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Biofuels for a sustainable future

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Summary

Rapid increase of energy consumption and human dependency on fossil fuels have led to the accumulation of greenhouse gases and consequently, climate change; as such, major efforts have been taken to develop, test, and adopt clean renewable fuel alternatives. Production of bioethanol and biodiesel from crops is well developed, while other feedstock resources and processes have also shown high potential to provide efficient and cost-effective alternatives, such as landfill and plastic waste conversion, algal photosynthesis, as well as electrochemical carbon fixation. In addition, the downstream microbial fermentation can be further engineered to not only increase the product yield but also expand the chemical space of biofuels through rational design and fine-tuning of biosynthetic pathways towards the realization of 'designer fuels' and diverse future applications.

Key words: Biofuels, alternative energy, climate change, metabolic engineering, synthetic biology

Introduction

There is a clear need to transition energy dependence from fossil fuels to renewable energy sources to address the unprecedented pace of climate change due to the accumulation of greenhouse gases (GHG) in the atmosphere. Overwhelming evidence has shown that human activity is the major driver of climate change and that its consequences are impacting food production, migration patterns, economic, and political stability on a global scale. In the US alone, 6.677 gigatons of GHG were emitted in 2018 with the largest fractions being attributed to transportation (28%), electricity generation (27%), industry (22%), commercial and residential applications (12%), as well as agriculture (10%) (US Environmental Protection Agency, 2020). As all these activities are largely dependent on fossil fuels, technological advances and diversification of alternative energy sources hold promise to significantly reduce carbon emissions and alleviate climate change.

Predating the use of petroleum itself, biofuels such as vegetable oils, animal fats, and ethanol were used for heat and illumination (Figure 1). This is exemplified by the first mass-produced car, the Ford model T, which ran on corn-derived ethanol. As automobile production became increasingly industrialized in the early 20th century, it became evident that the ethanol production scheme could no longer meet the ever-growing fuel demand for internal combustion engines.

Today, with environmental policies pushing for a reduction of GHG emission, aided by recent advances in crop engineering and fermentation processes, bioethanol and biodiesel production have once again become viable and sustainable surrogates for petroleum-based fuels. Bioethanol is derived from corn and sugar cane in the United States and Brazil, respectively, which together account for 84% of the total global production. In the United States bioethanol production has reached a volume of 15.7 billion gallons in 2019 (US Department of Energy, 2020), thus meeting the mandatory 10% supplementation requirement for gasoline (109th Congress, 2005). In Europe, the lack of cultivable land and the ban on genetically modified crops has largely limited bioethanol production. As such, 75% of the biofuel market in the European Union is comprised of biodiesels derived from rapeseed, palm oil, soybean, and used cooking oil. As of 2015, biofuels have reduced carbon emissions by 589.3 million tons (Figure 1) and will continue to play an important role in renewable energies (Biotechnology innovation organization, 2014).

Recent advances in battery technology have substantially increased the power density of electrical energy storage devices, thus accelerating the development of electric vehicles. However, to date, electricity in the US is still predominantly derived from fossil fuels such as gas and coal (38% and 23%, respectively) (US Department of Energy, 2020). Furthermore, limitations in driving range, capital cost, the lack of infrastructure, and power-to-weight ratios preclude the implementation of electric long-haul vehicles and aviation. To reach a carbon-neutral to -negative transportation scheme a more diversified approach therefore requires the use of both electric vehicles and biofuels alike. Specifically, electric vehicles hold promise in short-range and light-weight configurations, whereas the use of biofuels offers significant advantages for conventional long-distance ground transportation and aircraft.

To mitigate GHG emissions while meeting the global fuel demand, biofuel technology advancements need to focus on: i) optimization of current biofuel production technology for higher productivity and efficiency of lignocellulosic biomass conversion, ii) diversification of feedstocks to ensure the viability of biofuel production within existing ecological and economic constraints (e.g. carbon fixation through photosynthetic and electrochemical means and conversion of biowaste into value-added products), and iii) expansion of the chemical space towards designer molecules that improve fuel economy and performance while reducing carbon emissions. Major efforts need to be devoted to overcoming not only technological barriers but also to integrate social, economic, and environmental factors to provide long-term, cost-effective, and reliable production systems for the biofuel industry.

