acid biosynthetic genes, ACC1, FAS1, and FAS2 in a strain lacking POX1 (Runguphan and Keasling 2014). Alternatively, deletion of enzymes responsible for acyl-CoAs peroxisomal import, acyl-CoA transporter PXA2 and acyl-CoA synthetase FAA2, enabled blocking of β-oxidation pathway, as well as deletion of ACB1, which can increase transcription levels of fatty acid biosynthetic genes, resulted in enhanced cytosolic acyl-CoAs levels and FAEEs production (Thompson and Trinh 2014). Another work to produce FAEEs in yeast includes introducing an orthogonal route for fatty acid synthesis through heterologous expression of a type-I fatty acid synthase from *Brevibacterium ammoniagenes* coupled with ws2 (Eriksen et al. 2015), reversing the β-oxidation cycle in the yeast cytosol which enabled endogenous acyl-CoA/ethanol O-acyltransferases, EEB1, and EHT1 to catalyze the synthesis of medium-chain FAEEs (Lian and Zhao 2015) and using glycerol as well as sodium oleate as substrates (Yu et al. 2012).

Fatty acid esters with branched-chain alcohol moieties have superior fuel properties. This includes improved cold flow characteristics, as one of the major problems associated with biodiesel use is poor low-temperature flow properties (Knothe 2005). By expressing ws2 or Maqu_0168 from *Marinobacter sp.*, fatty acid short- and branched-chain alkyl esters, including ethyl, isobutyl, isoamyl, and active amyl esters, were produced using endogenously synthesized fatty acids and alcohols (Teo et al. 2015). Deletion of OPI1, a negative regulator of the INO1 gene in phospholipid metabolism, and expression of isobutanol pathway enzymes (ILV2, ILV5, ILV3, ARO10, and ADH7) targeted into the mitochondria enhanced fatty acid esters production.

4 Isoprenoids

Isoprenoids isoprenoidsare a diverse class of natural products derived from the sequential condensation of the basic C_5 isoprene units, isopentenyl pyrophosphate (IPP), and dimethylallyl pyrophosphate (DMAPP) via the mevalonate (MVA) pathway in *S. cerevisiae.* Due to their lower hygroscopy, higher energy content, and good fluidity at lower temperature (Gupta and Phulara 2015), recent years have seen much interest in the exploration of the fuel potential of the C_5 hemiterpenes, the C_{10} monoterpenes, and the C_{15} sesquiterpenes (Fig. 4 and Table 3). Specifically, the hemiterpene isoprene is a potential fuel additive for gasoline, diesel, or jet fuel (Bentley et al. 2014), while monoterpenes including limonene, cineole, linalool, sabinene, and geraniol are good candidates of biofuel precursor and aviation fuel alternative or additive (Meylemans et al. 2011; Ryder 2012). Moreover, sesquiterpenes such as farnesol, farnesene, and bisabolene are prospective biosynthetic biodiesel either on its own or in its hydrogenated form (Renninger and McPhee 2008, 2010; Peralta-Yahya et al. 2011).

