Chapter 8

Metabolic Engineering of Hydrocarbon Biosynthesis for Biofuel Production

Anne M. Ruffing

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/52050

1. Introduction

The world's supply of petroleum hydrocarbons, which serve as feedstock for the fuel and chemical industries, is rapidly diminishing to satisfy the global demand for energy and consumer goods. In response to this increasing demand and limited supply, the cost of crude oil has risen to over $100 per barrel in 2012, a 10-fold increase compared to prices in the late 1990s [1]. As fossil fuels are nonrenewable resources, the price of oil is only expected to increase in the future. This unavoidable reality necessitates the development of renewable energy sources in order to maintain the current standard of living. Among the alternative energy options under development, biofuels are anticipated to supplement and eventually replace the petroleum-based fuels that supply the transportation and chemical industries. Currently, first generation biofuels like corn-based ethanol are blended into conventional petroleum fuels, with biofuels supplying 2.7% of the world's transportation fuel in 2010 [2]. It appears that biofuels are on their way to becoming a viable renewable energy source, yet technological and biological advancements are necessary for sustainable and economical biofuel production at the scales necessary to support the world's energy needs.

The current practice of using food crops, like corn or soybean, as feedstocks for biofuel production is not a viable, long-term solution to the energy crisis. In fact, to replace our current petroleum usage with crop-based ethanol production, the entire surface area of land on Earth would be needed for corn production [3]. In addition to this shortcoming, first generation biofuels compete with food production for arable land, require significant nutrient resources (fertilizer and fresh water), and typically have low net energy yields due to the low energy density of the product fuel (i.e. ethanol) and the energy input required to harvest the feedstock and convert it into fuel [4]. Second and third generation biofuels address these limitations. Second generation biofuels use lignocellulosic biomass as the feedstock for fuel production.

Lignocellulose, the main component of plant biomass, is the most abundant form of renewable carbon on the Earth, making it an ideal feedstock for renewable hydrocarbon production. The cellulose and hemicellulose components of lignocellulose can be degraded into fermentable sugars to serve as the carbon source for microbial-based fuel production. The carbon feedstocks for both first and second generation biofuels are ultimately derived from carbon dioxide (CO_2) fixation through the process of photosynthesis. Third generation biofuels use photosynthetic microorganisms (i.e. microalgae) to directly convert CO_2 into fuel molecules or fuel precursors, eliminating the biomass intermediate (Figure 1). While both second and third generation biofuels require land, nutrients, and energy investment for harvesting and fuel production, the fuel production yields from these processes are predicted to be capable of meeting energy needs. However, these technologies have yet to be demonstrated at scale and still require further improvement before they can be economically competitive with fossil fuels.

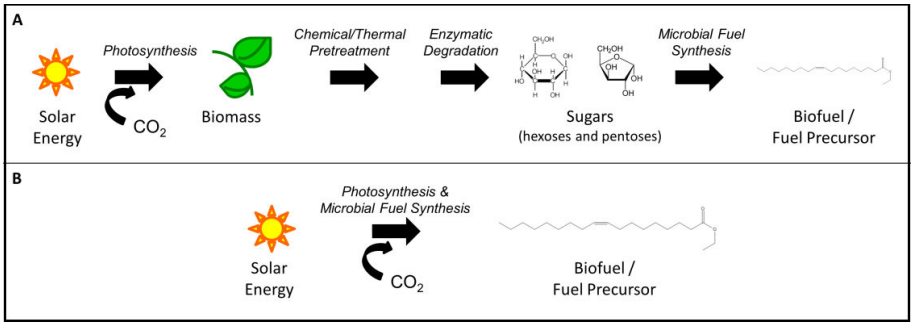

Figure 1. Process steps for (A) second (i.e. lignocellulosic feedstock) and (B) third (i.e. inorganic carbon feedstock) generation biofuels.

Both second and third generation biofuels rely on microbes to convert the carbon feedstock into the desired hydrocarbon fuels. Microorganisms have been identified that are capable of producing a range of fuel molecules and fuel precursors, yet the natural rates of microbial fuel synthesis are typically too low to support industrial-scale production. Metabolic engineering is a powerful tool to improve microbial fuel production, either through engineering the metabolic pathways within the native microorganism to encourage high fuel synthesis or though transferring the fuel production pathway into a model organism for optimization. This chapter will focus on the application of metabolic engineering to increase hydrocarbon fuel production. Within this chapter, hydrocarbon-based fuels are defined to include oxygen-containing fuel molecules with long hydrocarbon chains, such as fatty alcohols and fatty acid ethyl esters (FAEE), in addition to pure hydrocarbons like alkanes, alkenes, and isoprenoid-based molecules: hemiterpene (C5), monoterpenes (C10), and sesquiterpenes (C15). Hydrocarbon-based fuel precursors will also be considered, including free fatty acids (FFAs) and triacylglycerol (TAG). The structures of these hydrocarbon-based fuels and precursors are illustrated in Figure 2. Hydrocarbon-based fuels and precursors can be produced by both second and third generation biofuel processes. Therefore, the first section in this chapter will

http://dx.doi.org/10.5772/52050

discuss the metabolic pathways for hydrocarbon fuel production and common metabolic engineering strategies for improving fuel synthesis. Because second and third generation biofuel processes rely on different carbon sources, sugars and CO_2 respectively, the remaining sections will focus on the use of organic carbon (heterotrophy) and inorganic carbon (autotrophy) as feedstocks for biofuel production. This division, based on carbon source, is important from both the biofuel production and metabolic engineering perspectives. The chapter will conclude with a discussion of the future outlook for microbial-based, hydrocarbon fuel synthesis.

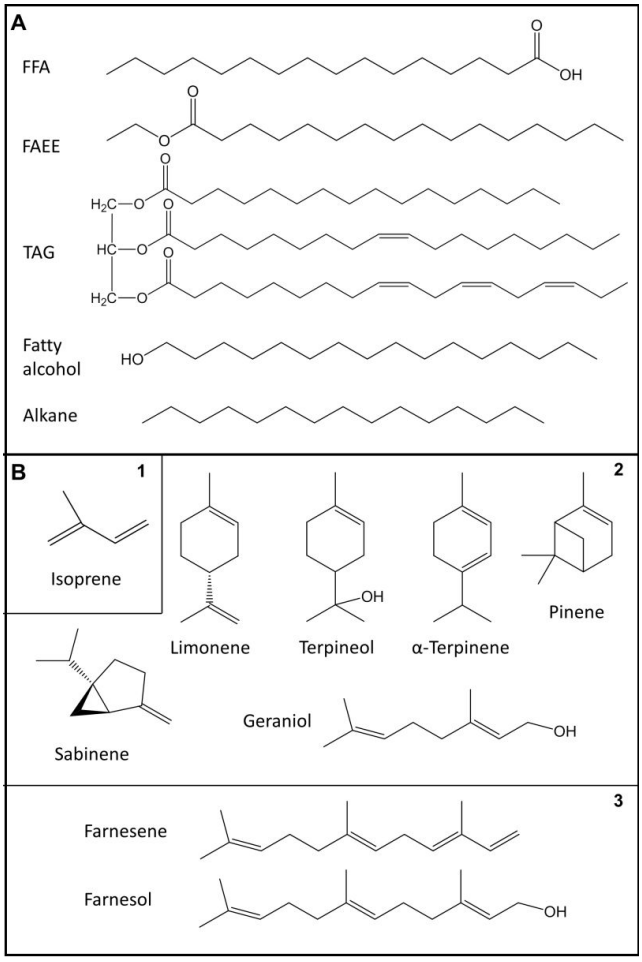

Figure 2. Chemical structures of hydrocarbon-based biofuels and fuel precursors. (A) Fuels derived from fatty acid biosynthesis and (B) fuels derived from isoprenoid biosynthesis, including (1) hemiterpene, (2) monoterpenes, and (3) sesquiterpenes.

2. Engineering hydrocarbon biosynthesis pathways

The hydrocarbon-based biofuels considered in this chapter (Figure 2) are all derived from two metabolites: fatty acids and isoprenoids. Thus, the two metabolic pathways commonly targeted by metabolic engineering strategies are the fatty acid biosynthesis pathway and the two pathways for isoprenoid production (Figure 3).

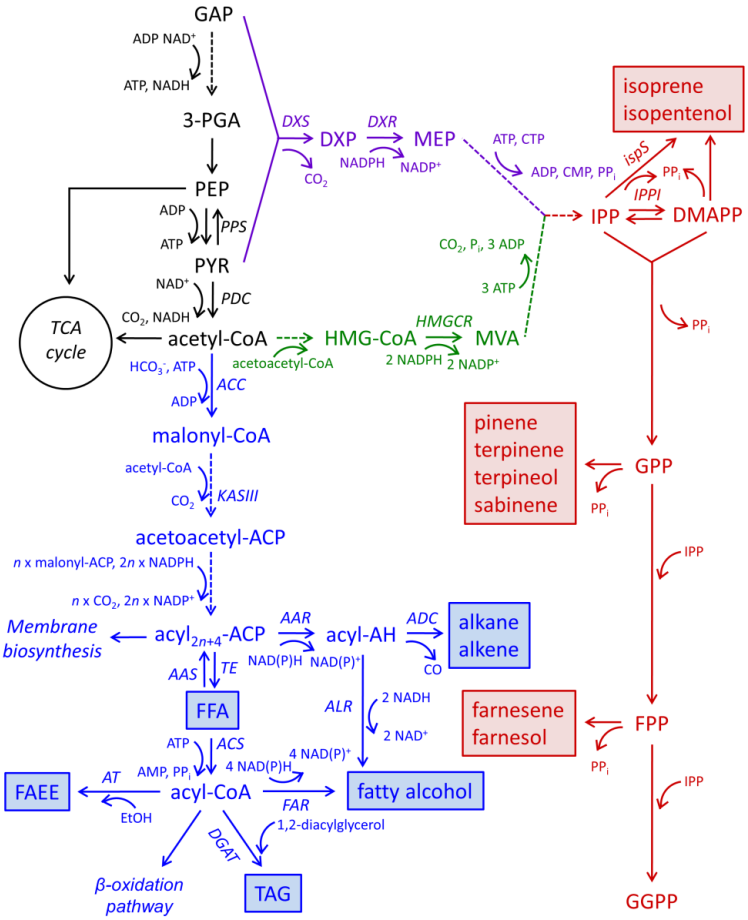

Figure 3. Hydrocarbon biosynthesis pathways for the production of biofuels, with the fatty acid biosynthesis pathway in blue, isoprenoid pathway in red, mevalonate pathway in green, and methylerythritol phosphate pathway in purple. Biofuels and biofuel precursors are highlighted in the colored boxes. Enzymes are in italics. Solid arrows represent a single enzymatic step, while dashed arrows represent multiple enzymatic steps. Abbreviations for metabolites and enzymes are listed at the end of the chapter.

http://dx.doi.org/10.5772/52050

2.1. Fatty acid derived biofuels

As shown in Figure 3, fatty acid biosynthesis interfaces with the primary metabolism at the acetyl-CoA node. Fatty acid biosynthesis is initiated by the formation of acetoacetyl-ACP, the substrate for fatty acid chain elongation. The conversion of acetyl-CoA to acetoacetyl-ACP includes two key enzymatic steps: (1) the conversion of acetyl-CoA to malonyl-CoA, catalyzed by acetyl-CoA carboxylase (ACC) and (2) the conversion of malonyl-ACP to acetoacetyl-ACP via β-ketoacyl-ACP synthase III (KASIII). These two enzymes are common metabolic engineering targets for improving fatty acid biosynthesis. In fact, ACC has been shown to be a rate-limiting step of fatty acid synthesis in *Escherichia coli*, and overexpression of ACC has been shown to yield more than a 5-fold increase in FFA production [5]. Overexpression of KASIII in *E. coli* also improved FFA synthesis, increasing lipid production by 20-60% [6]. After acetoacetyl-ACP formation, fatty acid chain elongation proceeds by an iterative process, whereby the hydrocarbon chain is elongated in increments of 2 carbons. Once the elongation process terminates, the final acyl-ACP is divided among three possible paths: one leading to membrane biosynthesis, an essential pathway for cell growth, and the other two yielding hydrocarbon fuels or fuel precursors (Figure 3).

To produce biofuels with an even-numbered carbon chain, the acyl-ACP is cleaved by a thioesterase (TE), releasing the FFA. The TE is yet another key target for metabolic engineering. The final fuel properties, including viscosity, cloud point, flash point, oxidative stability, ignition delay, and combustion quality, are largely determined by the hydrocarbon chain length and degree of saturation [7]. Accordingly, numerous TEs have been cloned and characterized, predominantly from plant sources, to control the carbon chain length of the FFAs. Engineering strategies often exploit this collection of TEs to tailor the biofuel product. Favored TEs include a truncated TE (‘*tesA*) from *E. coli* and acyl-ACP TEs from *Umbellularia californica* and *Cuphea hookeriana*, producing FFAs with carbon lengths of 16:0, 12:0, and 10:0 and 8:0, respectively [8-10]. The FFAs themselves can be extracted as fuel precursors and converted into biodiesel (FAMEs or FAEEs) using acid-catalyzed chemical processes [11]. To allow for FFA accumulation, the β-oxidation pathway and free fatty acid recycling are often eliminated by gene knockout of acyl-CoA synthetase (*acs*) and acyl-ACP synthetase (*aas*) [12]. An alternative strategy was recently demonstrated, whereby FFAs were synthesized through an engineered reversal of the β-oxidation cycle [13]. In this strategy, acetyl-CoA is used directly for fatty acid chain elongation, allowing for improved carbon and energy efficiency compared to the fatty acid biosynthesis pathway which requires activation of acetyl-CoA to malonyl-CoA. Engineering a reversed β-oxidation cycle required modification of multiple regulatory mechanisms, knockout of other fermentative pathways, expression of a TE or other fuel producing enzyme, and overexpression of key enzymes in the β-oxidation pathway [13]. While this strategy yielded the highest reported concentration of FFAs in *E. coli* (7 g/L), its application to other host organisms may be restricted by inadequate knowledge of the native regulatory mechanisms.