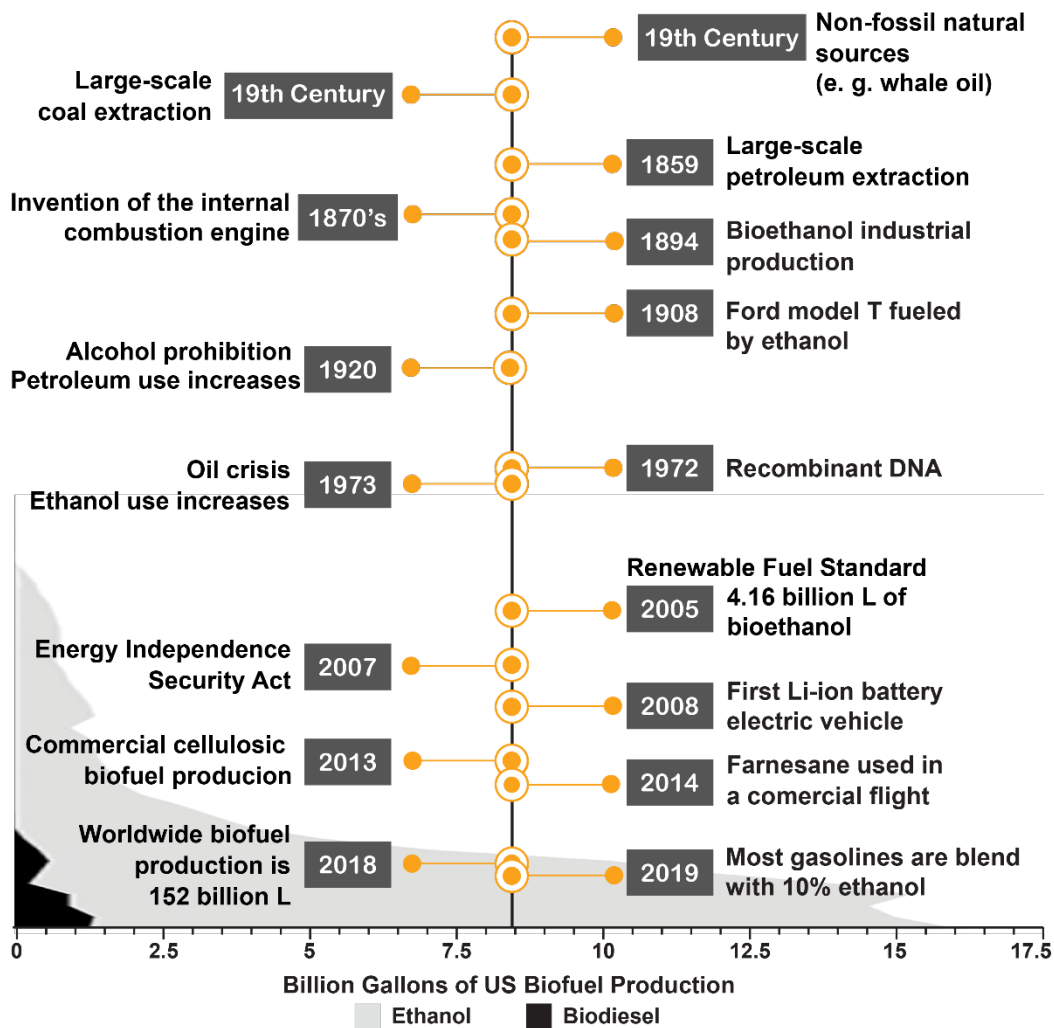


Figure 1. A timeline of biofuel production. Non-fossil fuels have been used long before the beginning of the oil era in the late 19th century. Scientific advancements, legislation and the pressing need to lower CO₂ emissions has led to an increase in the production of biofuels, reaching 15.8 billion gallons and 1.7 billion gallons per year of ethanol and biodiesel, respectively.

Advancing lignocellulosic feedstocks for biofuel production

Biofuel technology has evolved through several generations of significant advancements. The predominant problem with first-generation biofuels is that they are derived from food crops (e.g. corn and sugar cane) which require fertilization, water, and soil, and thus directly compete with food production. Tight regulations on the use of pesticides and genetically modified crops further limit their utilization in sustainable transportation. In order to mitigate these shortcomings, second-generation biofuels are derived from non-edible lignocellulose remnants of plants, which consist of up to 70% polymerized sugars and constitute the most abundant form of biomass on Earth (Isikgor and Becer, 2015). These biofuels are attractive because their net carbon footprint (emitted carbon – consumed carbon) can be neutral or even negative (Field et al. 2020; Tilman et al. 2006) and their generation from agricultural and forest residues or white wood chips lowers the price compared to crops. However, using lignocellulose for biofuel production requires energetically and financially costly extraction of fermentable sugars such as thermal, chemical and/or biochemical pre-treatment. As a result, despite the fact that the Energy Independence and Security Act (EISA) of 2007 set an annual blending target of 16 billion gallons of cellulosic biofuels by 2022 for the US (110th Congress, 2007), by 2017 production had amounted to less than 2% of this benchmark (US Department of Energy, 2020). Significant technological progress has since been made in the production of lignocellulosic biofuel towards a clean and economically viable process, including advances in energy crop engineering strategies, efficient degradation of lignocellulose, as well as simultaneous manufacturing of higher-value products.

The climate benefits of large-scale lignocellulosic biofuel production have been questioned due to its potential competition with land use for reforestation; it is believed that energy crop cultivation may result in less carbon capture efficiency than reforestation, leading to a carbon debt that must be compensated by the carbon negativity of the resulting biofuels. However, recent analysis of switchgrass production on transitioning crop/pasture land showed that in fact its GHG mitigation potential is comparable with reforestation of this land, and has several times more mitigation potential than grassland restoration (Field et al., 2020). Additionally, the ability of energy crops like sorghum to grow on marginal lands provides an avenue for production that limits the competition for farmable land needed to support the growing population (Jiang et al., 2019; Diallo et al., 2019).

In order to maximize the land use for lignocellulosic biofuel production, crops have been engineered to be more productive by optimizing photosynthetic capacity and carbon fixation for maximum biomass accumulation (Figure 2). Biological processes like non-photochemical quenching (NPQ) and photorespiration are suboptimal for the bioconversion of photon energy into fixed carbon. The NPQ process dissipates excess photon energy as heat (unproductive), and the transition from an NPQ state to a carbon fixation state (productive) is generally slow, leading to mass energy loss in field conditions. It has been shown that overexpressing the genes responsible for NPQ relaxation in the model crop *Nicotiana tabacum* accelerated the switching process, resulting in ~15% increases in plant height, leaf area, and total biomass accumulation (Kromdijk et al., 2016). Additionally, plants have evolved to maximize light capture with much of the energy wasted. While counterintuitive, diminishing plant's light harvesting capacity in dense field conditions has a drastic and beneficial effect on biomass accumulation. In fact, truncation of light-

harvesting complex antenna components decreased the capacity for light capture in engineered lines resulting in a 20% increase in total biomass accumulation under these conditions (Kirst et al., 2017). Photorespiration is another process that limits productivity due to Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase's (RuBisCO's) capacity to react with molecular oxygen in place of CO₂ thus leading to a net loss of carbon sequestration efficiency. Therefore, introducing an engineered photorespiratory bypass pathway into biofuel crops has the potential to increase the energy conversion efficiency of the plant (Shih et al., 2014; South et al., 2019). Eventually, many of these traits can be stacked into individual biofuel crops to increase plant productivity and maximize biomass production as technologies for advanced plant engineering and gene regulation are developed (Belcher et al., 2020).