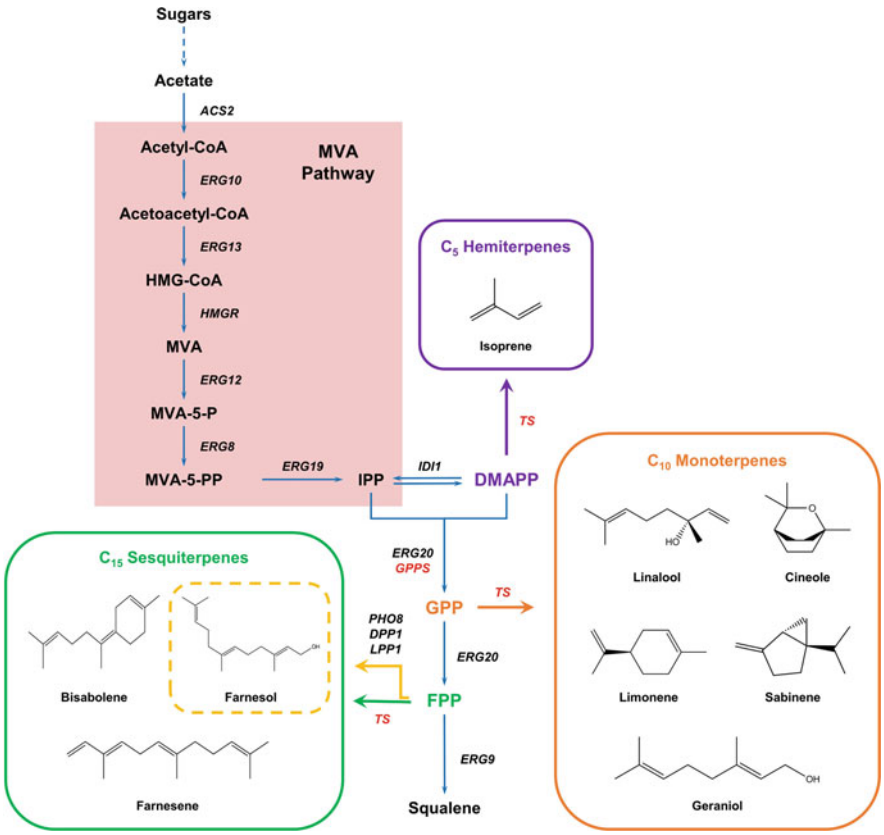

Fig. 4 Biosynthetic pathways of isoprenoid biofuels in engineered *S. cerevisiae*. Heterologous genes are colored in *red*. *Dotted lines* indicate multistep reactions. *MVA* mevalonate, *HMG* 3-hydroxy-3-methylglutaryl, *IPP* isopentenyl pyrophosphate, *DMAPP* dimethylallyl pyrophosphate, *GPP* geranyl pyrophosphate, *FPP* farnesyl pyrophosphate, *ACS2* acetyl-CoA synthetase, *ERG10* acetyl-CoA acetyltransferase, *ERG13* HMG-CoA synthase, *HMGR* HMG-CoA reductase, *ERG12* mevalonate kinase, *ERG8* phosphomevalonate kinase, *ERG19* mevalonate pyrophosphate decarboxylase, *IDI1* IPP isomerase, *ERG20* FPP synthase, *ERG9* squalene synthase, *TS* terpene synthase, *GPPS* GPP synthase, *PHO8* alkaline phosphatase, *DPP1* diacylglycerol pyrophosphate phosphatase, *LPP1* lipid phosphate phosphatase

Efficient microbial production of isoprenoids requires first accumulation of the corresponding branch-point intermediates, namely, IPP/DMAPP, geranyl pyrophosphate (GPP), and farnesyl pyrophosphate (FPP) for hemiterpenes, monoterpenes, and sesquiterpenes, respectively, and their subsequent conversion into desired isoprenoids by specific terpene synthase. As such, engineering strategies for isoprenoid production in *S. cerevisiae* are mostly devoted toward increasing these intermediate pools. These strategies include (i) upregulation of the upstream MVA pathway to improve precursor supply and (ii) downregulation of downstream competing metabolic branches to minimize metabolic flux loss. To improve precursor supply, the rate-

Table 3 Isoprenoid biofuels produced by engineered *S. cerevisiae*

Biofuel	Titer (mg/L)	Carbon source (g/L)	References
Isoprene	0.5	Galactose (20)	Hong et al. (2012)
	37	Sucrose (6)	Lv et al. (2014)
		Galactose (14)	
Limonene	1.48	Galactose (18)	Behrendorff et al. (2013)
		Glucose (2)	
	0.49	Galactose (20)	Jongedijk et al. (2015)
Cineole	~100 mg/gDCW	Galactose (20)	Ignea et al. (2011)
		Raffinose (10)	
Linalool	0.077	Glucose (20)	Herrero et al. (2008)
	0.132	Glucose (20)	Rico et al. (2010)
	0.127	n.i.	Sun et al. (2013)
	0.095	Galactose (20)	Amiri et al. (2016)
	0.241	n.i.	Deng et al. (2016)
Sabinene	17.5	n.i.	Ignea et al. (2014)
Geraniol	1	Glucose (10)	Oswald et al. (2007)
	5.4	Glucose 10	Fischer et al. (2011)
	36	Glucose (20)	Liu et al. (2013)
	8	Glucose (20)	Pardo et al. (2015)
	11.4	n.i.	Campbell et al. (2016)
	293	n.i.	Zhao et al. (2016a)
Farnesol	4950	n.i.	Millis and Maurina-Brunker (2004)
	~80	n.i.	Takahashi et al. (2007)
	22.6	Galactose (20)	Asadollahi et al. (2008)
	146	Glucose (50)	Ohto et al. (2009)
	79	Glucose (20)	Zhuang and Chappell (2015)
Farnesene	762	n.i.	Renninger and Mcphee (2010)
	170	n.i.	Tippmann et al. (2016)
Bisabolene	994	Galactose (18)	Peralta-Yahya et al. (2011)
		Glucose (2)	
	5200	n.i.	Ozaydin et al. (2013)