With an intact *acs*, FFAs can be converted into acyl-CoA, a precursor for other fuel products including the biodiesel precursor, TAG, and fuels such as FAEEs and fatty alcohols (Figure 3). The conversion of acyl-CoA to TAG requires the provision of 1,2-diacylglycerol and a

diacylglycerol acyltransferase (DGAT) to catalyze transfer of the acyl chain. While DGAT has been overexpressed to improve TAG production in plants [14], the utility of this strategy still remains to be tested in microorganisms. Most metabolic engineering strategies for microbial TAG synthesis focus on improving the supply of the precursors: FFA and glycerol-3-phosphate (G3P) [15, 16]. Microbial production of FAEEs typically involves heterologous expression of both the pathway for ethanol production and an acyltransferase (AT) [17-19]. Selection of the two genes required for ethanol synthesis, pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase (*adh*), will largely depend on the host organism, but generally, efforts involving prokaryotic hosts such as *E. coli* and cyanobacteria will use *pdc* and *adh* from *Zymomonas mobilis* due to their capacity for high ethanol production [20]. To date, only one AT has been heterologously expressed for FAEE production: the wax synthase gene (*aftA*) from *Acinetobacter baylyi* ADP1 [17-19]. A third biofuel product derived from acyl-CoA is fatty alcohols. The enzymatic conversion of acyl-CoA to a fatty alcohol is dependent upon whether the fatty acyl-CoA reductase (*far*) is of prokaryotic or eukaryotic origin. Most prokaryotic FARs reduce acyl-CoA to a fatty aldehyde, requiring another enzyme, fatty aldehyde reductase (ALR), for conversion to the fatty alcohol product. On the other hand, eukaryotic FARs catalyze the direct conversion of acyl-CoA to fatty alcohol without release of an aldehyde intermediate [21]. Metabolic engineering strategies for fatty alcohol production include: expression of a prokaryotic FAR, *acr1* from *Acinetobacter calcoaceticus* BD413, with reliance on native fatty aldehyde reductases for fatty alcohol synthesis [19]; expression of 5 different eukaryotic FAR homologs from the model plant organism *Arabidopsis thaliana* [22]; and expression of a eukaryotic FAR, *far1* from mouse [23]. The recent discovery of a prokaryotic FAR from *Marinobacter aquaeolei* VT8, capable of catalyzing the direct conversion of acyl-CoA to fatty alcohol, may be a beneficial alternative to the use of eukaryotic FARs for fatty alcohol production in prokaryotic hosts such as *E. coli* and cyanobacteria [24]. An alternative strategy used by Dellomonaco and colleagues identifies surrogates for *far* and *adh* in the native *E. coli* genome based on sequence homology [13]. With the numerous biofuel products derived from acyl-CoA and the natural enzymatic diversity for these conversions, we have only just begun to explore and develop the metabolic engineering tools essential to enable large-scale synthesis.

In addition to oxygen-containing biofuels, acyl-ACP can also be converted into pure hydrocarbon fuels in the form of alkanes and alkenes (Figure 3). In 2010, the discovery of an alkane synthesis pathway in cyanobacteria provided the genetic knowledge necessary for engineering microbial alkane production [25]. The pathway consists of two enzymatic steps: (1) reduction of acyl-ACP to a fatty aldehyde by means of an acyl-ACP reductase (AAR) and (2) decarbonylation of the aldehyde to an alkane or alkene, catalyzed by an aldehyde decarbonylase (ADC). Due to the recent discovery of this pathway, few metabolic engineering strategies have been applied for alkane production. Some strategies focus on improving supply of the acyl-ACP precursor, relying on the native cyanobacterial pathway for alkane synthesis [23], while others have simply transferred the alkane pathway (AAR and ADC) into another host organism [25-27]. With the rapidly growing database of genome sequence information, numerous homologs of AAR and ADC have been identified [26, 27], representing a diverse range of targets for metabolic engineering. Future optimization of the alkane biosynthesis pathway may result in the high alkane yields needed for biofuel production.

http://dx.doi.org/10.5772/52050

2.2. Isoprenoid-based biofuels

The chemical composition of petroleum-based fuels: gasoline, diesel, and jet fuel, includes linear, branched, and cyclic alkanes, aromatics, and chemical additives [28]. Isoprenoid-based biofuels have the structural diversity to mimic these petroleum compounds, with up to 50,000 known isoprenoid structures including branched and cyclic hydrocarbons with varying degrees of unsaturation [29, 30]. Isoprenoids reported to be potential fuel candidates include: the hemiterpene (C5) isoprene; monoterpenes (C10): terpinene, pinene, limonene, and sabinene; the sesquiterpene (C15) farnesene, and their associated alcohols: isopentenol, terpineol, geraniol, and farnesol [12, 31]. Two metabolic pathways are capable of producing the isoprenoid building blocks isopentenyl pyrophosphate (IPP) and dimethylallyl diphosphate (DMAPP): the mevalonate (MVA) pathway [32] and the methylerythritol phosphate (MEP) pathway, also known as the 1-deoxy-D-xylulose-5-phosphate (DXP) pathway and the non-mevalonate pathway (Figure 3) [33]. In general, the MVA pathway is found in eukaryotes and archaea while the MEP pathway is utilized by prokaryotes. In agreement with the proposed evolutionary origin of plants, they contain both isoprenoid pathways with the MEP pathway localized in the plastid and the MVA pathway in the cytosol [34]. The MVA and MEP pathways differ with respect to their requirement for carbon, energy, and reducing equivalents; this is illustrated by the net balances for IPP biosynthesis from glyceraldehyde-3-phosphate (GAP):

$$\text{MVA:3 GAP} + 3\ \text{ADP} + 4\ \text{NAD(P)}^{+} + 2\ \text{P}_{\text{i}} \rightarrow \text{IPP} + 4\ \text{CO}_2 + 3\ \text{ATP} + 4\ \text{NAD(P)H} \quad (1)$$

$$\text{MEP:2 GAP} + \text{ADP} + \text{CTP} + \text{P}_{\text{i}} \rightarrow \text{IPP} + \text{CO}_2 + \text{ATP} + \text{CMP} + \text{PP}_{\text{i}} \quad (2)$$

Based on these balances, IPP production via the MEP pathway is more efficient at carbon utilization, as only 2 GAPs are required and 1 CO_2 is emitted, compared to 3 GAPs and 4 CO_2 for the MVA pathway. On the other hand, IPP production via the MVA pathway is more energy efficient overall, resulting in ATP generation and yielding a net gain in reducing equivalents (NAD(P)H). These carbon, energy, and reducing equivalent requirements should be considered when designing a metabolic engineering strategy for isoprenoid biosynthesis.

The MVA pathway interfaces with the primary metabolism at the acetyl-CoA node (Figure 3), and it can be divided into two parts: the top, which involves 3 enzymatic steps to convert acetyl-CoA to MVA, and the 3 enzymatic conversions of the bottom portion to produce IPP from MVA. One novel metabolic engineering strategy compared the efficiencies of the top and bottom portions of the MVA pathway in *E. coli* using heterologously expressed pathways from 5 different eukaryotic sources. The most efficient top and bottom portions were combined to maximize the yield of isoprenoid building blocks [35]. Accumulation of an intermediate metabolite, 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA), is a known bottleneck in the top MVA pathway, and HMG-CoA was also shown to inhibit cell growth in *E. coli* [36]. Thus, overexpression of the HMG-CoA reductase (HMGCR) increased MEV production and synthesis of subsequent FPP-derivatives in both *E. coli* and *S. cerevisiae* [36-38]. Whole pathway

expression and elimination of the HMGCR bottleneck have proven to be successful techniques for enhancing the metabolic throughput of the MVA pathway.

The MEP pathway requires two primary metabolites as precursors: GAP and pyruvate (PYR) (Figure 3). Compared to the 6 enzymatic steps of the MVA pathway, the MEP pathway is comprised of 7 steps. Metabolic engineering strategies for the MEP pathway have primarily focused on the first two enzymatic steps. Overexpression of 1-deoxy-D-xylulose-5-phosphate synthase (*dxs*), catalyzing the conversion of GAP and PYR to 1-deoxy-D-xylulose-5-phosphate (DXP), resulted in 6-10-fold increases in the final isoprenoid product [39, 40]. Targeting the next enzymatic step through overexpression of DXP reductoisomerase (*dxr*) was shown to have little effect on isoprenoid production using the native gene; however, expression of *dxs* and *dxr* from *Bacillus subtilis* improved isoprenoid production 2.3-fold in *E. coli* [41]. The final step of the MEP pathway was also shown to be rate-limiting, as heterologous expression of IPP isomerases (IPPI) enhanced isoprenoid production in *E. coli* [42]. Based on its rate-limiting steps, the MEP pathway is a prime candidate for a push-pull metabolic engineering strategy, whereby overexpression of the first step 'pushes' carbon flux into the MEP pathway and overexpression of the final step 'pulls' the metabolic flux towards the end product. This strategy yielded nearly 2-fold improvements in isoprenoid production in *E. coli* [43, 44]. Lastly, overexpression of the entire MEP pathway can increase isoprenoid biosynthesis. In fact, Leonard and colleagues demonstrated that 5 additional copies of the MEP pathway genes yielded the highest production, while further increasing the gene copy number to 10 produced lower titers [45].

While targeted gene overexpression may alleviate pathway bottlenecks, the pathway is still subject to native regulatory mechanisms which may limit isoprenoid biosynthesis from either the MVA or MEP pathways. A highly successful strategy for overcoming regulatory limitations is overexpression of the non-native isoprenoid pathway. Expression of the MVA pathway from *Saccharomyces cerevisiae* in *E. coli* has enabled higher levels of isoprenoid synthesis compared to engineering the native MEP pathway as the sole isoprenoid pathway [46-50]. The success of this strategy has made it a favorite among metabolic engineers seeking to improve isoprenoid biosynthesis. Farmer and Liao presented a clever approach for regulating the carbon flux into an engineered MEP pathway in *E. coli* [51]. In this work, a native regulatory circuit was used to control the carbon flux into and through the MEP pathway by regulating expression of two key enzymes: phosphoenolpyruvate synthase (PPS) and isopentenyl diphosphate isomerase (IPPI). Under excess carbon flux, expression of *pps* and *idi* was activated using the regulatory circuit, redirecting carbon flux into and through the MEP pathway, yet when the carbon flux was growth limiting, expression of these genes was reduced. This strategy allows for high isoprenoid production without negatively impacting cell growth. As evidence, the regulated pathway improved isoprenoid titers by 50%, while simply placing *pps* and *ippi* under control of strong *tac* promoters resulted in growth inhibition [51]. Native regulatory mechanisms are often obstacles limiting isoprenoid biosynthesis, yet they can also be exploited to optimize the flux balance to support both cell growth and isoprenoid production.

Additional targets for improving isoprenoid-based fuel production include precursor supply, cofactor supply, and optimization of the downstream fuel synthesis pathway. Acetyl-CoA is

http://dx.doi.org/10.5772/52050

the precursor for isoprenoid production via the MVA pathway. Overexpression of acetaldehyde dehydrogenase (ALDH) and acetyl-CoA synthetase (ACS), both of which produce acetyl-CoA, increased the acetyl-CoA supply and subsequently isoprenoid biosynthesis in *S. cerevisiae* [52]. On the other hand, the MEP pathway requires two precursors from the glycolysis pathway: PYR and GAP. The supply of these metabolites is complicated by the fact that PYR is derived from GAP, and consequently, the PYR/GAP balance is an important metabolic engineering target. The supply of GAP was shown to be limiting in *E. coli*, as modifying the conversion between PEP and PYR to redistribute the flux toward GAP synthesis increased isoprenoid production [53]. In addition to the carbon precursors, co-factors in the form of energy (ATP, CTP) and reducing equivalents (NADPH) are also required for isoprenoid synthesis. Co-factor supply is often overlooked in strategies for isoprenoid production, yet by improving the availability of NADPH in *S. cerevisiae*, isoprenoid synthesis through the MVA pathway increased by 85% [54]. This result emphasizes the importance of co-factor availability. Despite optimizing production of the isoprenoid building blocks, the downstream efficiency of assembling the final fuel product may still limit the overall yield. Successful strategies for improving downstream efficiency include overexpression of GPP and FPP synthases [47], overexpression and codon optimization of hemiterpene, monoterpene, and sesquiterpene synthases [41, 47, 48], fusion proteins to localize FPP synthesis and its conversion to sesquiterpene [47], and downregulation of competing products like squalene [37, 48]. The optimized production of isoprenoid-based fuels requires strategies to address limitations throughout the metabolic pathway, from precursor and co-factor supply to end product synthesis.