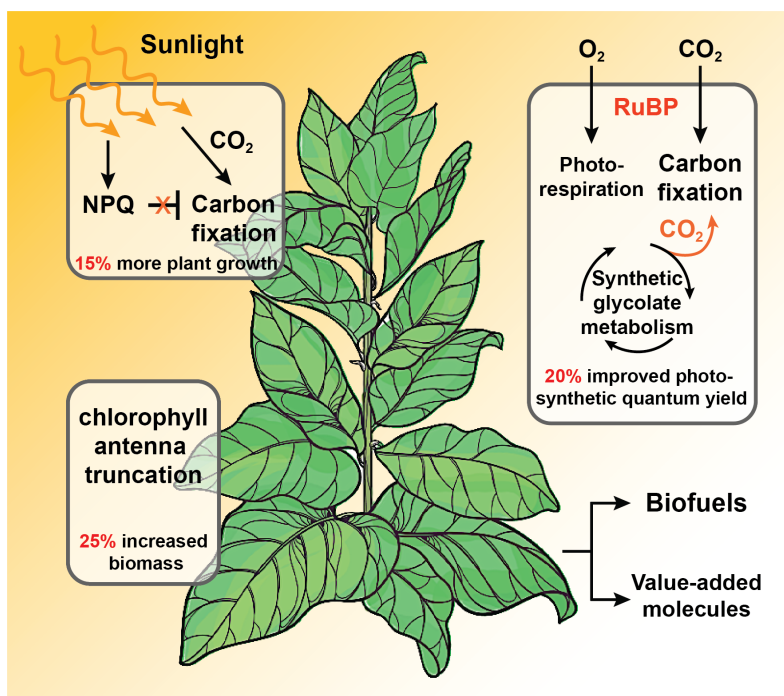


Figure 2. Engineering strategies *in planta* to improve CO₂ fixation. Acceleration of NPQ relaxation, truncation of the chlorophyll antenna, and introduction of a photorespiratory bypass are promising strategies to increase CO₂ fixation and biomass yield in plants. Metabolic pathways to synthesize products with higher values can also be incorporated in the meantime to increase the economic feasibility of lignocellulosic biofuels. As fossil fuels are currently produced at a far lower price than biofuels, simultaneous manufacturing of higher-value products with biofuels would increase their economic viability. 'Molecular farming', which couples agriculture with the production of high-value small molecules and proteins including therapeutics and antibodies, is a promising strategy for increasing the value of lignocellulosic biomass (Buyel, 2018; Yang et al., 2020). Accumulating these molecules after integrating their biosynthetic pathways in a biofuel crop background and introducing efficient extraction schemes into the processing pipeline can drastically decrease production costs, thus increasing profits. This has additional implications for medicine, as cancer biologics and viral antibodies can be produced *in planta* at high levels in the field without the need for sterile manufacturing systems (Dent and Matoba, 2020; Donini and Marusic, 2019; Mortimer, 2019;

Capell et al., 2020). This is one of the most promising strategies to achieve economic viability for biofuel production, although transgene biocontainment strategies will need to be implemented to prevent unwanted transgene flow from engineered crops (Clark and Maselko, 2020). Because of the mismatch in the volumes of fuel needed versus the volume of each individual therapeutic needed, it will be necessary to have a large number of crops, each producing the same biofuel precursor and different high-value products, which will be agronomically challenging.

Biofuel production from lignocellulosic biomass relies on the microbial bioconversion of cell-wall sugars and components into fuels and products (Figure 3A) (Baral et al., 2019; Perez-Pimienta et al., 2019). A major hurdle to efficient bioconversion is the recalcitrance of the feedstock material and the inhibitory effect that lignin has on this process (dos Santos et al., 2019). Cell-wall engineering has shown promise for decreasing overall recalcitrance by increasing the ratio of C6/C5 sugars, reducing lignin content, and reducing the acetylation of cell-wall polymers that limit the conversion efficiency of the feedstock material (Aznar et al., 2018; Eudes et al., 2015; Yan et al., 2018). While lignin is a major contributor of feedstock recalcitrance, it is also a promising substrate for specialized microbes that convert these aromatic polymers into usable products (Fang et al., 2020; Incha et al., 2020). The introduction of specialized microbial hosts into various processing systems has the potential to optimize the conversion of all lignocellulosic feedstock components into products with economic value, limiting the waste streams for biofuel production and increasing the viability for their use on a global scale.

The synergistic application of these various strategies has the potential to make lignocellulosic biofuels economically viable while shifting the current paradigm of what an effective biofuel/bioproduct production system achieves. Through a multidisciplinary approach across all sectors, we have the potential to revolutionize the manufacturing of biofuels/bioproducts from lignocellulosic biomass ushering in a new era of green technologies.