n.i. not indicated in the publication, *gDCW* g dry cell weight

limiting 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) (Ohto et al. 2009), its truncated, cytosolic variant, tHMGR engineered to bypass feedback inhibition (Ohto et al. 2009; Rico et al. 2010; Behrendorff et al. 2013; Sun et al. 2013; Lv et al. 2014; Amiri et al. 2016; Tippmann et al. 2016), or its stabilized variant, $HMG2^{K6R}$ (Ignea et al. 2011), was overexpressed. In addition, expression of the IPP isomerase, IDI1 (Ignea et al. 2011; Liu et al. 2013; Sun et al. 2013; Zhao et al. 2016) and the mutated global transcriptional regulator of the sterol biosynthetic pathway, UPC2-1 (Peralta-Yahya et al. 2011; Behrendorff et al. 2013; Zhao et al. 2016) were also upregulated to improve precursor supply. To divert flux away from the competing yet growth-essential ergosterol biosynthesis into the corresponding intermediate pools, the

expression of the FPP synthase, ERG20 (for DMAPP), or the squalene synthase, ERG9 (for GPP and FPP), was attenuated, either through promoter replacement (e.g., P_{MET3}, P_{HXT1}, or P_{CYC1}) (Asadollahi et al. 2008; Peralta-Yahya et al. 2011; Ozaydin et al. 2013; Lv et al. 2014; Amiri et al. 2016; Tippmann et al. 2016) or complete knockout in a sterol uptake enhancement background strain (Takahashi et al. 2007; Zhuang and Chappell 2015).

Apart from these, other genes constituting the acetyl-CoA formation and the MVA pathways are also prospective manipulation targets for improved intermediate pools. Overexpression of ACS2 and ERG10, along with deregulation of tHMGR and ERG20 improved isoprene production through enhanced acetyl-CoA precursor supply (Lv et al. 2014). In addition, upregulation of the whole MVA pathway through overexpression of 3-hydroxy-3-methylglutaryl-CoA synthase (ERG13), tHMGR, mevalonate kinase (ERG12), and phosphomevalonate kinase (ERG8) has also been undertaken to improve flux toward GPP accumulation for geraniol production (Campbell et al. 2016). Besides, ERG20 overexpression has been suggested for improved FPP accumulation (Peralta-Yahya et al. 2011; Ozaydin et al. 2013). To direct flux away from the undesirable tRNA biosynthesis into the DMAPP pool, overexpression of MAF1, the negative regulator of tRNA biosynthesis has also been attempted for improved geraniol production (Liu et al. 2013).

The metabolic engineering of *S. cerevisiae* for monoterpene production is largely hindered by the low cytosolic availability of GPP, given that GPP is a transitory intermediate of the two-step FPP biosynthesis catalyzed by ERG20 (Rico et al. 2010; Fischer et al. 2011; Ignea et al. 2014). To circumvent this, efforts have been directed toward the identification of ERG20 variants with improved GPP-synthesizing ability. Overexpression of the protein variants, $ERG20^{K197E}$ (Oswald et al. 2007; Campbell et al. 2016; Deng et al. 2016), $ERG20^{K197G}$ (Fischer et al. 2011; Liu et al. 2013; Jongedijk et al. 2015), or $ERG20^{F96W\text{-}N127W}$ (Ignea et al. 2014; Zhao et al. 2016) along with specific terpene synthase resulted in the production of a range of monoterpenes including limonene, linalool, sabinene, and geraniol. $ERG20^{F96W\text{-}N127W}$ was reported to yield slightly higher geraniol production in comparison to $ERG20^{K197G}$ in a recent study (Zhao et al. 2016). Furthermore, expression of a heterologous GPP synthase (GPPS) that exclusively produces GPP, such as that from *Abies grandis*, was also undertaken to enhance the GPP pool (Campbell et al. 2016). However, in another study, decreased geraniol yield was reported for heterologous expression of GPPS from *A. grandis*, *Picea abies*, or *Catharanthus roseus* (Zhao et al. 2016).

Apart from engineering the accumulation of intermediates, the identification of an efficient terpene synthase to catalyze the final step of isoprenoid formation from the corresponding branch-point intermediate is of equal significance and is regarded as one of the key limiting steps in the microbial production of isoprenoids (Kirby et al. 2014). Such enzymes are generally endogenously absent in *S. cerevisiae*, and thus, heterologous sources, especially of plant origin, are usually sought. The use of plant-derived enzymes necessitates additional protein modifications including codon optimization and removal of the plastid-targeting sequence, to improve heterologous protein expression and to avoid potential protein misfolding and inclusion body