3. Influence of feedstock on hydrocarbon-based biofuel production

While hydrocarbon-based biofuel production relies on the biosynthetic pathways discussed in the previous section, the source of feedstock plays an important role in the overall production process. As discussed in the Introduction to this chapter, there are two main feedstocks for biofuel production: lignocellulosic biomass and gaseous CO_2, supporting the production of second and third generation biofuels, respectively (Figure 1). Both processes ultimately rely on CO_2 and sunlight as the carbon and energy source, but the microbial conversion processes are distinctly different between the two feedstocks. Lignocellulosic biomass deconstruction produces organic carbon, mostly in the form of hexoses and pentoses (C5 and C6 sugars); this feedstock requires heterotrophic microorganisms to convert the organic carbon into biofuel. Alternatively, the fixation of inorganic carbon feedstock (CO_2/HCO_3^-) into biofuel is reliant upon autotrophic microbes. The heterotroph vs. autotroph requirement of the respective feedstocks is an important distinction from both the metabolic engineering and biofuel production perspectives. Only a few model microorganisms are capable of both heterotrophy and autotrophy, resulting in different host candidates for second and third generation biofuel production. The feedstock will also influence the metabolic engineering targets, as heterotrophs utilize glycolysis and oxidative phosphorylation pathways for carbon consumption and energy production while oxygen-generating autotrophs utilize the Calvin-Benson-Bassham cycle and photosynthesis under light conditions (Figure 4). This section will discuss the host

organisms, engineering strategies, and biofuel production processes specific to each carbon feedstock.

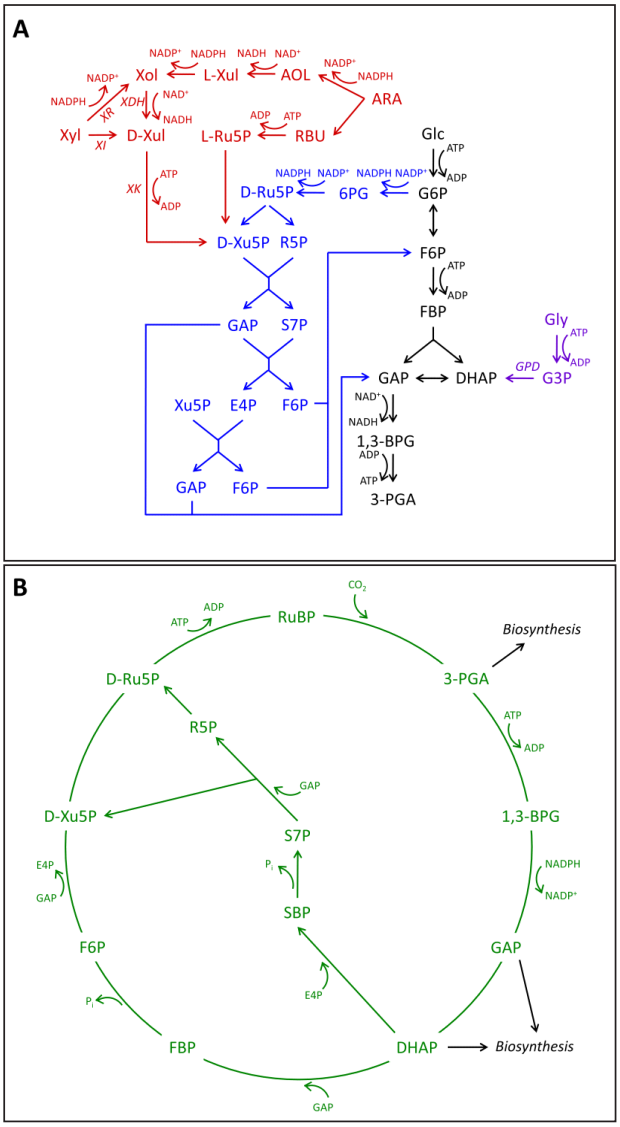

Figure 4. Heterotrophic (A) and autotrophic (B) pathways for carbon utilization, with the Embden-Meyerhof-Parnas (EMP) pathway (glycolysis) in black, the pentose phosphate pathway (PPP) in blue, pentose utilization pathways in red, glycerol metabolism in purple, and the Calvin-Benson-Bassham cycle in green. Abbreviations for metabolites and enzymes are listed at the end of the chapter.

http://dx.doi.org/10.5772/52050

3.1. Hydrocarbon biofuel production from organic carbon feedstocks

The release of C5 and C6 sugars from lignocellulosic biomass deconstruction supports the growth of heterotrophic microorganisms and the metabolic conversion of sugars into biofuel. Representative hydrocarbon-based fuel titers produced by engineered, heterotrophic hosts are listed in Table 1. The most common heterotrophic hosts for biofuel production are the model organisms *Escherichia coli* and *Saccharomyces cerevisiae*. These hosts are attractive candidates for fuel production due to their fast growth rates, well-known genetics and regulation, advanced molecular tools for genetic engineering, and established use in the industrial setting. Neither *E. coli* nor *S. cerevisiae* naturally produce significant amounts of hydrocarbon-based fuels, necessitating the application of metabolic engineering techniques. Heterotrophic organisms that naturally produce hydrocarbon-based fuels are also potential hosts for large-scale biofuel production. For example, *Bacillus subtilis* naturally produces higher concentrations of isoprene than other commonly known bacteria like *E. coli* [55]. *B. subtilis* is also a model organism for Gram-positive bacteria with established tools for genetic modification, advancing its appeal as a host for isoprene production. Similarly, heterotrophic algae can produce significant quantities of TAG. This has motivated some preliminary investigation into engineering the model green alga, *Chlamydomonas reinhardtii*, for TAG production [56-58]. While most metabolic engineering efforts have focused on these model heterotrophic hosts, genetic tools can be developed for other organisms with desirable fuel production traits.

Hydrocarbon Fuel/ Fuel Precursor	Concentration Range	Microbial Hosts	References
		Heterotrophic Production	
FFA	0.5 – 7 g/L	*Escherichia coli*	[5, 12, 13, 19, 59, 60]
	0.024 – 0.2 g/L	*Saccharomyces cerevisiae*	[61, 62]
TAG	20 - 32.6% dcw, 0.12 g/L	*Chlamydomonas reinhardtii*	[56-58]
	0.4 – 0.7 g/L	*Saccharomyces cerevisiae*	[63, 64]
FAEE	0.07 – 1.5 g/L	*Escherichia coli*	[18, 19, 65-67]
	N/A	*Saccharomyces cerevisiae*	[17]
Fatty alcohols	0.001 – 1.67 g/L	*Escherichia coli*	[13, 19, 22, 27, 59, 66, 68]
Alkanes/Alkenes	0.042 – 0.32 g/L	*Escherichia coli*	[25, 27]
Other Isoprenoids (lycopene, β-carotene, amorphadiene,	0.002 – 1 g/L	*Escherichia coli*	[35, 39, 42, 45, 50, 69]
	0.01 g/L	*Saccharomyces cerevisiae*	[37, 52]

Hydrocarbon Fuel/ Fuel Precursor	Concentration Range	Microbial Hosts	References
levopimaradiene, cubebol)			
Isoprene	0.31 – 0.53 g/L	*Escherichia coli*	[41, 49]
	0.002 g/L	*Bacillus subtilis*	[55]
Farnesol	N/A	*Escherichia coli*	[48]
	0.009 – 0.15 g/L	*Saccharomyces cerevisiae*	[37, 38, 70, 71]
Farnesene	0.38 – 1.1 g/L	*Escherichia coli*	[47, 72]
Autotrophic Production			
FFA	0.11 - 0.20 g/L	*Synechocystis* sp. PCC 6803	[73-75]
	0.015 - 0.06 g/L	*Synechococcus elongatus* PCC 7942	[73, 75, 76]
	0.051 g/L	*Synechococcus* sp. PCC 7002	[77]
TAG	28.5% dcw	*Chlamydomonas reinhardtii*	[57]
FAEE	0.077 – 0.086 g/L	*Synechococcus* sp. PCC 7002	[77]
Fatty alcohols	200 µg/L	*Synechocystis* sp. PCC 6803	[23]
Alkanes/Alkenes	150 µg/L/OD730	*Synechocystis* sp. PCC 6803	[23]
	0.05 g/L	*Synechococcus* sp. PCC 7002	[26]
	N/A	*Thermosynechococcus elongatus* BP-1	[26]
Isoprene	0.5 mg/L	*Synechocystis* sp. PCC 6803	[78]

Table 1. Hydrocarbon fuels and fuel precursors produced by genetically engineered microorganisms.

Most heterotrophic hosts for biofuel production utilize the Embden-Meyerhof-Parnas (EMP) pathway for sugar catabolism (Figure 4). The EMP pathway has evolved for efficient carbon utilization and is typically not rate-limiting for fuel production. As such, EMP pathway enzymes are not often targeted for genetic manipulation. However, the organic feedstock from lignocellulose deconstruction is comprised of a range of sugars, including hexoses: glucose, mannose, and galactose, and pentoses: xylose and arabinose [79]. A major concern in converting these sugars into fuel is the efficient utilization of all available hexoses and pentoses. While some organisms like *E. coli* can naturally metabolize these different forms of sugar, others, like *S. cerevisiae*, can only utilize specific forms [80]. *S. cerevisiae* does not naturally express pathways for catabolizing pentoses. There are two known pathways for xylose catabolism, both of which have been expressed in *S. cerevisiae* [81-83]. Xylose can be converted into xylulose-5-phosphate (Xu5P), an intermediate in the pentose phosphate pathway (PPP), through expression of a xylose isomerase (XI) and xylulose kinase (XK) [82]. Alternatively, the XI can be replaced by a xylose reductase (XR) and xylitol dehydrogenase (XDH) [81, 82]. Complications

http://dx.doi.org/10.5772/52050

in these two xylose utilization pathways include the inhibition of XI by xylitol (Xol) and the reducing equivalents required by XR and XDH [80]. Successful strategies for engineering xylose utilization in *S. cerevisiae* include expression of a fungal XI from *Piromyces* sp. E2 along with overexpression of the non-oxidative PPP pathway [84] and expression of XR and XDH from the xylose-fermenting yeast *Pichia stipitis* [85]. Two pathways have also been expressed in *S. cerevisiae* for arabinose utilization [86, 87]. The bacterial pathway for arabinose catabolism consists of 3 enzymatic steps, while the fungal pathway involves 5 enzymatic steps, 4 of which require cofactors of NADPH or NAD^+ (Figure 4). Efficient arabinose utilization in *S. cerevisiae* has been achieved through heterologous expression of a bacterial arabinose catabolism pathway along with overexpression of the non-oxidative PPP and evolutionary engineering [88]. While most of these metabolic engineering examples focus on utilizing sugars for fermentation to ethanol, the strategies for engineering carbon utilization can also be applied for hydrocarbon-based fuel production.

Unlike *S. cerevisiae*, *E. coli* can utilize the hexoses and pentoses derived from lignocellulose; however, the carbon catabolite repression (CCR) system in *E. coli* leads to inefficient, diauxic growth [89]. Through CCR, *E. coli* sequentially consumes different sources of organic carbon based on substrate preference, leading to delayed and often incomplete utilization of unpreferred sugars like xylose and arabinose. This translates into lower productivities and yields along with downstream complications due to the presence of unmetabolized sugars [80]. As a result, CCR is often targeted by metabolic engineering to alleviate these undesired effects. A common engineering strategy is to use mutants of the transcriptional activator CRP (cyclic AMP receptor protein) which have been modified to eliminate the allosteric requirement for cAMP, thereby leading to expression of the pentose catabolizing pathways in the presence of the preferred substrate, glucose [90]. The phosphotransferase system (PTS), responsible for the preferential uptake of glucose, has also been deleted to encourage simultaneous utilization of mixed sugars [91]. Lastly, deletion of methylglyoxyal synthase was shown to improve the co-metabolism of sugars, ostensibly due to elimination of methylglyoxyal, an inhibitor of sugar metabolism [92]. Through modifying the components of CCR, *E. coli* can be engineered to efficiently utilize the organic carbon mixture resulting from lignocellulose degradation.

In addition to the hexoses and pentoses derived from lignocellulosic biomass, glycerol may soon become an inexpensive organic carbon source for fuel production. Glycerol is a byproduct of the conversion of TAG into biodiesel during algal biofuel processing, and thus, large quantities of glycerol may be available for use as an organic carbon source. The main pathway for aerobic glycerol utilization involves a two-step conversion to produce the glycolytic metabolite DHAP [93]. The glycerol utilization pathway is not a common target for metabolic engineering, yet glycerol has been reported as a supplementary carbon source for the production of isoprenoid-based fuels, farnesol and α-farnesene [47, 48]. Future metabolic engineering efforts may focus more on glycerol utilization as the availability of glycerol increases.