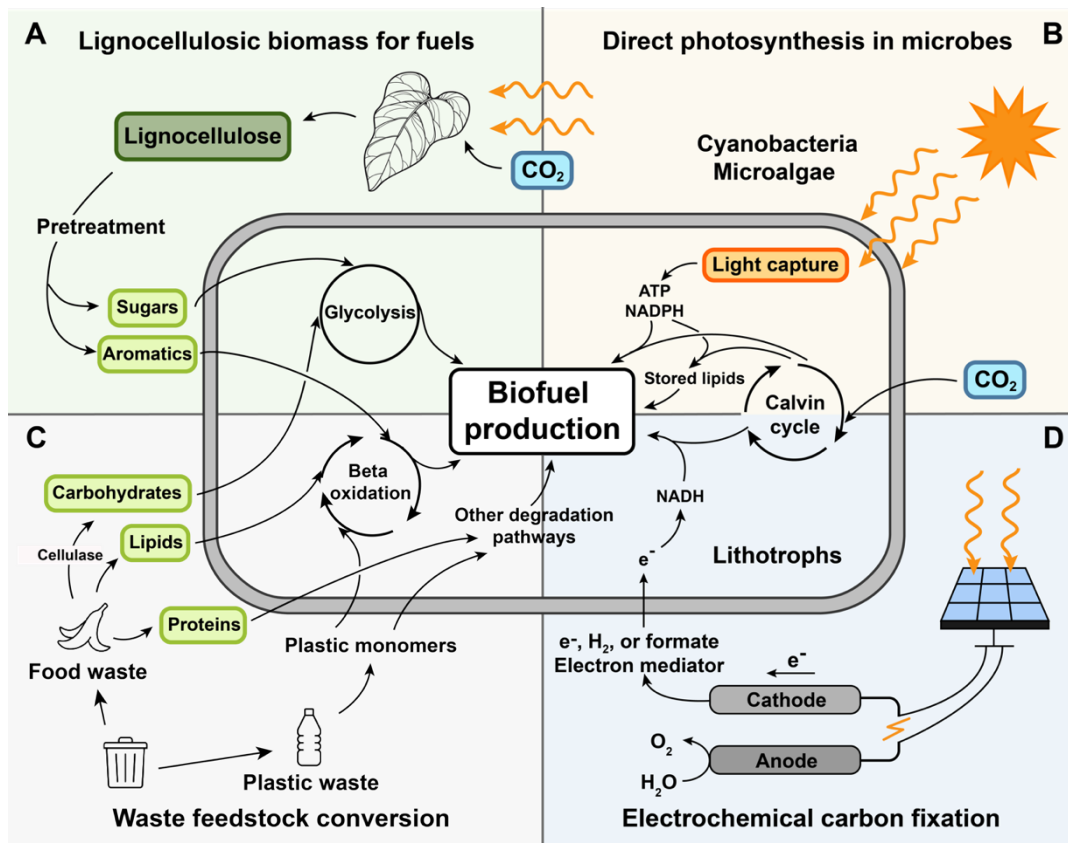


Figure 3. Diverse feedstock conversion to biofuels using engineered microbial metabolic pathways. (A) and (C) Upon pretreatment, sugars and aromatic molecules are extracted from lignocellulosic biomass, which together with the carbohydrates, lipids, proteins, and plastic monomers from waste can be used as carbon source for microbial production through glycolysis and beta oxidation pathways to make biofuels and biogas. (B) Instead of feeding biomass to microbes, photosynthesis and conversion of biofuels can be achieved in a single cell in the case of cyanobacteria and microalgae. (D) Lithotrophs are coupled to the cathode of an electrochemical cell, with delivery of electrons from the electrode driving CO₂ reduction and conversion.

Diversifying feedstocks for biofuel production

Photosynthetic carbon fixation

While the first and second generations of biofuels use light and CO₂ to produce biomass in crops that is later fed to microbes, third generation, or algal biofuels, combine energy capture and fuel production within a single cell by using photosynthetic cyanobacteria and algae (Figure 3B). Having the entire fuel production process taking place in one organism makes the process more direct and efficient with no energy invested in non-fermentable parts such as plant stems, roots and leaves. The solar energy conversion in cyanobacteria and algae is higher than in plants, reaching an efficiency of 3% in microalgae as opposed to less than 1% in most crops (Wijffels and Barbosa, 2010). Furthermore, many species can grow in wastewater or marine environments with simple nutritional requirements and therefore do not compete for land use with agriculture. It is estimated that microalgae can produce oil at a yield of 100,000 L/hectare/year, while palm and

sunflower oil can only reach 1000 to 6000 L/hectare/year. Algal fermentation could also lead to 9000 L/hectare/year of bioethanol production, compared to 600 L/hectare/year derived from corn (Alalwan et al., 2019).

Despite these favorable comparisons, attempts at large scale cultivations have struggled with high production costs. Unlike agriculture which has been optimized over millennia by humans, the technology for mass scale cultivation of photosynthetic microorganisms is still in its early developmental stage. The cultivation can be done in either an open system like a raceway pond, or in a closed system like a photobioreactor. Open ponds have lower operating costs but run the risk of contamination, as well as strict regulation against genetically modified organisms in an uncontained system (Abdullah et al., 2019). Closed systems, on the other hand, can have more tightly controlled cultivation conditions and have a low risk of contamination but the operating costs are high: \$2743 per ton of biomass vs. \$1227 per ton of biomass in open ponds (algae farm cost model) (Zhu et al., 2018). These costs can be lowered if the following limitations are addressed: i) light dissipation in the NPQ process, ii) narrow usable light spectrum, and iii) poor carbon fixation efficiency of microbes, in conjunction with efficient host engineering strategies.

Light dissipation in NPQ in algae is akin to that of energy crops, which can also be addressed by a similar engineering strategy: truncation of the chlorophyll A in green algae (Melis et al., 1998), as demonstrated in *N. tabacum* (Kromdijk et al., 2016) and modification of the light-harvesting complex antenna size of the cyanobacterial photosystems has been shown to improve the solar-to-product conversion efficiency up to 3-fold (Melis, 2009). The second issue is due to the fact that cyanobacteria and microalgae only capture the visible light range (400 – 700 nm), which makes ~50% of incident solar energy inaccessible (Blankenship et al., 2011). However, some terrestrial cyanobacteria when grown under far-red light environments express chlorophyll F (Chen et al., 2010). Heterologous expression of this pigment in *Synechococcus sp.* PCC7002 successfully extended light absorption up to 750 nm, thereby broadening the usable solar spectrum for fuel production (Tros et al., 2020).

The issue with poor carbon fixation is more complex in nature since RuBisCO, the main CO₂ fixing enzyme, suffers from poor catalytic activity and its promiscuity towards O₂. In fact, attempts to improve the activity of RuBisCO have had limited success due to a trade-off between CO₂ affinity and carboxylation rate (Savir et al., 2010). As an alternative, efforts to engineer the Calvin cycle for increased regeneration of RuBisCO's substrate, ribulose-1,5-biphosphate (RuBP), resulted in a 69% increase in ethanol yield (Liang et al., 2018). Alternatively, CO₂ fixation can be improved by engineering a carbon sink that pulls fixed carbon away from the Calvin cycle. By introducing the 2,3-butanediol pathway into *Synechococcus elongatus* PCC 7942, as well as enzymes generating pyruvate from 3-phosphoglycerate, the total carbon yield was increased by 1.8-fold (Oliver and Atsumi, 2015). Unnatural carbon fixation pathways have also been introduced to circumvent the poor properties of RuBisCO and its complicated regulation. Some archaea use a reductive acetyl-CoA pathway to fix carbon with formate dehydrogenase and CO dehydrogenase/acetyl-CoA synthase. This pathway is ATP efficient, requiring 2 ATPs to synthesize acetyl-CoA, compared to 7 in the Calvin cycle (Claassens et al., 2016). However, due to the pathway normally operating under anaerobic conditions, it might have limited transferability

to organisms with oxygenic photosynthesis. Nevertheless, an *in vitro* study has demonstrated that enoyl-CoA carboxylases/reductases with better properties than RuBisCO, along with 16 other enzymes from all three domains of life can be incorporated in a circular pathway where cofactors can be regenerated (Schwander et al., 2016).