formation, respectively (Behrendorff et al. 2013). The significance of these was well evident by improved limonene, geraniol, and bisabolene production utilizing modified terpene synthases in comparison to their wild-type counterparts (Peralta-Yahya et al. 2011; Liu et al. 2013; Jongedijk et al. 2015; Zhao et al. 2016). Besides, an additional strategy has been suggested to improve the capacity of a plant terpene synthase by co-expressing its plant-interacting proteins that are involved in its correct folding, stability, or localization. Using a two-hybrid screen, a luminal binding protein, a SEC14 homologous protein, and a heat shock protein 90 (HSP90) were isolated for their specific interaction with the *Salvia fruticosa* cineole synthase. Co-expression of the cineole synthase and HSP90 protein increased cineole production in *S. cerevisiae* (Ignea et al. 2011).

The choice of terpene synthase utilized is also of particular importance and would have a significant impact on the final isoprenoid production. Comparison of the product formation efficiency of homolog enzymes from various sources was attempted to identify the best performing variant. In a recent study, three plant geraniol synthases were evaluated for geraniol production, with the highest titer observed from *Valeriana officinalis* geraniol synthase, in comparison to those from *Lippia dulcis* and *Ocimum basilicum* (Zhao et al. 2016). Notably, the *O. basilicum* enzyme variant was the most commonly used geraniol synthase in earlier studies, of which unsatisfactory production titers were reported (Oswald et al. 2007; Fischer et al. 2011; Liu et al. 2013; Pardo et al. 2015; Campbell et al. 2016). Similar enzyme comparison approaches were also undertaken for limonene synthase (production level with enzyme from *Perilla frutescens* > *Citrus limon*), farnesene synthase (*Malus domestica* > *C. junos* > *Artemisia annua*), and bisabolene synthase (*A. grandis* > *Pseudotsuga menziesii* > *Arabidopsis thaliana*) (Peralta-Yahya et al. 2011; Jongedijk et al. 2015; Tippmann et al. 2016).

To enhance the efficiency of substrate's channeling between enzymes of two sequential reactions, a fusion protein strategy was employed. The fusion of ERG20 (native or mutated variants) with specific terpene synthase resulted in improved linalool, sabinene, geraniol, and bisabolene production (Ozaydin et al. 2013; Ignea et al. 2014; Deng et al. 2016; Zhao et al. 2016). In addition, a strategy to improve protein expression by modulating mRNA stability and translation was also demonstrated. Using a Tween-20 surfactant-based screening method, an alternative 3′ coding sequence of *A. grandis* bisabolene synthase that yielded higher bisabolene synthase protein level and elevated bisabolene production was isolated (Kirby et al. 2014).

Differing from other isoprenoids, farnesol production was achieved mainly via engineering strategies that elevate the FPP pool without the need to express any heterologous terpene synthase (Takahashi et al. 2007; Asadollahi et al. 2008; Ohto et al. 2009; Zhuang and Chappell 2015). Farnesol is a dephosphorylated product of FPP, and its formation was suggested to result from promiscuous hydrolysis by endogenous phosphatases including PHO8, LPP1, and DPP1 (Faulkner et al. 1999; Song 2006). Noteworthy, deletion of LPP1 and DPP1 was suggested to improve sesquiterpene production through enhanced FPP pool (Scalcinati et al. 2012).

However, negative results were seen when such an approach was applied for sabinene production (Ignea et al. 2014).

5 Research Needs

While drop-in biofuels can potentially replace conventional fuels in our daily lives, much work remains to be done to make the current production processes commercially viable. Achieving theoretical maximum yields while coupling the technology with cheap biomass feedstock fermentation is crucial for cost considerations. Bottlenecks that limit the productivity of biofuels will need to be solved in order to attain more efficient production pathways. Work that needs to be done include the discovery and design of new enzymes and pathways, engineering efficient use of cellular resources, and development of microbial host strains capable of withstanding high biofuel concentrations.

Here, we have reviewed the progress of engineering yeast to produce drop-in biofuels including short-chain alcohols, fatty acid derivatives, and isoprenoids. Efforts to produce fuels with properties most suitable for substituting conventional fuels will be centered on producing fuel molecules of a range of chain lengths, increasing the branch structure of the molecules as well as production of saturated hydrocarbons. Using synthetic biology tools and high-throughput iterative design-build-test-analyze cycles, yeast variants can be constructed and characterized to deliver synthetic yeast strains capable of powering the future.