Second generation biofuel production still remains to be demonstrated at large scales, yet the overall process is easily integrated with current technologies. Equipment and practices used for agricultural harvesting can be directly applied to harvesting lignocellulosic biomass. In fact, some agricultural processes already produce biomass waste streams that can be utilized

for feedstock, such as corn stover. Moreover, commercial fermenters can be employed as bioreactors for the microbial fuel conversion. The main technical difficulties in large-scale lignocellulosic fuel production center on provision of the carbon source. The quantities of biomass needed to support industrial-scale fuel production will require a significant investment of land and nutrient resources, and the supply will be subject to varying climate conditions. A supply chain infrastructure must also be constructed to harvest the biomass and transport it to the production facilities. A primary technical focus of current research on lignocellulosic-derived fuels is the deconstruction of biomass into useable sugars. The thermal, chemical, and enzymatic processes for biomass deconstruction have been a limiting factor for economical second generation biofuel production [94, 95]. As the cost of biomass deconstruction is reduced with new technology, the large-scale production of second generation biofuels will begin to contribute to the world's supply of renewable energy.

3.2. Hydrocarbon biofuel production from inorganic carbon feedstocks

The direct conversion of CO_2 into hydrocarbon-based fuels could greatly simplify the overall production process and reduce the cost of biofuel production (Figure 1). The search for autotrophic microorganisms capable of performing this CO_2-to-fuel conversion started in the late 1970's with the U.S. Department of Energy's Aquatic Species Program (ASP) [96]. The ASP isolated and screened over 3,000 species of microalgae from a diverse range of environmental habitats. The program focused mainly on eukaryotic algae, as they naturally produce significant amounts of TAG. During the course of the program, the recombinant DNA technology used in metabolic engineering was developed, yet due to the infancy of this technology, it was not applied to microalgae for fuel applications until near the end of the ASP [15]. With the development of recombinant DNA technology, prokaryotic microalgae (i.e. cyanobacteria, previously known as blue-green algae) were recognized as potential hosts for fuel production, and the successful engineering of cyanobacteria for ethanol production confirmed their potential [97]. Unfortunately, research funding for microalgal fuel production waned as crude oil prices fell in the 1990's. However, in the late 2000's, the cost of crude oil soared, spurring a resurgence of interest in microalgae for fuel production and in the application of metabolic engineering to enhance fuel yields. In general, both eukaryotic microalgae (referred to as algae in the subsequent text) and prokaryotic microalgae (referred to as cyanobacteria in the subsequent text) utilize photosynthesis for energy generation and the Calvin-Benson-Bassham cycle for CO_2 fixation (Figure 4). However, due to the cellular differences between algae and cyanobacteria, the strategies for engineering autotrophic fuel production will be discussed based on this host division.

3.2.1. Engineering algae for biofuel production

Algae are predicted to have first appeared approximately 1.5 billion years ago from an endosymbiotic event in which a eukaryotic cell engulfed a cyanobacterium [98]. The cyanobacterium evolved into the modern day chloroplast, the algal organelle responsible for photosynthesis and carbon fixation. Today, algae can be found in a wide-range of environmental habitats from freshwater lakes and oceans to deserts and even the snow of the Antarctic

http://dx.doi.org/10.5772/52050

[99]. Along with this diversity of habitat, algae have evolved diverse cellular physiologies and genetics, resulting in a wealth of potential hosts and genetic sources for engineering fuel production. Many types of algae are currently under consideration for fuel production due to their natural TAG synthesis, including diatoms, green algae, eustigmatophytes, prymnesiophytes, and red algae [100]. While many types of algae produce the fuel precursor TAG, few algal species have well-developed genetic tools available for engineering improved lipid production [101, 102]. Consequently, there are only a few reported examples of engineering algae for biofuel production.

To date, the only genetic mutation shown to improve lipid production in algae is the elimination of starch biosynthesis, a competing carbon sink. The generation of mutants with impaired starch synthesis using random mutagenesis techniques resulted in up to a 10-fold increase in cellular lipid production in *C. reinhardtii* [56-58, 103]. Other targeted metabolic engineering attempts, such as overexpression of ACC in the diatoms *Cyclotella cryptic* and *Navicula saprophila*, failed to improve TAG biosynthesis [15, 96]. In addition to targeting overall TAG production, metabolic engineering strategies have been applied to influence the chemical composition of the fatty acid side chains. By expressing two heterologous TEs, the diatom *Phaeodactylum tricornutum* produced TAG with increased levels of lauric acid (C12:0) and myristic acid (C14:0) [104]. These shorter chain length fatty acids are more desirable for fuel production, and this demonstrates the potential to control the chemical composition of the fuel product and its associated properties with metabolic engineering. While examples of engineering algal TAG production are sparse, many engineering strategies have proven successful at improving the fatty acid content in plants. These strategies include expression of ACC and KASIII involved in fatty acid biosynthesis, expression of G3P dehydrogenase (GPD) for production of the glycerol backbone of TAG, expression of ATs such as DGAT, expression of TEs to release FFAs, and deletion of desaturases to alter the fatty acid composition [105]. Similar strategies may also be successful at improving TAG production in algae.

The metabolic engineering of algae is complicated by several factors. Most algae have a rigid cell wall structure that makes transformation difficult. A common transformation technique uses glass beads (or silicon carbide whiskers) along with a cell wall-deficient algal strain [106]. The cell wall can be removed using enzymatic techniques or through genetic mutation. Alternatively, a microparticle bombardment technique has been applied successfully to transform many different algal species [107]. In this technique, the recombinant DNA is coated onto a metal microparticle and 'shot' into the algal cell using a helium-powered 'gun'. Other transformation methods include electroporation and the traditional plant transformation technique of *Agrobacterium tumefaciens* T-DNA-mediated transfer [107]. Once the recombinant DNA enters the cell, it must integrate into one of 3 algal genomes: nuclear, chloroplast, or mitochondrial (assuming the transformed DNA is not a stably maintained plasmid). DNA has been successfully integrated into the chloroplast genome via homologous recombination, whereby the recombinant gene and marker are flanked by homologous (i.e. matching) regions of the targeted chloroplast DNA, and the recombinant DNA replaces the matching region in the chloroplast. Unfortunately, homologous recombination does not occur in the nuclear genomes of many algae [108], and instead, the recombinant DNA is randomly integrated into

the nuclear genome. This complicates metabolic engineering strategies due to the possibility of detrimental genetic effects resulting from the random integration and the lack of a technique for targeted gene knockout. Lastly, algal engineering attempts are often plagued by low gene expression. It has been discovered that many algae, like the model alga *C. reinhardtii*, employ RNA-mediated gene silencing [109]. Numerous strategies have been applied to combat the low gene expression brought about by gene silencing in algae, including codon optimization, the use of 5′ and 3′ untranslated regions which may participate in regulatory functions, and the inclusion of native intron sequences [108]. Knowledge of the gene silencing mechanisms in algae has led to the development of RNA interference (RNAi) technology for gene knockdown. RNAi exploits the native cellular machinery for gene silencing to reduce the expression of target genes [109]. As we continue to expand our knowledge of algal genetics, the list of engineered algae will rapidly increase. As evidence, the biofuel-relevant alga, *Nannochloropsis* sp., was recently shown to have a high efficiency of homologous recombination in the nuclear genome [110]. This will simplify future strategies for genetic engineering in *Nannochloropsis* sp. Another promising development is the construction of a plasmid for gene expression in *C. reinhardtii* that is now commercially available through Life Technologies [111]. The greater availability and standardization of tools for the genetic manipulation of algae will move algal engineering towards the advanced stages currently seen with other industrial organisms like *E. coli* and *S. cerevisiae*.

3.2.2. Engineering cyanobacteria for biofuel production

Cyanobacteria are predicted to be the first microorganisms to develop the capability of oxygenic photosynthesis, some 2.7 billion years ago [112]. Similar to algae, cyanobacteria have a great range of diverse morphologies, cellular functions, and genetics, presumably due to their long evolutionary history and their diverse habitats. As discussed previously, the ASP initially deemed cyanobacteria unfit for fuel production due to their lack of natural TAG accumulation. Since they are amenable to genetic manipulation, however, cyanobacteria can be engineered to produce a range of biofuel products (Table 1). As prokaryotes, cyanobacteria are subject to the traditional methods employed for engineering other well-developed bacterial hosts like *E. coli*. Some strains of cyanobacteria are even naturally transformable, uptaking exogenous DNA from their environment without the use of cell permeablization techniques [113]. As progenitors of the algal chloroplast, cyanobacteria also integrate DNA into their chromosomes using homologous recombination. Moreover, cyanobacteria do not possess the cellular components for gene silencing. The genetic tools for engineering some model strains of cyanobacteria are well developed and have been used to genetically modify cyanobacteria for several decades [113]. Another advantage of using cyanobacteria as the microbial host for hydrocarbon-based fuel production is that they have been shown to excrete potential fuel precursors such as FFAs [73]. Fuel excretion enables a continuous production process, eliminating the cost associated with harvesting the algal biomass and the time and nutrients needed to repeatedly grow new batches of algae for fuel production. The advantages of straightforward genetic manipulation and fuel excretion make cyanobacteria contenders for large-scale biofuel production despite the disadvantage of low natural lipid yields.

http://dx.doi.org/10.5772/52050

After the initial demonstration of engineering cyanobacteria for ethanol production [97], the production of hydrocarbon-based fuels in engineered cyanobacteria has expanded to include isoprene, FFAs, FAEEs, fatty alcohols, and alkanes/alkenes (Table 1). Isoprene biosynthesis was established in the model cyanobacterium, *Synechocystis* sp. PCC 6803, through expression of the isoprene synthase (*ispS*) from kudzu [78]. Codon optimization of *ispS* and the use of a strong promoter (*psbA2*) increased isoprene production. Engineering strategies targeting the upstream MEP pathway for isoprenoid biosynthesis, as described in Section 2.2 of this chapter, will likely further improve isoprene productivity. The remaining four hydrocarbon-based fuels are all derived from the fatty acid biosynthesis pathway. Common strategies for improving FFA production (see Section 2.1) have proven successful in cyanobacteria [74-76]. Eliminating non-essential, competing pathways such as polyhydroxybutyrate (PHB), cyanophycin, and acetate biosynthesis also improved FFA production [74]. Liu and colleagues engineered a more permeable peptidoglycan layer to improve FFA excretion in *Synechocystis* sp., yet this weakened cell membrane resulted in slower growth rates and may also make the engineered cyanobacterium more susceptible to external predators and toxins that may be present in large-scale cultivations. Initial engineering attempts for fatty alcohol and alkane/alkene production entail expression of a heterologous FAR and overexpression of AAR and ADC, respectively [23, 26]. Alkane/alkene synthesis was also observed with ACC overexpression and native AAR and ADC activities in cyanobacteria [23]. Despite being derived from fatty acids, the synthesis of fatty alcohols and alkanes/alkenes is up to 1000-fold lower than that observed with FFA production (Table 1), suggesting that the conversion of acyl-ACP to the final fuel product is rate limiting. These inaugural proof-of-concept reports illustrate the potential of cyanobacteria as hosts for autotrophic biofuel production, but additional metabolic engineering will be required to achieve the fuel titers necessary for large-scale synthesis.

3.3. Heterotrophic vs. autotrophic biofuel production

The selection of organic or inorganic carbon feedstock for biofuel production has downstream ramifications on host selection, product yields, and process requirements. Clearly, the feedstock choice will determine whether a heterotrophic or autotrophic host is required, and in turn, this will influence the metabolic engineering strategy. In general, heterotrophic hosts have generated higher fuel titers than autotrophic hosts, with more than 10-fold higher concentrations of FFAs, FAEEs, fatty alcohols, and alkanes/alkenes (Table 1). This does not imply that heterotrophic production is more advantageous than autotrophic production, for the entire production process must be considered (Figure 1). The sugars from lignocellulosic biomass deconstruction (heterotrophic feedstock) have a higher energy content compared to inorganic carbon (autotrophic feedstock). The overall balances for obtaining one molecule of GAP from heterotrophic and autotrophic metabolisms provide evidence for this:

$$\text{Heterotrophic: } \tfrac{1}{2}\ \text{Glc} + \text{ATP} \rightarrow \text{GAP} + \text{ADP} \tag{3}$$

$$\text{Autotrophic: } 3\ CO_2 + 9\ \text{ATP} + 6\ \text{NADPH} + 5\ H_2O \rightarrow \text{GAP} + 9\ \text{ADP} + 6\ NADP^+ + 8\ P_i \tag{4}$$

While autotrophic GAP generation requires a significant investment of energy (9 ATP) and reducing equivalents (6 NADPH), heterotrophic GAP production only requires one energy equivalent. However, if a life cycle perspective is considered, the carbon from lignocellulosic feedstocks is ultimately derived from photosynthesis, requiring the same energy and reducing equivalent input as autotrophic microorganisms. Overlooking this fact will bias a direct comparison between heterotrophic and autotrophic fuel production.