One key parameter for viability of algal biofuels is the productivity of the host strain. Certain microalgae can store up to 80% of their dry weight as lipids, making them an attractive target for biodiesel production while other strains accumulate carbohydrates that can be fermented to make ethanol (Alalwan et al., 2019). Further engineering strategies have demonstrated the feasibility to upregulate lipid production (*e.g.*, triacylglycerol, de Bhowmick et al., 2018) and convert residual proteins and carbohydrates towards further fermentation (Rashid et al., 2013). The lipid length and level of saturation can also be altered through metabolic engineering, which can yield biodiesel for direct use in vehicles without extensive engine modification (Kings et al., 2016).

To produce a specific biofuel directly by photosynthetic microorganisms, metabolic engineering is often required, and cyanobacteria are typically used due to their genetic tractability. A range of different fuel molecules have been produced, including alcohols, free fatty acids, molecular hydrogen, and alkanes (Knoot et al., 2018). Standout examples include 5.5 g/L (212 mg/L/day) of ethanol and 4.8 g/L (302 mg/L/day) of butanol, both in the cyanobacterial model strain *Synechocystis sp.* PCC6803 (Gao et al., 2012; Liu et al., 2019). In these cases, the most efficient strategy to reach high titers was to use the strongest promoters available to drive overexpression of pathway enzymes. However, a better understanding of the native metabolism and the development of more sophisticated genetic tools will likely lead to more efficient approaches. Indeed, transcriptomics studies have revealed the importance of small RNAs for cellular control (Kopf et al., 2014), and genome scale models have allowed coupling production with growth (Shabestary and Hudson, 2016). Still, the field has a long road ahead before cyanobacteria can reach the productivity of model heterotrophic hosts.

Electrochemical carbon fixation

Man-made devices, such as photovoltaics, are more efficient than photosynthesis at capturing sunlight, partly due to their ability to capture most of the solar spectrum (Blankenship et al., 2011). The problem with photovoltaics is energy storage, which in photosynthesis can easily be achieved through the formation of chemical bonds. Microbial bioelectrocatalysis is a hybrid between the two approaches (Figure 3D). This is a novel research area that uses electricity as a source of electrons, and CO₂ fixation plus energy storage from autotrophic microbes to make fuels and other products. The energy transfer can either be done through a direct connection with the electrode, or by using an electron mediator. It has been known for decades that oxidoreductase enzymes can use electrodes as either a source or a sink of electrons (Cooney et al., 2008). While the membrane of living cells has low conductivity, a number of microorganisms have the ability to shuttle electrons across the membrane for anaerobic respiration using conductive *pili* or outer membrane cytochromes (Logan et al., 2019). In one study, multiple acetogenic bacteria, including species from the genera *Geobacter* and *Clostridium*, were shown to reduce CO₂ to acetate as well as a small amount of 2-oxobutyrate with a faradaic efficiency of more than 85% (Nevin et al., 2011). This electrosynthetic system mimics the natural process of photosynthesis with oxygen

evolution at the anode and CO₂ reduction to value-added molecules at the cathode. It should be noted that in contrast to photosynthesis, carbon and electron flux is directed primarily to the synthesis of reduced carbon products instead of biomass (Nevin et al., 2010). A drawback of this method is the limited available surface area on the cathode where a biofilm can be formed, thereby making scale up difficult.

An alternative approach is to use an electron mediator that shuttles electrons from the electrode to the cells. Immobilized *Desulfovibrio vulgaris* have been used to produce H₂ with methyl viologen as the redox mediator between the cathode and hydrogenases (Tatsumi et al., 1999). In another study, *Ralstonia eutropha* (also known as *Cupriavidus necator*) was engineered to fix CO₂ using electrochemically-generated formate to synthesize isobutanol and 3-methyl-1-butanol (Lu et al., 2012). H₂ generated by electrochemical water splitting was used as an electron mediator to fuel production of methane in *Methanosarcina barkeri* (Nichols et al., 2015). Although methane can be generated directly by nonorganic electrocatalysis, higher efficiency and lower overpotential was achieved with this hybrid bioinorganic approach, demonstrating a fully solar-driven methane production system. In another study, a cobalt-phosphorus water-splitting catalyst, more compatible with bacterial growth was used to evolve *Ralstonia eutropha* to grow on H₂ (Liu et al., 2016). Their setup captured 180 g of CO₂ with 1 kWh of electricity. An issue with using H₂ as electron mediator is its low solubility, which hinders the maximum throughput of reducing equivalents being transferred to the cells. To overcome that problem, a biocompatible nanoemulsion has been used to increase the kinetics of H₂ transfer and subsequent oxidation by more than three-fold (Rodrigues et al., 2019).

Conversion of food waste into biogas

Turning waste into fuel is a solution to two pressing problems faced by biofuels: i) the cost of food waste is low, especially if collection schemes are routinely practiced, and it is a more sustainable alternative than burying it in landfills; ii) food waste has high content for carbohydrates, proteins, and lipids that can be readily consumed by microbes, forgoing the need of extensive pretreatment. According to the Food and Agriculture Organization of the United Nations, a third of all food produced for human consumption is lost or wasted worldwide *i.e.*, 1.3 billion wet tons per year which is equivalent to \$161 billion USD (FAO, 2011). Conversion of food waste is not only favorable for economic feasibility of biofuels, but also has a positive impact on land diversity as well as in the environment, as nearly 400 kg of CO₂ emission per ton of organic waste in landfilling can be avoided (Smith et al., 2001).