References

Amiri P, Shahpiri A, Asadollahi MA, Momenbeik F, Partow S (2016) Metabolic engineering of *Saccharomyces cerevisiae* for linalool production. Biotechnol Lett 38:503–508

Asadollahi MA, Maury J, Moller K, Nielsen KF, Schalk M, Clark A, Nielsen J (2008) Production of plant sesquiterpenes in *Saccharomyces cerevisiae*: effect of ERG9 repression on sesquiterpene biosynthesis. Biotechnol Bioeng 99:666–677

Avalos JL, Fink GR, Stephanopoulo SG (2013) Compartmentalization of metabolic pathways in yeast mitochondria improves the production of branched-chain alcohols. Nat Biotechnol 31:335–341

Bailey JE (1991) Toward a science of metabolic engineering. Science 252:1668–1675

Behrendorff JB, Vickers CE, Chrysanthopoulos P, Nielsen LK (2013) 2,2-Diphenyl-1-picrylhydrazyl as a screening tool for recombinant monoterpene biosynthesis. Microb Cell Factories 12:76

Bentley FK, Zurbriggen A, Melis A (2014) Heterologous expression of the mevalonic acid pathway in cyanobacteria enhances endogenous carbon partitioning to isoprene. Mol Plant 7:71–86

Branduardi P, Longo V, Berterame NM, Rossi G, Porro D (2013) A novel pathway to produce butanol and isobutanol in *Saccharomyces cerevisiae*. Biotechnol Biofuels 6:1–12

Brat D, Weber C, Lorenzen W, Bode HB, Boles E (2012) Cytosolic re-localization and optimization of valine synthesis and catabolism enables increased isobutanol production with the yeast *Saccharomyces cerevisiae*. Biotechnol Biofuels 5:1–16

Buijs NA, Zhou YJ, Siewers V, Nielsen J (2015) Long-chain alkane production by the yeast *Saccharomyces cerevisiae*. Biotechnol Bioeng 112:1275–1279

Campbell A, Bauchart P, Gold ND, Zhu Y, De Luca V, Martin VJ (2016) Engineering of a nepetalactol-producing platform strain of *Saccharomyces cerevisiae* for the production of plant seco-iridoids. ACS Synth Biol 5:405–414

Chen X, Nielsen KF, Borodina I, Kielland-Brandt MC, Karhumaa K (2011) Increased isobutanol production in *Saccharomyces cerevisiae* by overexpression of genes in valine metabolism. Biotechnol Biofuels 4:21

Chen B, Lee DY, Chang MW (2015) Combinatorial metabolic engineering of *Saccharomyces cerevisiae* for terminal alkene production. Metab Eng 31:53–61

de Jong BW, Shi S, Siewers V, Nielsen J (2014) Improved production of fatty acid ethyl esters in *Saccharomyces cerevisiae* through up-regulation of the ethanol degradation pathway and expression of the heterologous phosphoketolase pathway. Microb Cell Factories 13:1–10

de Jong BW, Shi S, Valle-Rodriguez JO, Siewers V, Nielsen J (2015) Metabolic pathway engineering for fatty acid ethyl ester production in *Saccharomyces cerevisiae* using stable chromosomal integration. J Ind Microbiol Biotechnol 42:477–486

Deng Y, Sun M, Xu S, Zhou J (2016) Enhanced (S)-linalool production by fusion expression of farnesyl diphosphate synthase and linalool synthase in *Saccharomyces cerevisiae*. J Appl Microbiol 121:187–195

Eriksen DT, Hamedirad M, Yuan Y, Zhao H (2015) Orthogonal fatty acid biosynthetic pathway improves fatty acid ethyl ester production in *Saccharomyces cerevisiae*. ACS Synth Biol 4:808–814

Faulkner A, Chen X, Rush J, Horazdovsky B, Waechter CJ, Carman GM, Sternweis PC (1999) The LPP1 and DPP1 gene products account for most of the isoprenoid phosphate phosphatase activities in *Saccharomyces cerevisiae*. J Biol Chem 274:14831–14837

Feng X, Lian J, Zhao H (2015) Metabolic engineering of *Saccharomyces cerevisiae* to improve 1-hexadecanol production. Metab Eng 27:10–19

Fischer MJ, Meyer S, Claudel P, Bergdoll M, Karst F (2011) Metabolic engineering of monoterpene synthesis in yeast. Biotechnol Bioeng 108:1883–1892

Foo JL, Susanto AV, Keasling JD, Leong SS, Chang MW (2015) Whole-cell biocatalytic and de novo production of alkanes from free fatty acids in *Saccharomyces cerevisiae*. Biotechnol Bioeng 114:232–237

Generoso WC, Schadeweg V, Oreb M, Boles E (2015) Metabolic engineering of *Saccharomyces cerevisiae* for production of butanol isomers. Curr Opin Biotechnol 33:1–7