One major difference between heterotrophic and autotrophic fuel production is the design considerations for the bioreactor. Heterotrophic microbes, such as *E. coli* and *S. cerevisiae*, are traditional industrial microorganisms with well-established, large-scale cultivation practices and bioreactors. On the other hand, autotrophic hosts like algae and cyanobacteria require light as the energy source to drive photosynthesis and inorganic carbon fixation. This can have a dramatic effect on bioreactor design. Transparent materials can be used with traditional bioreactor designs to allow for light penetration. Light availability, however, will ultimately limit the cell densities of photosynthetic microalgae, and the surface area of light exposure with traditional bioreactor designs is not optimal. Some have proposed to use fiber-optics within the liquid culture to improve light availability [114], but a costly solution such as this is not feasible for a low-value, commodity product like fuel. A wide-range of photobioreactor (PBR) designs have been proposed [115], yet generally, PBRs are characterized by the use of transparent materials, high surface area to volume ratios, and a relatively short pathlength for light. Other PBR design factors include a mechanism for air/CO_2 delivery, dissipation of radiative heat, and removal of inhibitory O_2 [115]. Due to the low value of fuel products, PBRs for fuel synthesis favor low-tech designs and inexpensive materials to reduce both capital and operating costs. In fact, NASA has proposed to float plastic bags of algal cultures in wastewater to allow for nutrient exchange [116]. Alternatively, open pond systems, traditionally a raceway configuration with a paddle-wheel for mixing, have proven successful for cultivating microalgae at scale [117]. Unlike PBRs, ponds are open to the environment, allowing for evaporative water loss and pond crash due to contamination by predators and competitors. However, the low capital cost of an open pond system makes this design a contender for fuel production. Clearly, the large-scale cultivation techniques for autotrophic fuel production still require additional development and optimization compared to heterotrophic cultivation.

4. Other metabolic engineering strategies for industrial production of hydrocarbon fuels

In addition to improving hydrocarbon-based fuel synthesis, metabolic engineering strategies can also be applied to address other factors affecting large-scale production. Two main issues will be addressed in this section: product toxicity and industrial strain robustness.

Product toxicity was shown to be a limiting factor in the production of first generation biofuels like ethanol. Since the interest in hydrocarbon-based fuels has developed only during the past decade, the toxicities of these fuels have not been fully explored, particularly with respect to autotrophic hosts. Fortunately, interest in hydrocarbon inhibition of microbial growth dates

http://dx.doi.org/10.5772/52050

back almost a century [118], and we can capitalize on this wealth of information to engineer improved product tolerance in microbial hosts. Most fatty acid derived fuel molecules have shown some antimicrobial activity. FFAs, with a diverse range of carbon chain lengths and degrees of unsaturation, impart inhibitory effects on organisms including algae, Gram-negative and Gram-positive bacteria, fungi, protozoans, and various cell types of multicellular organisms [119]. Medium chain fatty alcohols such as pentanol, hexanol, heptanol, and octanol inhibited the biological activity of several algal and cyanobacterial strains, including fuel-relevant hosts *C. reinhardtii* and *Dunaliella salina* [120]. Interestingly, long-chain fatty alcohols (>C14) did not exhibit inhibitory effects on yeasts, suggesting that targeting longer chain fatty alcohols may eliminate the toxicity concern [121]. Similarly, medium-length alkanes (hexane, heptane, and isooctane) were toxic to microalgae while long-chain alkanes (C12-C16) elicited no effect [120, 121]. Microbial TAG and FAEE toxicities have not been reported. However, the phospholipid membrane surrounding algal TAGs may mask potential inhibitory effects, and FAEE production has been linked to the toxic effects of alcohol consumption in humans [122]. Isoprenoid-based fuel molecules have also illustrated inhibitory effects. Cyclic terpenes, such as pinene and limonene (Figure 2), inhibited the growth of bacteria and *S. cerevisiae* [123, 124], while branched isoprenoids, such as farnesyl hexanoate and geranyl acetate, were shown to be toxic to *E. coli* [125]. In fact, *E. coli*'s tolerance to isoprenoid-derived biodiesels and bioaviation fuels only ranged from 0.025 – 1% (v/v) [125]. Based on these previous studies, product toxicity is a major limiting factor and should be integrated into the metabolic engineering strategy.

A variety of strategies can be adopted to address product toxicity. The easiest way to avoid complications from product toxicity is to select non-toxic fuel targets. Toxicity studies can be conducted for each potential host organism, and generally, fatty alcohols longer than C14, alkanes longer than C9, and alkenes longer than C12 have shown minimal microbial inhibition [120, 121]. Alternatively, metabolic engineering techniques can be applied to allow for a more diverse range of hydrocarbon fuel targets. Many cellular modifications have been shown to improve microbial solvent tolerance: changes in membrane lipid composition; altered enzymatic activities of membrane repair and energy transduction enzymes; solvent expulsion via efflux pump activity; and cellular stress responses including heat shock, phage shock, and general stress responses [118, 125, 126]. These natural mechanisms offer a range of engineering targets: expression of a cis-trans isomerase to alter lipid composition; overexpression of enzymes involved in membrane repair and energy transduction; expression of efflux pumps such as *tolC*, *mar*, *rob*, *soxS*, and *acrAB*; and overexpression of stress-induced enzymes such as phage shock protein, heat shock proteins, catalases, and superoxide dismutases [125, 126]. While few metabolic engineering efforts have focused on enhancing product tolerance, a recent study explored improving hydrocarbon-based fuel tolerance in *E. coli* by testing a library of 43 efflux pumps [127]. This work identified efflux pumps that improved tolerance to five potential isoprenoid derived fuels. This preliminary success at engineering solvent tolerance should inspire additional efforts to improve the microbial production of both fatty acid and isoprenoid derived fuels.

In addition to product tolerance, other host traits are desirable for industrial biofuel production, particularly for autotrophic microorganisms. As discussed in the previous section, light availability is often a growth limiting factor in microalgal cultures. Microalgae construct light harvesting complexes (LHC) to capture the available light for use in photosynthesis, and natural species actually absorb more light than is needed for photosynthesis under light intensities > 400 μmol photons m^{-2} s^{-1} [128]. As the sun can generate light intensities as high as 2,000 μmol photons m^{-2} s^{-1} during peak hours, it is estimated that as much as 80% of light absorbed by microalgae is 'wasted' as re-emitted fluorescence and heat [129]. In addition to this loss of energy, the excess energy can also cause cellular damage, known as photoinhibition [128]. In nature, this over-absorption of light will give the microalga a competitive advantage, but from a biofuel production perspective, this excess light harvesting will lead to lower culture cell densities and therefore lower biofuel productivities. Thus, there have been many attempts to engineer microalgae to absorb only the amount of light needed for photosynthesis. These efforts target genes of the light harvesting antenna complexes. Most LHC mutants were generated using random mutagenesis techniques including chemical, UV, and transposon mutagenesis [128, 130-134]. Many of these studies focus on the model alga *C. reinhardtii*, but other microalgal species, such as the diatom *Cyclotella* sp. and the cyanobacterium *Synechocystis* sp., have been mutated to reduce the size of their photosynthetic antennae [130, 133]. Several recent works have applied RNAi technology in *C. reinhardtii* to reduce the expression of targeted LHC genes in a more controlled manner [129]. In general, the antenna mutants have shown improved photosynthetic quantum yields, reduced photoinhibition, enhanced productivity under high light conditions, and increased light penetration within the culture [128, 129, 131-134]. While these results are promising, several questions remain to be addressed: Are the photosynthetic antenna mutants genetically stable, or will they revert back to their more competitive and less efficient forms over time? And are these mutants less fit and therefore more susceptible to predators and competitors in open pond systems?

Open pond systems are subject to a variety of changing environmental conditions, and as such, the optimal autotrophic host will have the necessary cellular mechanisms to adapt to these changing conditions. Desirable host traits may include temperature tolerance, salt tolerance, and resistance to predators. Open ponds are exposed to both daily and seasonal temperature fluctuations which often exceed the normal temperature ranges for optimal cell growth and may even cause cell death. Engineering efforts have successfully altered the temperature tolerance of cyanobacteria though either gene knockout or heterologous overexpression of desaturases which influence the viscosity of both the cell and photosynthetic membranes [135]. Alternatively, microalgae with different temperature optima can be rotated seasonally in the open ponds, similar to seasonal crop rotations in agricultural practices. As mentioned previously, open pond systems are complicated by evaporative water loss, particularly for the sunny, arid regions that are ideal for microalgal biofuel production. Evaporation can lead to fluctuations in the salt concentration within the pond culture, and many have proposed to utilize marine or brackish water sources to reduce the cost associated with freshwater systems. Moreover, high salt and saturated salt systems will have lower evaporative water loss compared to freshwater cultures. Naturally salt-toler-

http://dx.doi.org/10.5772/52050

ant microalgae, such as those isolated from marine or even hypersaline environments, may be selected as host for biofuel production, or efficient fuel-producing hosts can be engineered for increased salt tolerance. For example, the cyanobacterium *Synechococcus elongatus* PCC 7942, modified with expression of a Δ12 acyl-lipid desaturase (*desA*), showed improved resistance to salt and osmotic stress compared to the wildtype [136]. Lastly, pond crash due to microalgal predators like rotifers and chytrids is a major problem for open pond biofuel production systems. While there have not been any reported attempts at engineering predator-resistant microalgae, there have been reports of natural defense mechanisms such as palmelloid formation by *C. reinhardtii*, which produces non-motile cell aggregates that are simply too large to be consumed by grazing rotifers [137]. Once the genetic mechanism responsible for palmelloid formation is deciphered, it may be possible to transfer this resistance mechanism to other microalgae using genetic engineering techniques. When devising a metabolic engineering strategy for biofuel production, it is essential to consider the entire genomic landscape and the natural diversity of genetically-driven traits to design the optimal host for the specific industrial constraints.

5. Conclusions and future outlook

The microbial production of drop-in replacement fuels faces unprecedented challenges. The sheer quantity of hydrocarbon product required to meet the world's ever increasing demand for energy dwarfs the supply of any current microbially synthesized product. Moreover, both second (lignocellulosic feedstock) and third (microalgal feedstock) generation biofuels ultimately rely on sunlight and photosynthesis to supply the energy and carbon feedstocks necessary for production. This requires the development of new technology and infrastructure to facilitate the construction of this new supply chain. Finally, the low value of the final fuel product places additional financial restrictions on the development of large-scale biofuel production processes. For example, previous reports include the addition of exogenous metabolic precursors like mevalonate for isoprenoid production or FFA for FAEE biosynthesis [18, 50]. While these exogenous metabolites boost production of the desired hydrocarbon-based product, this practice is too expensive for large-scale biofuel applications. These challenges currently limit the industrial production of second and third generation biofuels.

Fortunately, new biological and technological tools are rapidly being developed and applied to overcome the obstacles in biofuel production. In addition to the metabolic engineering strategies previously described in this chapter, new global strategies are being applied to engineer microbes for biofuel production. With the affordability of next-generation DNA sequencing technologies, new microbial genomes are being reported at an unprecedented rate, and this information can be used to generate metabolic models for biofuel-producing hosts. In turn, these models can be leveraged to analyze proposed metabolic engineering strategies *in silico*, reducing the number of costly and time-intensive strain constructions and experiments. This technique was shown to be successful at increasing lycopene production, an isoprenoid derivative, in *E. coli* [69, 138]. The advancement of synthetic DNA technology

enables new engineering approaches such as multiplex automated genome engineering (MAGE) [139]. In MAGE, synthetic oligomers, consisting of degenerate DNA sequences flanked by regions homologous to the target sequences, are simultaneously transformed into *E. coli*, and the modified strains are screened for improvements. MAGE was used to target ribosome binding sites, for optimization of protein translation, and to inactivate genes by inserting nonsense mutations; this technique can also be applied to target promoters for improved gene transcription and enzyme active sites for enhanced activities. The technique does have some limitations, however. MAGE will likely require modification of the host organism to allow for efficient integration of the single-stranded oligonucleotides, and a high-throughput screening method is essential for screening the billions of genetic variants that are generated with MAGE. Global or systems-level technologies can also be applied to advance our fundamental understanding of genetic and regulatory mechanisms within a microbial host; this is vital to host development of non-model organisms and newly isolated strains. Omics technologies including genomics, transcriptomics, metabolomics, and proteomics provide global insight at the cellular level, which can be compared across different conditions or time points to identify the native mechanisms that control the cell metabolism. Integration of omics data can identify bottlenecks at the transcriptional, translational, and protein levels, and as such, can be applied to inform the metabolic engineering strategy for biofuel production [34]. Systems-level tools for engineering microbial hosts, including metabolic modeling, MAGE, and omics technologies, will be integral to the successful development of hosts for biofuel production.

Commercial interest in the production of second and third generation biofuels has developed rapidly in the past decade. As evidence of this, there has been a flurry of activity in patent applications regarding microbial hydrocarbon production. Companies invested in heterotrophic hydrocarbon-based fuel production include LS9 [27, 59, 65, 66, 140, 141] and Amyris Biotechnologies [72, 142], which focus mainly on *E. coli* as the host, and Solazyme [143, 144], which initially focused on fuels derived from algae but has since moved toward more high-value markets, such as cosmetics and nutraceuticals. Most companies interested in algae and cyanobacteria are focused on autotrophically-produced hydrocarbon fuels. Notable companies in this industry include Sapphire Energy [145, 146], Joule Unlimited [26, 77, 147], and Synthetic Genomics [68, 75]. The hydrocarbon-based fuels targeted by these companies span the entire gamut of fatty acid and isoprenoid derived fuel products. Despite this commercial interest, hydrocarbon biofuel production still remains to be demonstrated at scale and in a sustainable manner.