Bacterial anaerobic digestion (AD) (Figure 3C) is a common practice to convert food waste to biogas that is 50-75% methane and 25-50% CO₂ (Atelge et al., 2018). This process can be further upgraded to produce renewable natural gas (RNG) matching the quality of fossil natural gas while effectively lowering carbon emissions ranging from 17-70 kg CO₂/ton of waste (Slorach et al., 2019). AD is achieved through bacterial hydrolysis of materials with high molecular weight or high granular content into smaller soluble fragments (e.g., fatty acids, glucose, and amino acids), which are degraded into volatile fatty acids to be further digested into biogas acetate, CO₂, and H₂. This pool of molecules can then be utilized by methanogens to produce methane. Besides methane, biohydrogen is a non-carbon and pollution-free fuel with the highest energy yield of known fuels

-122 kJ/g (compared to 50.1 kJ/g of methane) (Han et al., 2016). By adding a dark fermentation stage to the existing AD, a mixture of methane and hydrogen (biohythane), can also be converted from waste. Several key parameters have been demonstrated to increase methane and hydrogen yield as well as system stability, including mesophilic temperature (30 - 40 °C) to increase metabolic rates as well as destruction of pathogens; pH 6.5 - 7.2 for favorable growth of methanogens; a carbon to nitrogen ratio of 20 - 30; and an optimal lipid concentration (Zhang et al., 2016). Co-digestion of food waste with other organic substrates has also been shown to improve the performance of anaerobic fermentation by 383% due to buffering capacity and nutrient balance (Misi and Forster, 2001), highlighting the need to engineer bacteria towards higher tolerance for pH and a wider spectrum of nutrient supplements.

Plastic waste degradation and conversion to biofuels

More than 350 million tons of plastic are produced in the world every year (Danso et al., 2019). Synthetic plastics have high resistance to many physical and chemical factors, and as such, the degradation in a natural setting is extremely slow. A few microbes have shown great potential towards the degradation and eventually, conversion of plastics (Figure 3C). For example, *Ideonella sakaiensis* is capable of metabolizing polyethylene terephthalate (PET) as a primary carbon and energy source, and two enzymes, PETase and MHETase, are responsible for degrading PET into its two monomers, terephthalic acid and ethylene glycol (Yoshida et al., 2016). Other bacteria and fungi have also been reported to degrade ester-linked polyurethane, polyethylene, polystyrene, and polyamide plastics. Despite the lack of well-studied depolymerizing enzymes, polystyrene, polypropylene, and polyethylene can be non-enzymatically treated e.g., via pyrolysis. The resulting long chain hydrocarbons can then be readily metabolized by microbes such as *Yarrowia lipolytica* (Mihreteab et al., 2019). The partial or complete plastic degradation pathways from existing microorganisms could potentially be incorporated into recombinant microbial hosts to not only break down polymers but also convert the degradation products into fuels, valuable chemicals, or biodegradable plastics.

The biofuel chemical and applications space

Petroleum-derived fuels are made of hundreds of hydrocarbons obtained by distillation. The differences in the average number of carbon atoms and content of aromatic, branched and aliphatic hydrocarbons defines their boiling point, freezing point, and combustion energy. This in turn defines their performance and application space: lighter fractions and gasoline are used in heating as well as light and medium duty transportation; kerosene is used in the aerospace industry; diesel is used for medium and heavy-duty transportation.

While bioethanol and biodiesel will likely remain the front runners in the near future of biofuels due to their existing market and technological advancement, the lack of chemical diversity compared to that of gasoline and diesel limits their use in a wide range of applications. In addition, new generations of biofuel molecules must meet the following criteria: i) higher energy content – pure ethanol energy content is only 70% of gasoline and pure biodiesel 90% of D2 diesel (US Department of energy, 2014); ii) low freezing temperatures – soybean biodiesel has a cloud point of 1°C, while D2 diesel has a cloud point of between -7 and -28 °C (US Department of agriculture,

2019), which makes biodiesel incompatible with the current distribution infrastructure, thus leading to geographical and seasonal constraints; and (iii) tailored molecular functionalities for highly specialized applications e.g., high isomeric carbon number and strained bond angles for aerospace applications (Holladay et al., 2020). To synthesize such molecules, novel bioproduction approaches that incorporate complex, unnatural biosynthetic pathways are needed. As synthetic biology has demonstrated the capability of delivering target molecules with precise control of their molecular structures and functionalities, such versatility can be translated into future biofuel production schemes.