Ghiaci P, Norbeck J, Larsson C (2014) 2-butanol and butanone production in *Saccharomyces cerevisiae* through combination of a B_{12} dependent dehydratase and a secondary alcohol dehydrogenase using a TEV-based expression system. PLoS ONE 9:e102774

Gupta P, Phulara SC (2015) Metabolic engineering for isoprenoid-based biofuel production. J Appl Microbiol 119:605–619

Hazelwood LA, Daeran J-M, Van Maris AJA, JT P, Dickinson JR (2008) The Ehrlich pathway for fusel alcohol production: a century of research on *Saccharomyces cerevisiae* metabolism. Appl Environ Microbiol 74:2259–2266

Herrero O, Ramon D, Orejas M (2008) Engineering the *Saccharomyces cerevisiae* isoprenoid pathway for de novo production of aromatic monoterpenes in wine. Metab Eng 10:78–86

Hong SY, Zurbriggen AS, Melis A (2012) Isoprene hydrocarbons production upon heterologous transformation of *Saccharomyces cerevisiae*. J Appl Microbiol 113:52–65

Ida K, Ishii J, Matsuda F, Kondo T, Kondo A (2015) Eliminating the isoleucine biosynthetic pathway to reduce competitive carbon outflow during isobutanol production by *Saccharomyces cerevisiae*. Microb Cell Factories 14:1–9

Ignea C, Cvetkovic I, Loupassaki S, Kefalas P, Johnson CB, Kampranis SC, Makris AM (2011) Improving yeast strains using recyclable integration cassettes, for the production of plant terpenoids. Microb Cell Factories 10:1–18

Ignea C, Pontini M, Maffei ME, Makris AM, Kampranis SC (2014) Engineering monoterpene production in yeast using a synthetic dominant negative geranyl diphosphate synthase. ACS Synth Biol 3:298–306

Jensen MK, Keasling JD (2014) Recent applications of synthetic biology tools for yeast metabolic engineering. FEMS Yeast Res 15:1–10

Jin Z, Wong A, Foo JL, Ng J, Cao YX, Chang MW, Yuan YJ (2016) Engineering *Saccharomyces cerevisiae* to produce odd chain-length fatty alcohols. Biotechnol Bioeng 113:842–851

Jongedijk E, Cankar K, Ranzijn J, Van Der Krol S, Bouwmeester H, Beekwilder J (2015) Capturing of the monoterpene olefin limonene produced in *Saccharomyces cerevisiae*. Yeast 32:159–171

Kalscheuer R, Luftmann H, Steinbuchel A (2004) Synthesis of novel lipids in *Saccharomyces cerevisiae* by heterologous expression of an unspecific bacterial acyltransferase. Appl Environ Microbiol 70:7119–7125

Kirby J, Nishimoto M, Chow RW, Pasumarthi VN, Chan R, Chan LJ, Petzold CJ, Keasling JD (2014) Use of nonionic surfactants for improvement of terpene production in *Saccharomyces cerevisiae*. Appl Environ Microbiol 80:6685–6693

Knothe G (2005) Dependence of biodiesel fuel properties on the structure of fatty acid alkyl esters. Fuel Process Technol 86:1059–1070

Kondo T, Tezuka H, Ishii J, Matsuda F, Ogino C, Kondo A (2012) Genetic engineering to enhance the Ehrlich pathway and alter carbon flux for increased isobutanol production from glucose by *Saccharomyces cerevisiae*. J Biotechnol 159:32–37

Krivoruchko A, Serrano-Amatriain C, Chen Y, Siewers V, Nielsen J (2013) Improving biobutanol production in engineered *Saccharomyces cerevisiae* by manipulation of acetyl-CoA metabolism. J Ind Microbiol Biotechnol 40:1051–1056

Lee WH, Seo SO, Bae YH, NAN H, Jin YS, Seo JH (2012) Isobutanol production in engineered *Saccharomyces cerevisiae* by overexpression of 2-ketoisovalerate decarboxylase and valine biosynthetic enzymes. Bioprocess Biosyst Eng 35:1467–1475

Lian J, Zhao H (2015) Reversal of the β-oxidation cycle in *Saccharomyces cerevisiae* for production of fuels and chemicals. ACS Synth Biol 4:332–341

Lian J, Si T, Nair NU, Zhao H (2014) Design and construction of acetyl-CoA overproducing *Saccharomyces cerevisiae* strains. Metab Eng 24:139–149

Liu J, Zhang W, Du G, Chen J, Zhou J (2013) Overproduction of geraniol by enhanced precursor supply in *Saccharomyces cerevisiae*. J Biotechnol 168:446–451