This chapter has described the challenges in microbial hydrocarbon production and presented metabolic engineering strategies to resolve these issues. As is evident from this discussion, microbial-based fuel production is only in the initial stages of exploration, and additional research and innovation is necessary to enable large-scale biofuel production. New metabolic engineering tools and techniques are currently being developed for engineering untraditional hosts like eukaryotic algae and cyanobacteria, and as our understanding of these new hosts matures, significant improvement in hydrocarbon yields is anticipated.

http://dx.doi.org/10.5772/52050

Abbreviations

1,3-BPG	1,3-bisphosphoglycerate	GGPP	geranylgeranyl pyrophosphate
3-PGA	3-phosphoglycerate	Glc	glucose
AAR	acyl-ACP reductase	Gly	glycerol
AAS	acyl-ACP synthetase	GPD	glycerol-3-phosphate dehydrogease
ACC	acetyl-CoA carboxylase	GPP	geranyl pyrophosphate
ACP	acyl carrier protein	HCO_3^-	bicarbonate
ACS	acetyl-CoA synthetase	HMG-CoA	3-hydroxy-3-methyl- glutaryl-CoA
ADC	aldehyde decarbonylase	HMGCR	HMG-CoA reductase
ADH	alcohol dehydrogenase	IPP	isopentenyl Pyrophosphate
ADP	adenosine diphosphate	IPPI	isopentenyl diphosphate isomerase
AH	aldehyde	*ispS*	isoprene synthase
ALDH	acetaldehyde dehydrogenase	KASIII	β-ketoacyl-ACP synthase
ALR	aldehyde reductase	LHC	light harvesting complex
AMP	adenosine monophosphate	L-Ru5P	L-ribulose-5-phosphate
AOL	arabitol	L-Xu5P	L-xylulose-5-phosphate
ARA	arabinose	L-Xul	L-xylulose
ASP	aquatic species program	MEP	methylerythritol phosphate
AT	acyltransferase	MVA	mevalonate
ATP	adenosine triphosphate	NAD^+	nicotinamide adenine dinucleotide (oxidized)
cAMP	cyclic AMP	NADH	nicotinamide adenine dinucleotide (reduced)
CCR	carbon catabolite repression	$NADP^+$	nicotinamide adenine dinucleotide phosphate (oxidized)
CMP	cytosine monophosphate	NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
CO_2	carbon dioxide	PBR	photobioreactor
CoA	coenzyme A	PDC	pyruvate decarboxylase
CRP	cyclic AMP receptor protein	PEP	phosphoenolpyruvate
CTP	cytosine triphosphate	P_i	phosphate
desA	Δ12 acyl-lipid desaturase	PP_i	pyrophosphate
DGAT	diacylglycerol acyltransferase	PPP	pentose phosphate pathway
DHAP	dihydroxyacetone phosphate	PPS	phosphoenolpyruvate synthase
DMAPP	dimethylallyl diphosphate	PTS	phosphotransferase system

D-Ru5P	D-ribulose-5-phosphate	PYR	pyruvate
DXP	1-deoxy-D-xylulose-5- phosphate	R5P	ribose-5-phosphate
DXR	1-deoxy-D-xylulose-5- phosphate reductoisomerase	RBU	ribulose
DXS	1-deoxy-D-xylulose-5- phosphate synthase	RNAi	ribonucleic acid interference
D-Xu5P	D-xylulose-5-phosphate	RuBP	ribulose-1,5-bisphosphate
D-Xul	D-xylulose	S7P	sedoheptulose-7- phosphate
E4P	erythrose-4-phosphate	SBP	sedoheptulose-1,7- bisphosphate
EMP	Embden-Meyerhof-Parnas	TAG	triacylglycerol
F6P	fructose-6-phosphate	TCA	tricarboxylic acid
FAEE	fatty acid ethyl ester	TE	thioesterase
FAR	fatty acyl-CoA reductase	XDH	xylitol dehydrogenase
FBP	fructose-1,6-bisphosphate	XI	xylose isomerase
FFA	free fatty acid	XK	xylulose kinase
FPP	farnesyl pyrophosphate	Xol	xylitol
G3P	glycerol-3-phosphate	XR	xylose reductase
G6P	glucose-6-phosphate	Xu5P	xylulose-5-phosphate
GAP	glyceraldehyde-3- phosphate	Xyl	xylose

Acknowledgements

This work was supported by the Harry S. Truman Fellowship in National Security Science and Engineering and the Laboratory Directed Research and Development program at Sandia National Laboratories. Sandia National Laboratories is a multi-program laboratory managed and operated by Sandia Corporation, a wholly owned subsidiary of Lockheed Martin Corporation, for the U.S. Department of Energy's National Nuclear Security Administration under contract DE-AC04-94AL85000.

Author details

Anne M. Ruffing

Sandia National Laboratories, Department of Bioenergy and Defense Technologies, Albuquerque, NM, USA

References

[1] Williams JL. (2011) Oil Price History and Analysis. WTRG Economics. Available: http://www.wtrg.com/prices.htm. Accessed 2012 April 16.

[2] Sawin JL, Martinot E, Barnes D, McCrone A, Roussell J, Sims R, et al. (2011) Renewables 2011 Global Status Report. Renewable Energy Policy Network for the 21st Century.

[3] Rittmann BE. (2008) Opportunities for Renewable Bioenergy Using Microorganisms. Biotechnol. Bioeng. 100(2):203-212.

[4] Christi Y. (2008) Biodiesel from Microalgae Beats Bioethanol. Trends Biotechnol. 26(3): 126-131.

[5] Davis MS, Solbiati J, Cronan JE. (2000) Overproduction of Acetyl-CoA Carboxylase Activity Increases the Rate of Fatty Acid Biosynthesis in *Escherichia coli*. J. Biol. Chem. 275(37):28593-28598.

[6] Lee S, Jeon E, Yun H, Lee J. (2011) Improvement of Fatty Acid Biosynthesis by Engineered Recombinant *Escherichia coli*. Biotech. Biopro. Eng. 16(4):706-713.

[7] Ramos MJ, Fernández CM, Casas A, Rodríguez L, Pérez Á. (2009) Influence of Fatty Acid Composition of Raw Materials on Biodiesel Properties. Bioresour. Technol. 100(1): 261-268.

[8] Dehesh K, Jones A, Knutzon DS, Voelker TA. (1996) Production of High Levels of 8:0 and 10:0 Fatty Acids in Transgenic Canola by Overexpression of Ch FatB2, a Thioesterase cDNA from *Cuphea hookeriana*. Plant J. 9(2):167-172.

[9] Jiang P, Cronan JE. (1994) Inhibition of Fatty Acid Synthesis in *Escherichia coli* in the Absence of Phospholipid Synthesis and Release of Inhibition by Thioesterase Action. J. Bacteriol. 176(10):2814-2821.

[10] Voelker TA, Davies HM. (1994) Alteration of the Specificity and Regulation of Fatty Acid Synthesis of *Escherichia coli* by Expression of a Plant Medium-Chain Acyl-Acyl Carrier Protein Thioesterase. J. Bacteriol. 176(23):7320-7327.

[11] Lotero E, Liu Y, Lopez DE, Suwannakarn K, Bruce DA, Goodwin JG. (2005) Synthesis of Biodiesel via Acid Catalysis. Ind. Eng. Chem. Res. 44(14):5353-5363.

[12] Liu T, Khosla C. (2010) Genetic Engineering of *Escherichia coli* for Biofuel Production. Annu. Rev. Genet. 44(1):53-69.

[13] Dellomonaco C, Clomburg JM, Miller EN, Gonzalez R. (2011) Engineered Reversal of the β-Oxidation Cycle for the Synthesis of Fuels and Chemicals. Nature. 476(7360): 355-359.

[14] Jako C, Kumar A, Wei Y, Zou J, Barton DL, Giblin EM, et al. (2001) Seed-Specific Over-Expression of an *Arabidopsis* cDNA Encoding a Diacylglycerol Acyltransferase Enhances Seed Oil Content and Seed Weight. Plant Physiol. 126(2):861-874.

[15] Dunahay T, Jarvis E, Dais S, Roessler P. (1996) Manipulation of Microalgal Lipid Production Using Genetic Engineering. Appl. Biochem. Biotechnol. 57-58(1):223-231.

[16] Nguyen HTT, Dieterich A, Athenstaedt K, Truong NH, Stahl U, Nevoigt E. (2004) Engineering of *Saccharomyces cerevisiae* for the Production of L-Glycerol 3-Phosphate. Metab. Eng. 6(2):155-163.

[17] Kalscheuer R, Luftmann H, Steinbüchel A. (2004) Synthesis of Novel Lipids in *Saccharomyces cerevisiae* by Heterologous Expression of an Unspecific Bacterial Acyltransferase. Appl. Environ. Microbiol. 70(12):7119-7125.

[18] Kalscheuer R, Stölting T, Steinbüchel A. (2006) Microdiesel: *Escherichia coli* Engineered for Fuel Production. Microbiol. 152(9):2529-2536.

[19] Steen EJ, Kang Y, Bokinsky G, Hu Z, Schirmer A, McClure A, et al. (2010) Microbial Production of Fatty-Acid-Derived Fuels and Chemicals from Plant Biomass. Nature. 463(7280):559-562.

[20] Ohta K, Beall DS, Mejia JP, Shanmugam KT, Ingram LO. (1991) Genetic Improvement of *Escherichia coli* for Ethanol Production: Chromosomal Integration of *Zymomonas mobilis* Genes Encoding Pyruvate Decarboxylase and Alcohol Dehydrogenase II. Appl. Environ. Microbiol. 57(4):893-900.

[21] Hofvander P, Doan TTP, Hamberg M. (2011) A Prokaryotic Acyl-CoA Reductase Performing Reduction of Fatty Acyl-CoA to Fatty Alcohol. FEBS Lett. 585(22): 3538-3543.

[22] Doan TTP, Carlsson AS, Hamberg M, Bülow L, Stymne S, Olsson P. (2009) Functional Expression of Five *Arabidopsis* Fatty Acyl-CoA Reductase Genes in *Escherichia coli*. J. Plant Physiol. 166(8):787-796.

[23] Tan X, Yao L, Gao Q, Wang W, Qi F, Lu X. (2011) Photosynthesis Driven Conversion of Carbon Dioxide to Fatty Alcohols and Hydrocarbons in Cyanobacteria. Metab. Eng. 13(2):169-176.

[24] Willis RM, Wahlen BD, Seefeldt LC, Barney BM. (2011) Characterization of a Fatty Acyl-CoA Reductase from *Marinobacter aquaeolei* VT8: A Bacterial Enzyme Catalyzing the Reduction of Fatty Acyl-CoA to Fatty Alcohol. Biochem. 50(48):10550-10558.

[25] Schirmer A, Rude MA, Li X, Popova E, del Cardayre SB. (2010) Microbial Biosynthesis of Alkanes. Science. 329(5991):559-562.

[26] Reppas NB, Ridley CP, inventors; Joule Unlimited, Inc., assignee. (2010) Methods and Compositions for the Recombinant Biosynthesis of *n*-Alkanes. patent US 7794969.

[27] Schirmer A, Rude MA, Brubaker S, inventors; LS9, Inc., assignee. (2010) Methods and Compositions for Producing Hydrocarbons. patent US 2010/0249470.

[28] Lee SK, Chou H, Ham TS, Lee TS, Keasling JD. (2008) Metabolic Engineering of Microorganisms for Biofuels Production: From Bugs to Synthetic Biology to Fuels. Curr. Opin. Biotechnol. 19(6):556-563.

[29] Chandran SS, Kealey JT, Reeves CD. (2011) Microbial Production of Isoprenoids. Process Biochem. 46(9):1703-1710.

[30] Fortman JL, Chhabra S, Mukhopadhyay A, Chou H, Lee TS, Steen E, et al. (2008) Biofuel Alternatives to Ethanol: Pumping the Microbial Well. Trends Biotechnol. 26(7):375-381.

[31] Rude MA, Schirmer A. (2009) New Microbial Fuels: A Biotech Perspective. Curr. Opin. Microbiol. 12(3):274-281.

[32] Rilling H, K B. (1959) On the Mechanism of Sqalene Biogenesis from Mevalonic Acid. J. Biol. Chem. 234(6):1424-1432.

[33] Rohmer M, Knani M, Simonin P, Sutter B, Sahm H. (1993) Isoprenoid Biosynthesis in Bacteria: A Novel Pathway for the Early Steps Leading to Isopentenyl Diphosphate. Biochem. J. 295:517-524.

[34] Muntendam R, Melillo E, Ryden A, Kayser O. (2009) Perspectives and Limits of Engineering the Isoprenoid Metabolism in Heterologous Hosts. Appl. Microbiol. Biotechnol. 84(6):1003-1019.

[35] Yoon S-H, Lee S-H, Das A, Ryu H-K, Jang H-J, Kim J-Y, et al. (2009) Combinatorial Expression of Bacterial Whole Mevalonate Pathway for the Production of β-Carotene in *E. coli*. J. Biotechnol. 140(3–4):218-226.

[36] Pitera DJ, Paddon CJ, Newman JD, Keasling JD. (2007) Balancing a Heterologous Mevalonate Pathway for Improved Isoprenoid Production in *Escherichia coli*. Metab. Eng. 9(2):193-207.

[37] Asadollahi MA, Maury J, Schalk M, Clark A, Nielsen J. (2010) Enhancement of Farnesyl Diphosphate Pool as Direct Precursor of Sesquiterpenes Through Metabolic Engineering of the Mevalonate Pathway in *Saccharomyces cerevisiae*. Biotechnol. Bioeng. 106(1): 86-96.

[38] 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(5):837-845.