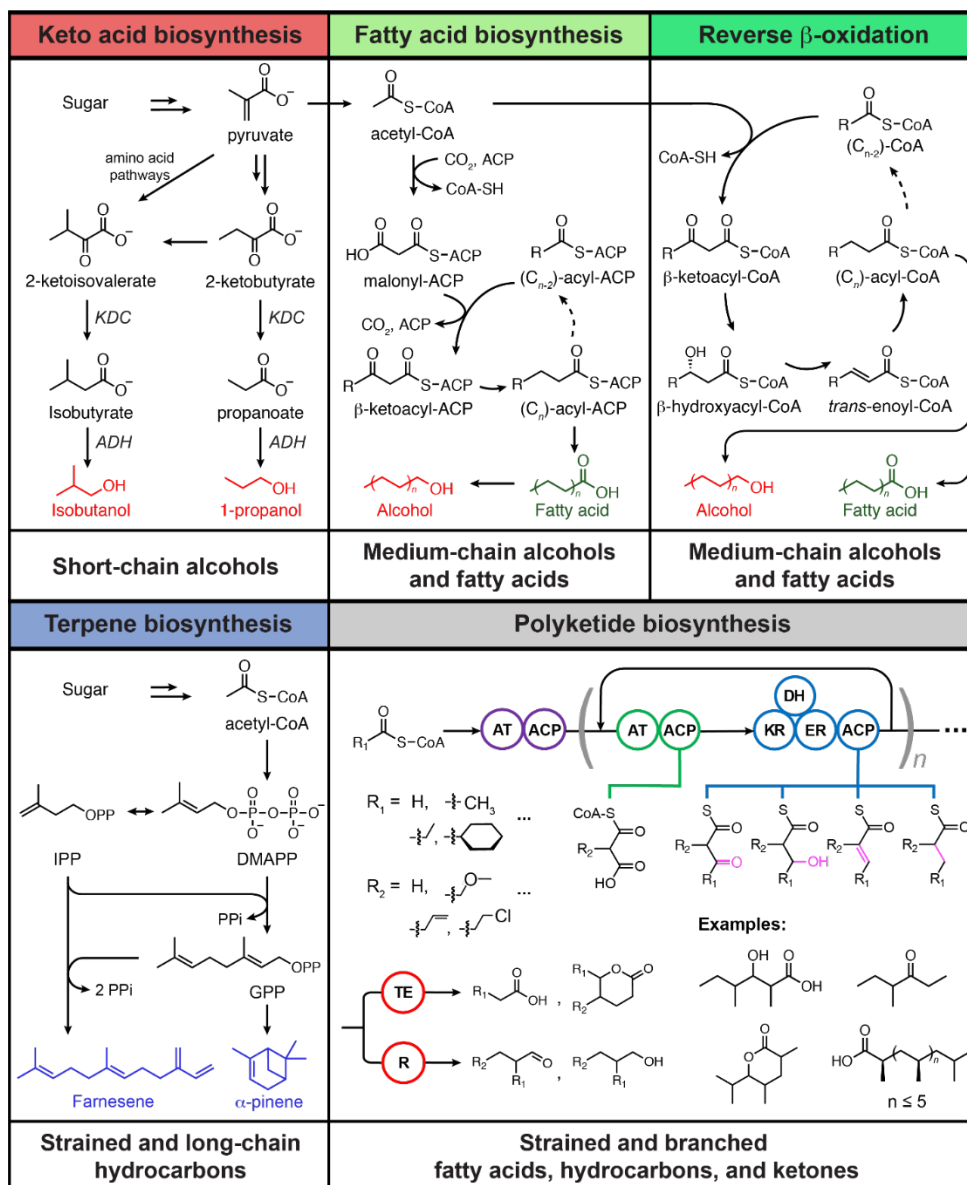


Figure 4. Engineering of microbial pathways to produce diverse biofuels and bioproducts with varying chain-length and structural functionalities. Short- and medium-chain alcohols and fatty acids can be made from the Keto acid pathway, as well as fatty acid and reverse β -oxidation pathways. Long-chain and strained hydrocarbons, such as farnesene and α -pinene, can be converted from sugar using the

terpene biosynthesis pathway. Engineering of multi-modular PKS systems have also been successfully adjusted to incorporate natural and unnatural units to produce pre-designed compounds as biofuel and bioproducts.

Biosynthetic pathways to produce alcohol, fatty acid and terpene biofuels

Extant biofuels are usually simple molecules such as short-chain alcohols, fatty acid esters and terpenes. These pathways are usually engineered from naturally occurring metabolism that converts carbohydrates, pyruvate, and other glycolytic intermediates to make biofuels, which requires additional reducing equivalents to convert these oxidized substrates into highly saturated products. Two structural changes are often needed: i) transformation of the natural precursor into burnable products by elimination of functional groups, *e.g.*, carboxyl and phosphate groups in lipids, or addition of simple functionalities such as alcohols, (m)ethyl-esters in fatty acids or methylene in unsaturated terpenes; ii) modification of the carbon number and density, which can be achieved by extending the length of the molecule or introducing modifications, such as branches and rings.

Short-chain alcohol production pathways directly convert primary metabolites to the fuel molecules without additional chain extension. For example, ethanol production pathways reduce pyruvate to ethanol, in the presence or absence of acetyl-CoA. (Liao et al., 2016) Due to their simplicity and natural occurrence in microorganisms, these pathways can be very efficient and are compatible with many microbial hosts, leading to ethanol's practical usage in gasoline blends. However, the short chain length and high oxygen content of ethanol results in low energy density and high hygroscopicity (Liu and Khosla, 2010). Alcohols produced by keto acid pathways, such as butanol and propanol, have longer chains and better fuel properties over ethanol (higher energy content and lower hygroscopicity). In these pathways (Figure 4), 2-keto acids, intermediates from amino acid biosynthesis, are converted to aldehydes by 2-keto-acid decarboxylases (KDCs) and then to alcohols by alcohol dehydrogenases (ADHs) (Liu and Khosla, 2010; Atsumi et al. 2008).

Iterative assembly of building blocks using a common mechanism such as fatty acid and isoprenoid biosynthesis is ideal for long-chain fuel molecules. For isoprenoids, C5 units are synthesized from acetyl-CoA through the mevalonate (MVA) pathway, or from acetyl-CoA and glyceraldehyde 3-phosphate (G3P) through methylerythritol 4-phosphate (MEP) pathway (Figure 4). These C5 units are condensed in linear pyrophosphate intermediates such as dimethylallyl pyrophosphate (GPP, C10) and farnesyl pyrophosphate (FPP, C15) (Peralta-Yahya et al., 2012). These pyrophosphate intermediates can be transformed by terpene synthases or chemical semi-synthesis to produce fuel molecules. Examples are isoprenol/prenol (C5) (Zheng et al., 2013) and farnesol (C15) (Wang et al., 2016) produced by phosphatases or pyrophosphatases, and pinene (C10) (Sarria et al., 2014), limonene (C10) (Chuck and Donnelly, 2014), farnesene (C15) (Brennan et al., 2015), and bisabolane (C15) (Peralta-Yahya et al., 2011) produced by terpene synthases and followed by optional chemical hydrogenation. These pathways can generate products with isomeric carbons and strained angles yielding better fuel properties for aviation. In

fact, farnesane, a jet fuel derived from farnesene has a freezing point of $-70\text{ }^{\circ}\text{C}$ and was approved for use as a 10% blend in jet fuel.