Lv X, Xie W, Lu W, Guo F, Gu J, Yu H, Ye L (2014) Enhanced isoprene biosynthesis in *Saccharomyces cerevisiae* by engineering of the native acetyl-CoA and mevalonic acid pathways with a push-pull-restrain strategy. J Biotechnol 186:128–136

Matsuda F, Kondo T, Ida K, Tezuka H, Ishii J, Kondo A (2012) Construction of an artificial pathway for isobutanol biosynthesis in the cytosol of *Saccharomyces cerevisiae*. Biosci Biotechnol Biochem 76:2139–2141

Matsuda F, Ishii J, Kondo T, Ida K, Tezuka H, Kondo A (2013) Increased isobutanol production in *Saccharomyces cerevisiae* by eliminating competing pathways and resolving cofactor imbalance. Microb Cell Factories 12:119–119

Meylemans HA, Quintana RL, Goldsmith BR, Harvey BG (2011) Solvent-free conversion of linalool to methylcyclopentadiene dimers: a route to renewable high-density fuels. ChemSusChem 4:465–469

Millis JR, Maurina-Brunker J (2004) Production of farnesol and geranylgeraniol. US Patent 6689593 B2

Milne N, Wahl SA, Van Maris AJA, Pronk JT, Daran JM (2016) Excessive by-product formation: a key contributor to low isobutanol yields of engineered *Saccharomyces cerevisiae* strains. Metab Eng Commun 3:39–51

Ohto C, Muramatsu M, Obata S, Sakuradani E, Shimizu S (2009) Overexpression of the gene encoding HMG-CoA reductase in *Saccharomyces cerevisiae* for production of prenyl alcohols. Appl Microbiol Biotechnol 82:837–845

Oswald M, Fischer M, Dirninger N, Karst F (2007) Monoterpenoid biosynthesis in *Saccharomyces cerevisiae*. FEMS Yeast Res 7:413–421

Ozaydin B, Burd H, Lee TS, Keasling JD (2013) Carotenoid-based phenotypic screen of the yeast deletion collection reveals new genes with roles in isoprenoid production. Metab Eng 15:174–183

Pardo E, Rico J, Gil JV, Orejas M (2015) De novo production of six key grape aroma monoterpenes by a geraniol synthase-engineered *S. cerevisiae* wine strain. Microb Cell Factories 14:136

Park SH, Kim S, Hahn JS (2014) Metabolic engineering of *Saccharomyces cerevisiae* for the production of isobutanol and 3-methyl-1-butanol. Appl Microbiol Biotechnol 98:9139–9147

Park S-H, Kim S, Hahn J-S (2016) Improvement of isobutanol production in *Saccharomyces cerevisiae* by increasing mitochondrial import of pyruvate through mitochondrial pyruvate carrier. Appl Microbiol Biotechnol 100:7591–7598

Peralta-Yahya PP, Ouellet M, Chan R, Mukhopadhyay A, Keasling JD, Lee TS (2011) Identification and microbial production of a terpene-based advanced biofuel. Nat Commun 2:483

Renninger NS, Mcphee DJ (2008) Fuel compositions comprising farnesane and farnesane derivatives and method of making and using same. US Patent 7399323 B2

Renninger NS, Mcphee DJ (2010) Fuel compositions comprising farnesane and farnesane derivatives and method of making and using same. US Patent 7846222 B2

Rico J, Pardo E, Orejas M (2010) Enhanced production of a plant monoterpene by overexpression of the 3-hydroxy-3-methylglutaryl coenzyme A reductase catalytic domain in *Saccharomyces cerevisiae*. Appl Environ Microbiol 76:6449–6454

Runguphan W, Keasling JD (2014) Metabolic engineering of *Saccharomyces cerevisiae* for production of fatty acid-derived biofuels and chemicals. Metab Eng 21:103–113

Ryder JA (2012) Jet fuel compositions and methods of making and using same. US Patent 8106247 B2

Scalcinati G, Knuf C, Partow S, Chen Y, Maury J, Schalk M, Daviet L, Nielsen J, Siewers V (2012) Dynamic control of gene expression in *Saccharomyces cerevisiae* engineered for the production of plant sesquitepene alpha-santalene in a fed-batch mode. Metab Eng 14:91–103

Schadeweg V, Boles E (2016) n-Butanol production in *Saccharomyces cerevisiae* is limited by the availability of coenzyme A and cytosolic acetyl-CoA. Biotechnol Biofuels 9:44

Sheng J, Stevens J, Feng X (2016) Pathway compartmentalization in peroxisome of *Saccharomyces cerevisiae* to produce versatile medium chain fatty alcohols. Sci Report 6:26884