[39] Kim S-W, Keasling JD. (2001) Metabolic Engineering of the Nonmevalonate Isopentenyl Diphosphate Synthesis Pathway in *Escherichia coli* Enhances Lycopene Production. Biotechnol. Bioeng. 72(4):408-415.

[40] Matthews PD, Wurtzel ET. (2000) Metabolic Engineering of Carotenoid Accumulation in *Escherichia coli* by Modulation of the Isoprenoid Precursor Pool with Expression of Deoxyxylulose Phosphate Synthase. Appl. Microbiol. Biotechnol. 53(4):396-400.

[41] Zhao Y, Yang J, Qin B, Li Y, Sun Y, Su S, et al. (2011) Biosynthesis of Isoprene in *Escherichia coli* via Methylerythritol Phosphate (MEP) Pathway. Appl. Microbiol. Biotechnol. 90(6):1915-1922.

[42] Kajiwara S, Fraser P, Kondo K, Misawa N. (1997) Expression of an Exogenous Isopentenyl Diphosphate Isomerase Gene Enhances Isoprenoid Biosynthesis in *Escherichia coli*. Biochem. J. 324:421-426.

[43] Ghimire GP, Lee HC, Sohng JK. (2009) Improved Squalene Production via Modulation of the Methylerythritol 4-Phosphate Pathway and Heterologous Expression of Genes from *Streptomyces peucetius* ATCC 27952 in *Escherichia coli*. Appl. Environ. Microbiol. 75(22):7291-7293.

[44] Morrone D, Lowry L, Determan M, Hershey D, Xu M, Peters R. (2010) Increasing Diterpene Yield with a Modular Metabolic Engineering System in *E. coli* Comparison of MEV and MEP Isoprenoid Precursor Pathway Engineering. Appl. Microbiol. Biotechnol. 85(6):1893-1906.

[45] Leonard E, Ajikumar PK, Thayer K, Xiao W-H, Mo JD, Tidor B, et al. (2010) Combining Metabolic and Protein Engineering of a Terpenoid Biosynthetic Pathway for Overproduction and Selectivity Control. Proceedings of the National Academy of Sciences.

[46] Martin VJJ, Pitera DJ, Withers ST, Newman JD, Keasling JD. (2003) Engineering a Mevalonate Pathway in *Escherichia coli* for Production of Terpenoids. Nat Biotech. 21(7): 796-802.

[47] Wang C, Yoon S-H, Jang H-J, Chung Y-R, Kim J-Y, Choi E-S, et al. (2011) Metabolic Engineering of *Escherichia coli* for α-Farnesene Production. Metab. Eng. 13(6):648-655.

[48] Wang C, Yoon S-H, Shah AA, Chung Y-R, Kim J-Y, Choi E-S, et al. (2010) Farnesol Production from *Escherichia coli* by Harnessing the Exogenous Mevalonate Pathway. Biotechnol. Bioeng. 107(3):421-429.

[49] Yang J, Zhao G, Sun Y, Zheng Y, Jiang X, Liu W, et al. (2012) Bio-Isoprene Production Using Exogenous MVA Pathway and Isoprene Synthase in *Escherichia coli*. Bioresour. Technol. 104(0):642-647.

[50] Yoon S-H, Lee Y-M, Kim J-E, Lee S-H, Lee J-H, Kim J-Y, et al. (2006) Enhanced Lycopene Production in *Escherichia coli* Engineered to Synthesize Isopentenyl Diphosphate and Dimethylallyl Diphosphate from Mevalonate. Biotechnol. Bioeng. 94(6):1025-1032.

[51] Farmer WR, Liao JC. (2000) Improving Lycopene Production in *Escherichia coli* by Engineering Metabolic Control. Nat Biotech. 18(5):533-537.

[52] Shiba Y, Paradise EM, Kirby J, Ro D-K, Keasling JD. (2007) Engineering of the Pyruvate Dehydrogenase Bypass in *Saccharomyces cerevisiae* for High-Level Production of Isoprenoids. Metab. Eng. 9(2):160-168.

[53] Farmer WR, Liao JC. (2001) Precursor Balancing for Metabolic Engineering of Lycopene Production in *Escherichia coli*. Biotechnol. Prog. 17(1):57-61.

[54] Asadollahi MA, Maury J, Patil KR, Schalk M, Clark A, Nielsen J. (2009) Enhancing Sesquiterpene Production in *Saccharomyces cerevisiae* Through in Silico Driven Metabolic Engineering. Metab. Eng. 11(6):328-334.

[55] Xue J, Ahring BK. (2011) Enhancing Isoprene Production by Genetic Modification of the 1-Deoxy-d-Xylulose-5-Phosphate Pathway in *Bacillus subtilis*. Appl. Environ. Microbiol. 77(7):2399-2405.

[56] Li Y, Han D, Hu G, Dauvillee D, Sommerfeld M, Ball S, et al. (2010) *Chlamydomonas* Starchless Mutant Defective in ADP-Glucose Pyrophosphorylase Hyper-Accumulates Triacylglycerol. Metab. Eng. 12(4):387-391.

[57] Li Y, Han D, Hu G, Sommerfeld M, Hu Q. (2010) Inhibition of Starch Synthesis Results in Overproduction of Lipids in *Chlamydomonas reinhardtii*. Biotechnol. Bioeng. 107(2): 258-268.

[58] Work VH, Radakovits R, Jinkerson RE, Meuser JE, Elliott LG, Vinyard DJ, et al. (2010) Increased Lipid Accumulation in the *Chlamydomonas reinhardtii* sta7-10 Starchless Isoamylase Mutant and Increased Carbohydrate Synthesis in Complemented Strains. Eukaryot. Cell. 9(8):1251-1261.

[59] Hu Z, Valle F, inventors; LS9, Inc, assignee. (2011) Enhanced Production of Fatty Acid Derivatives. patent US 2011/0256599.

[60] Lu X, Vora H, Khosla C. (2008) Overproduction of Free Fatty Acids in *E. coli*: Implications for Biodiesel Production. Metab. Eng. 10(6):333-339.

[61] Michinaka Y, Shimauchi T, Aki T, Nakajima T, Kawamoto S, Shigeta S, et al. (2003) Extracellular Secretion of Free Fatty Acids by Disruption of a Fatty Acyl-CoA Synthetase Gene in *Saccharomyces cerevisiae*. J. Biosci. Bioeng. 95(5):435-440.

[62] Scharnewski M, Pongdontri P, Mora G, Hoppert M, Fulda M. (2008) Mutants of *Saccharomyces cerevisiae* Deficient in Acyl-CoA Synthetases Secrete Fatty Acids due to Interrupted Fatty Acid Recycling. FEBS J. 275(11):2765-2778.

[63] Kamisaka Y, Tomita N, Kimura K, Kainou K, Uemura H. (2007) *DGA1* (Diacylglycerol Acyltransferase Gene) Overexpression and Leucine Biosynthesis Significantly Increase Lipid Accumulation in the Δ*snf*2 disruptant of *Saccharomyces cerevisiae*. Biochem. J. 408:61-68.

[64] Nojima Y, Kibayashi A, Matsuzaki H, Hatano T, Fukui S. (1999) Isolation and Characterization of Triacylglycerol-Secreting Mutant Strain from Yeast, *Saccharomyces cerevisiae*. The Journal of General and Applied Microbiology. 45(1):1-6.

[65] del Cardayre SB, Milner Cockrem MC, inventors; LS9, Inc., assignee. (2009) Systems and Methods for the Production of Fatty Esters. patent WO/2009/009391.

[66] Keasling JD, Hu Z, Somerville C, Church G, Berry D, Friedman L, et al., inventors; LS9, Inc., assignee. (2007) Production of Fatty Acids and Derivatives Thereof. patent WO/2007/136762.

[67] Zhang F, Carothers JM, Keasling JD. (2012) Design of a Dynamic Sensor-Regulator System for Production of Chemicals and Fuels Derived from Fatty Acids. Nat Biotech. 30(4):354-359.

[68] Roessler PG, Watts K, Liu B, inventors; Synthetic Genomics, Inc., assignee. (2011) Microbial Production of Fatty Alcohols. patent US 2011/0195469.

[69] Alper H, Miyaoku K, Stephanopoulos G. (2005) Construction of Lycopene-Overproducing *E. coli* Strains by Combining Systematic and Combinatorial Gene Knockout Targets. Nat Biotech. 23(5):612-616.

[70] Muramatsu M, Ohto C, Obata S, Sakuradani E, Shimizu S. (2008) Various Oils and Detergents Enhance the Microbial Production of Farnesol and Related Prenyl Alcohols. J. Biosci. Bioeng. 106(3):263-267.

[71] Takahashi S, Yeo Y, Greenhagen BT, McMullin T, Song L, Maurina-Brunker J, et al. (2007) Metabolic Engineering of Sesquiterpene Metabolism in Yeast. Biotechnol. Bioeng. 97(1):170-181.

[72] Renninger NS, McPhee DJ, inventors; Amyris Biotechnologies, Inc., assignee. (2008) Fuel Compositions Comprising Farnesane and Farnesane Derivatives and Methods of Making and Using Same. patent WO/2008/045555.

[73] Kaczmarzyk D, Fulda M. (2010) Fatty Acid Activation in Cyanobacteria Mediated by Acyl-Acyl Carrier Protein Synthetase Enables Fatty Acid Recycling. Plant Physiol. 152(3):1598-1610.

[74] Liu X, Sheng J, Curtiss III R. (2011) Fatty Acid Production in Genetically Modified Cyanobacteria. Proceedings of the National Academy of Sciences. Epub April 11, 2011.

[75] Roessler PG, Chen Y, Liu B, Dodge CN, inventors; Synthetic Genomics, Inc., assignee. (2009) Secretion of Fatty Acids by Photosynthetic Microorganisms. patent US 2009/0298143.

[76] Ruffing AM, Jones HDT. (2012) Physiological Effects of Free Fatty Acid Production in Genetically Engineered *Synechococcus elongatus* PCC 7942. Biotechnol. Bioeng. 109(9): 2190-2199.

[77] Berry DA, Afeyan NB, Skraly FA, Ridley CP, Robertson DE, Wilpiszeski R, et al., inventors; Joule Unlimited, Inc., assignee. (2011) Methods and Compositions for the Recombinant Biosynthesis of Fatty Acids and Esters. patent US 2011/0111470.

[78] Lindberg P, Park S, Melis A. (2010) Engineering a Platform for Photosynthetic Isoprene Production in Cyanobacteria, Using *Synechocystis* as the Model Organism. Metab. Eng. 12(1):70-79.

[79] Hahn-Hägerdal B. (1996) Ethanolic Fermentation of Lignocellulose Hydrolysates. Appl. Biochem. Biotechnol. 57-58(1):195-199.

[80] Dellomonaco C, Fava F, Gonzalez R. (2010) The Path to Next Generation Biofuels: Successes and Challenges in the Era of Synthetic Biology. Microb. Cell Fact. 9(1):3.

[81] Jin Y, Lee T, Choi Y, Ryu Y, Seo J. (2000) Conversion of Xylose to Ethanol by Recombinant *Saccharomyces cerevisiae* Containing Genes for Xylose Reductase and Xylitol Dehydrogenase from *Pichia stipitis*. J. Microbiol. Biotech. 10(4):564-567.

[82] van Maris A, Winkler A, Kuyper M, de Laat W, van Dijken J, Pronk J. (2007) Development of Efficient Xylose Fermentation in *Saccharomyces cerevisiae:* Xylose Isomerase as a Key Component Biofuels. Adv Biochem Eng Biotechnol. 108:179-204.

[83] Walfridsson M, Anderlund M, Bao X, Hahn-Hägerdal B. (1997) Expression of Different Levels of Enzymes from the *Pichia stipitis* XYL1 and XYL2 Genes in *Saccharomyces cerevisiae* and its Effects on Product Formation During Xylose Utilisation. Appl. Microbiol. Biotechnol. 48(2):218-224.

[84] Kuyper M, Hartog MMP, Toirkens MJ, Almering MJH, Winkler AA, van Dijken JP, et al. (2005) Metabolic Engineering of a Xylose-Isomerase-Expressing *Saccharomyces cerevisiae* Strain for Rapid Anaerobic Xylose Fermentation. FEMS Yeast Res. 5(4-5): 399-409.

[85] Walfridsson M, Hallborn J, Penttilä M, Keränen S, Hahn-Hägerdal B. (1995) Xylose-Metabolizing *Saccharomyces cerevisiae* Strains Overexpressing the TKL1 and TAL1 Genes Encoding the Pentose Phosphate Pathway Enzymes Transketolase and Transaldolase. Appl. Environ. Microbiol. 61(12):4184-4190.

[86] Becker J, Boles E. (2003) A Modified *Saccharomyces cerevisiae* Strain That Consumes l-Arabinose and Produces Ethanol. Appl. Environ. Microbiol. 69(7):4144-4150.

[87] Richard P, Verho R, Putkonen M, Londesborough J, Penttilä M. (2003) Production of Ethanol from L-Arabinose by *Saccharomyces cerevisiae* Containing a Fungal L-Arabinose Pathway. FEMS Yeast Res. 3(2):185-189.

[88] Wisselink HW, Toirkens MJ, del Rosario Franco Berriel M, Winkler AA, van Dijken JP, Pronk JT, et al. (2007) Engineering of *Saccharomyces cerevisiae* for Efficient Anaerobic Alcoholic Fermentation of L-Arabinose. Appl. Environ. Microbiol. 73(15):4881-4891.