Reverse β -oxidation (Dellomonaco et al., 2011), fatty acid synthases (FASs) (Steen et al., 2010), and polyketide synthases (PKSs) (Yuzawa et al., 2018) can be used to produce long-chain biofuels (Figure 4). These pathways have in common thiol carrier-bound intermediates derived from acyl-Coenzyme A (Acyl-CoA). In reverse β -oxidation, CoA acts directly as the thiol carrier, while in FASs and PKSs an acyl carrier protein (ACP) is activated with a phosphopantetheinyl group, which is in turn derived from CoA. Another common feature of these pathways is that the chain extension occurs via a Claisen condensation mechanism. After each condensation, the resulting β -ketone is fully reduced to form a methylene moiety, and the final products, fatty acids, are released from the carriers by a thioesterase (Figure 4). Esterification of these carboxylic acids yields fatty acid methyl ester (FAME) and ethyl ester (FAEE) biofuels such as ethyl-octanoate and ethyl-decanoate (Saerens et al., 2006). Hydride substitution, from NADPH or NADH, releases the chain to fatty aldehydes (Schirmer et al., 2010), further reduced to fatty alcohols (Steen et al., 2010), or decarbonylated to burnable hydrocarbons (Schirmer et al., 2010). Alternatively, thioesterases release the intermediates to carboxylic acids. These free fatty acids can be reduced to alcohols by the carboxylic acid reductase-aldehyde reductase pathway (Akhtar et al., 2013) or decarboxylated to hydrocarbons by enzymes like nonheme iron oxidases (Rui et al., 2014), ferulic acid decarboxylases, (Aleku et al., 2018) and light-driven fatty acid decarboxylases (Sorigué et al., 2017), which has been used for the production of pentadecane from palmitic acid.

Engineering of polyketide synthases for novel biofuels

Among these thiol carrier-dependent pathways, modular type I PKSs are the most versatile synthetic platform due to their modular biosynthetic logic. PKSs are very large proteins which consist of multiple domains that work together to extend the chain length while adding functionalities to the product: the acyltransferase (AT) domain loads acyl-coA precursors, such as malonyl-CoA and its α -substituted analogs, the ketosynthase (KS) domain catalyzes the Claisen condensation, while optional ketoreductase (KR), dehydratase (DH), and enoylreductase (ER) domains convert the resulting β -keto moiety to a β -hydroxyl group, α - β alkene, or a saturated β carbon (Figure 4). PKS synthetic pipelines often have a strong correspondence between module order, domain composition, and the chemical structure of the final products, which enables the prediction of gene clusters from chemical structures, and *vice versa*, allowing for retrosynthetic design for new molecules of various chain lengths and functionalities (Backman et al, 2018) and the potential production of 'designer' fuels.

The PKS biological design space is enormous considering i) a large number of starter and extender units with different α -groups in combination (*e.g.* methyl and ethyl) with ii) the varying degrees of β -carbon reduction, and iii) the variations in the number of modules (Pang et al., 2019). Engineering efforts to controllably manipulate these characteristics has led to the production of novel biomolecules, including new biofuels. Acyltransferase engineering through point mutations and AT domain swapping has enabled the incorporation of many non-natural extender units (Kalkreuter, Curr Opin, 2018). For example, the AT domain of borrelidin PKS module 1 has been demonstrated to not only take malonyl-CoA, but also its α -branched derivatives, such as

methylmalonyl-CoA into its assembly line, leading to highly branched C3-C15 molecules (Curran et al., 2018). AT domain swapping and further engineering have also led to the production of C6 and C7 ethyl ketones, as well as C5 and C6 methyl ketones at high titers of >1 g/L and several hundred mg/L, respectively (Yuzawa et al., 2018). These short-chain ketones can be added to gasoline as oxygenates to increase their octane number, and the fact that these molecules can be produced from plant biomass hydrolysates highlight the efficient and renewable biofuel production using PKSs.

As high degrees of reduction are a desirable biofuel property, reliable re-engineering of PKS reductive modules towards full reduction of the β -carbon is an important design principle. While this remains a significant challenge in PKS engineering, a recent study illustrated that chemoinformatic considerations of the donor and recipient modules could assist in successful reductive loop (RL) exchanges, producing 165 mg/L of the branched 2,4-dimethylpentanoic (C7) acid (Zargar et al., 2020).

Owing to its multi-modular nature, PKS provides a versatile synthetic platform where multiple modules can be assembled with complete fidelity towards rational design and synthesis of novel chemicals. Such multi-modular system has been successfully implemented to produce the pre-designed molecules (Kalkreuter et al. 2019), including the production of ethyl ketones, a potential biofuel with favorable properties (Zargar et al. 2020). However, side products are typically observed due to gatekeeping domains and other mismatched enzymatic considerations.

Conclusion and Future perspectives

As described above, there are many possible routes to renewable, carbon-neutral biofuels. And over the last two decades of research, many of these routes have been proven out and optimized. However, commercialization of biofuels has been a challenge due to the lack of a tax on carbon (CO₂ produced by burning fossil fuels) and/or mandates for the use of biofuels, the high cost of constructing large-scale biofuel production facilities and shifting agriculture production to bioenergy crops, and the low cost of petroleum-based fuels. Indeed, the advent of fracking and other methods to improve extraction of fossil fuels from the ground has made the situation even worse. Fortunately, many of the technologies originally created for producing advanced biofuels can be used to produce other products that might have otherwise been synthesized from petroleum and which have a favorable profit margin or products that would be difficult or impossible to synthesize from petroleum. As the methods for producing these products become more efficient, it will be easy to translate these gains to biofuel production when the world's governments decide to prioritize climate change over the petroleum industry.

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Competing Interests

JDK has a financial interest in Amyris, Lygos, Demetrix, Maple Bio, Napigen, Apertor Pharma, Ansa Biotechnologies, Berkeley Yeast, and Zero Acre Farms.

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