Shi S, Valle-Rodriguez JO, Khoomrung S, Siewers V, Nielsen J (2012) Functional expression and characterization of five wax ester synthases in *Saccharomyces cerevisiae* and their utility for biodiesel production. Biotechnol Biofuels 5:7

Shi S, Chen Y, Siewers V, Nielsen J (2014a) Improving production of malonyl coenzyme A-derived metabolites by abolishing Snf1-dependent regulation of Acc1. MBio 5:e01130–e01114

Shi S, Valle-Rodríguez JO, Siewers V, Nielsen J (2014b) Engineering of chromosomal wax ester synthase integrated *Saccharomyces cerevisiae* mutants for improved biosynthesis of fatty acid ethyl esters. Biotechnol Bioeng 111:1740–1747

Shi S, Si T, Liu Z, Zhang H, Ang EL, Zhao H (2016) Metabolic engineering of a synergistic pathway for n-butanol production in *Saccharomyces cerevisiae*. Sci Report 6:25675

Si T, Luo Y, Xiao H, Zhao H (2014) Utilizing an endogenous pathway for 1-butanol production in *Saccharomyces cerevisiae*. Metab Eng 22:60–68

Song L (2006) A soluble form of phosphatase in *Saccharomyces cerevisiae* capable of converting farnesyl diphosphate into E,E-farnesol. Appl Biochem Biotechnol 128:149–158

Steen EJ, Chan R, Prasad N, Myers S, Petzold CJ, Redding A, Ouellet M, Keasling JD (2008) Metabolic engineering of *Saccharomyces cerevisiae* for the production of n-butanol. Microb Cell Factories 7:36

Sun M, Liu J, Du G, Zhou J, Chen J (2013) Regulation of isoprenoid pathway for enhanced production of linalool in *Saccharomyces cerevisiae*. Sheng Wu Gong Cheng Xue Bao = Chin J Biotechnol 29:751–759

Takahashi S, Yeo Y, Greenhagen BT, Mcmullin T, Song L, Maurina-Brunker J, Rosson R, Noel JP, Chappell J (2007) Metabolic engineering of sesquiterpene metabolism in yeast. Biotechnol Bioeng 97:170–181

Tang X, Chen W (2015) Enhanced production of fatty alcohols by engineering the TAGs synthesis pathway in *Saccharomyces cerevisiae*. Biotechnol Bioeng 112:386–392

Teo WS, Ling H, Yu A-Q, Chang MW (2015) Metabolic engineering of *Saccharomyces cerevisiae* for production of fatty acid short- and branched-chain alkyl esters biodiesel. Biotechnol Biofuels 8:1–9

Thompson RA, Trinh CT (2014) Enhancing fatty acid ethyl ester production in *Saccharomyces cerevisiae* through metabolic engineering and medium optimization. Biotechnol Bioeng 111:2200–2208

Tippmann S, Scalcinati G, Siewers V, Nielsen J (2016) Production of farnesene and santalene by *Saccharomyces cerevisiae* using fed-batch cultivations with RQ-controlled feed. Biotechnol Bioeng 113:72–81

Valle-Rodríguez JO, Shi S, Siewers V, Nielsen J (2014) Metabolic engineering of *Saccharomyces cerevisiae* for production of fatty acid ethyl esters, an advanced biofuel, by eliminating non-essential fatty acid utilization pathways. Appl Energy 115:226–232

Williams TC, Pretorius IS, Paulsen IT (2016) Synthetic evolution of metabolic productivity using biosensors. Trends Biotechnol 34:371–381

Yu K, Jung J, Kim SW, Park C, Han S (2012) Synthesis of FAEEs from glycerol in engineered *Saccharomyces cerevisiae* using endogenously produced ethanol by heterologous expression of an unspecific bacterial acyltransferase. Biotechnol Bioeng 109:110–115

Yuan J, Ching CB (2015) Combinatorial assembly of large biochemical pathways into yeast chromosomes for improved production of value-added compounds. ACS Synth Biol 4:23–31

Zhang J, Jensen MK, Keasling JD (2015) Development of biosensors and their application in metabolic engineering. Curr Opin Chem Biol 28:1–8

Zhao J, Bao X, LI C, Shen Y, Hou J (2016) Improving monoterpene geraniol production through geranyl diphosphate synthesis regulation in *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 100:4561–4571

Zhou YJ, Buijs NA, Zhu Z, Qin J, Siewers V, Nielsen J (2016) Production of fatty acid-derived oleochemicals and biofuels by synthetic yeast cell factories. Nat Commun 7:11709

Zhuang X, Chappell J (2015) Building terpene production platforms in yeast. Biotechnol Bioeng 112:1854–1864