[89] Stülke J, Hillen W. (1999) Carbon Catabolite Repression in Bacteria. Curr. Opin. Microbiol. 2(2):195-201.

[90] Nichols NN, Dien BS, Bothast RJ. (2001) Use of Catabolite Repression Mutants for Fermentation of Sugar Mixtures to Ethanol. Appl. Microbiol. Biotechnol. 56(1):120-125.

[91] Hernández-Montalvo VH-M, Valle FV, Bolivar FB, Gosset GG. (2001) Characterization of Sugar Mixtures Utilization by an *Escherichia coli* Mutant Devoid of the Phosphotransferase System. Appl. Microbiol. Biotechnol. 57(1):186-191.

[92] Yomano L, York S, Shanmugam K, Ingram L. (2009) Deletion of Methylglyoxal Synthase Gene (*mgsA*) Increased Sugar Co-Metabolism in Ethanol-Producing *Escherichia coli*. Biotechnol. Lett. 31(9):1389-1398.

[93] da Silva GP, Mack M, Contiero J. (2009) Glycerol: A Promising and Abundant Carbon Source for Industrial Microbiology. Biotechnol. Adv. 27(1):30-39.

[94] Houghton J, Weatherwax S, Ferrell J. (2006) Breaking the Biological Barriers to Cellulosic Ethanol. Rockville, Maryland: Office of Energy Efficiency and Renewable Energy, Office of the Biomass Program, U.S. Department of Energy.

[95] NSF. (2008) Breaking the Chemical and Engineering Barriers to Lignocellulosic Biofuels: Next Generation Hydrocarbon Biorefineries. Washington, D.C.: University of Massachusetts Amerst. National Science Foundation. Chemical, Bioengineering, Environmental, and Transport Systems Division.

[96] Sheehan J, Dunahay T, Benemann J, Roessler P. (1998) A Look Back at the U.S. Department of Energy's Aquatic Species Program - Biodiesel from Algae. National Renewable Energy Laboratory.

[97] Deng M-D, Coleman JR. (1999) Ethanol Synthesis by Genetic Engineering in Cyanobacteria. Appl. Environ. Microbiol. 65(2):523-528.

[98] Falkowski PG, Katz ME, Knoll AH, Quigg A, Raven JA, Schofield O, et al. (2004) The Evolution of Modern Eukaryotic Phytoplankton. Science. 305(5682):354-360.

[99] Seckbach J, Oren A, Pattanaik B, Reisser W, Klaveness D, Lovhoiden F, et al. (2007) Algae and Cyanobacteria in Extreme Environments. Seckbach J, editor. The Netherlands: Springer.

[100] Singh J, Gu S. (2010) Commercialization Potential of Microalgae for Biofuels Production. Renew. Sustain. Ener. Rev. 14(9):2596-2610.

[101] León R, Fernández E. (2007) Nuclear Transformation of Eukaryotic Microalgae: Historical Overview, Achievements and Problems. In: León R, Galván A, Fernández E, editors.: Springer New York; p. 1-11.

[102] León-Bañares R, González-Ballester D, Galván A, Fernández E. (2004) Transgenic Microalgae as Green Cell-Factories. Trends Biotechnol. 22(1):45-52.

[103] Wang ZT, Ullrich N, Joo S, Waffenschmidt S, Goodenough U. (2009) Algal Lipid Bodies: Stress Induction, Purification, and Biochemical Characterization in Wild-Type and Starchless *Chlamydomonas reinhardtii*. Eukaryot. Cell. 8(12):1856-1868.

[104] Radakovits R, Eduafo PM, Posewitz MC. (2011) Genetic Engineering of Fatty Acid Chain Length in *Phaeodactylum tricornutum*. Metab. Eng. 13(1):89-95.

[105] Yu W-L, Ansari W, Schoepp N, Hannon M, Mayfield S, Burkart M. (2011) Modifications of the Metabolic Pathways of Lipid and Triacylglycerol Production in Microalgae. Microb. Cell Fact. 10(1):91.

[106] Stevens DR, Purton S. (1997) Genetic Engineering of Eukaryotic Algae: Progress and Prospects. J. Phycol. 33(5):713-722.

[107] Rosenberg JN, Oyler GA, Wilkinson L, Betenbaugh MJ. (2008) A Green Light for Engineered Algae: Redirecting Metabolism to Fuel a Biotechnology Revolution. Curr. Opin. Biotechnol. 19(5):430-436.

[108] Radakovits R, Jinkerson RE, Darzins A, Posewitz MC. (2010) Genetic Engineering of Algae for Enhanced Biofuel Production. Eukaryot. Cell. 9(4):486-501.

[109] Cerutti H, Ma X, Msanne J, Repas T. (2011) RNA-Mediated Silencing in Algae: Biological Roles and Tools for Analysis of Gene Function. Eukaryot. Cell. 10(9):1164-1172.

[110] Kilian O, Benemann CSE, Niyogi KK, Vick B. (2011) High-Efficiency Homologous Recombination in the Oil-Producing Alga *Nannochloropsis* sp. Proceedings of the National Academy of Sciences. 108(52):21265-21269.

[111] (2012) *Chlamydomonas* Engineering Kits. Life Technologies Corporation. Available: https://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Protein-Expression-and-Analysis/Protein-Expression/algae-engineering-kits/chlamydomonas-engineering-kits.html. Accessed 2012 April 16.

[112] Rasmussen B, Fletcher IR, Brocks JJ, Kilburn MR. (2008) Reassessing the First Appearance of Eukaryotes and Cyanobacteria. Nature. 455(7216):1101-1104.

[113] Koksharova OA, Wolk CP. (2002) Genetic Tools for Cyanobacteria. Appl. Microbiol. Biotechnol. 58(2):123-137.

[114] Ogbonna JC, Soejima T, Tanaka H. (1999) An Integrated Solar and Artificial Light System for Internal Illumination of Photobioreactors. J. Biotechnol. 70(1–3):289-297.

[115] Kunjapur AM, Eldridge RB. (2010) Photobioreactor Design for Commercial Biofuel Production from Microalgae. Ind. Eng. Chem. Res. 49(8):3516-3526.

[116] Bullis K. (2012) NASA Wants to Launch Floating Algae Farms. Technology Review.

[117] Chaumont D. (1993) Biotechnology of Algal Biomass Production: A Review of Systems for Outdoor Mass Culture. J. Appl. Phycol. 5(6):593-604.

[118] Sikkema J, de Bont JA, Poolman B. (1995) Mechanisms of Membrane Toxicity of Hydrocarbons. Microbiol. Rev. 59(2):201-222.

[119] Desbois A, Smith V. (2010) Antibacterial Free Fatty Acids: Activities, Mechanisms of Action and Biotechnological Potential. Appl. Microbiol. Biotechnol. 85(6):1629-1642.

[120] León R, Garbayo I, Hernández R, Vigara J, Vilchez C. (2001) Organic Solvent Toxicity in Photoautotrophic Unicellular Microorganisms. Enzyme Microb. Technol. 29(2–3): 173-180.

[121] Gill C, Ratledge C. (1972) Toxicity of *n*-Alkanes, *n*-Alk-1-enes, *n*-Alkan-1-ols and *n*-Alkyl-1-bromides Towards Yeasts. J. Gen. Microbiol. 72(1):165-172.

[122] Best CA, Laposata M. (2003) Fatty Acid Ethyl Esters: Toxic Non-Oxidative Metabolites of Ethanol and Markers of Ethanol Intake. Front. Biosci. 8:202-217.

[123] Andrews RE, Parks LW, Spence KD. (1980) Some Effects of Douglas Fir Terpenes on Certain Microorganisms. Appl. Environ. Microbiol. 40(2):301-304.

[124] Uribe S, Ramirez J, Peña A. (1985) Effects of β-Pinene on Yeast Membrane Functions. J. Bacteriol. 161(3):1195-1200.

[125] Dunlop MJ. (2011) Engineering Microbes for Tolerance to Next-Generation Biofuels. Biotech. Biofuels. 4(32).

[126] Sardessai Y, Bhosle S. (2002) Tolerance of Bacteria to Organic Solvents. Res. Microbiol. 153(5):263-268.

[127] Dunlop MJ, Dossani ZY, Szmidt HL, Chu HC, Lee TS, Keasling JD, et al. (2011) Engineering Microbial Biofuel Tolerance and Export Using Efflux Pumps. Mol Syst Biol. 7.

[128] Melis A. (2009) Solar Energy Conversion Efficiencies in Photosynthesis: Minimizing the Chlorophyll Antennae to Maximize Efficiency. Plant Sci. 177(4):272-280.

[129] Mussgnug JH, Thomas-Hall S, Rupprecht J, Foo A, Klassen V, McDowall A, et al. (2007) Engineering Photosynthetic Light Capture: Impacts on Improved Solar Energy to Biomass Conversion. Plant Biotechnol. J. 5(6):802-814.

[130] Huesemann M, Hausmann T, Bartha R, Aksoy M, Weissman J, Benemann J. (2009) Biomass Productivities in Wild Type and Pigment Mutant of *Cyclotella* sp. (Diatom). Appl. Biochem. Biotechnol. 157(3):507-526.

[131] Lee J, Mets L, Greenbaum E. (2002) Improvement of Photosynthetic CO_2 Fixation at High Light Intensity Through Reduction of Chlorophyll Antenna Size. Appl. Biochem. Biotechnol. 98-100(1):37-48.

[132] Nakajima Y, Tsuzuki M, Ueda R. (2001) Improved Productivity by Reduction of the Content of Light-Harvesting Pigment in *Chlamydomonas perigranulata*. J. Appl. Phycol. 13(2):95-101.

[133] Nakajima Y, Ueda R. (1997) Improvement of Photosynthesis in Dense Microalgal Suspension by Reduction of Light Harvesting Pigments. J. Appl. Phycol. 9(6):503-510.

[134] Polle JEW, Kanakagiri S-D, Melis A. (2003) *tla1*; a DNA Insertional Transformant of the Green Alga *Chlamydomonas reinhardtii* with a Truncated Light-Harvesting Chlorophyll Antenna Size. Planta. 217(1):49-59.

[135] Gombos Z, Murata N. (2004) Genetic Engineering of the Unsaturation of Membrane Glycerolipid: Effects on the Ability of the Photosynthetic Machinery to Tolerate Temperature Stress Lipids in Photosynthesis: Structure, Function and Genetics. In: Paul-André S, Norio M, editors.: Springer Netherlands; p. 249-262.

[136] Allakhverdiev SI, Kinoshita M, Inaba M, Suzuki I, Murata N. (2001) Unsaturated Fatty Acids in Membrane Lipids Protect the Photosynthetic Machinery against Salt-Induced Damage in *Synechococcus*. Plant Physiol. 125(4):1842-1853.

[137] Lurling M, Beekman W. (2006) Palmelloids Formation in *Chlamydomonas reinhardtii*: Defence Against Rotifer Predators? Ann. Limnol. - Int. J. Limnol. 42:65-72.

[138] Jin Y-S, Stephanopoulos G. (2007) Multi-Dimensional Gene Target Search for Improving Lycopene Biosynthesis in *Escherichia coli*. Metab. Eng. 9(4):337-347.

[139] Wang HH, Isaacs FJ, Carr PA, Sun ZZ, Xu G, Forest CR, et al. (2009) Programming Cells by Multiplex Genome Engineering and Accelerated Evolution. Nature. 460(7257): 894-898.

[140] Friedman L, Rude MA, inventors; LS9, Inc., assignee. (2008) Process for Producing Low Molecular Weight Hydrocarbons from Renewable Resources. patent WO/2008/113041.

[141] Friedman L, da Costa B, inventors; LS9, Inc., assignee. (2008) Hydrocarbon-Producing Genes and Methods of Their Use. patent WO/2008/147781.

[142] Renninger NS, Ryder JA, Fisher KJ, inventors; Amyris Biotechnologies, Inc., assignee. (2008) Jet Fuel Compositions and Methods of Making and Using Same. patent WO/ 2008/140492.

[143] Trimbur DE, Im C-S, Dillon HF, Day AG, Franklin S, Coragliotti A, inventors; Solazyme, Inc., assignee. (2009) Lipid Pathway Modification in Oil-Bearing Microorganisms. patent US 2009/0061493.

[144] Trimbur D, Im C-S, Dillon HF, Day AG, Franklin S, Coragliotti A, inventors; Solazyme, Inc., assignee. (2009) Use of Cellulosic Materials for Cultivation of Microorganisms. patent US 2009/0011480.

[145] Mendez M, Fang S-C, Richard S, inventors; Sapphire Energy, Inc., assignee. (2010) Engineering Salt Tolerance in Photosynthetic Microorganisms. patent WO 2010/105095.

[146] Heaps NA, Behnke CA, Molina D, inventors; Sapphire Energy, Inc., assignee. (2010) Biofuel Production in Prokaryotes and Eukaryotes. patent WO/2010/104763.

[147] Berry DA, Robertson DE, Skraly FA, Green BD, Ridley CP, Kosuri S, et al., inventors; Joule Unlimited, Inc., assignee. (2011) Engineered CO_2 Fixing Microorganisms Producing Carbon-Based Products of Interest. patent US 2011/0008861.