Recent progress in the synthesis of advanced biofuel and bioproducts

Brian F. Pfleger[1,2,3,4,*] and Ralf Takors[4,*]

1. Department of Chemical and Biological Engineering, University of Wisconsin-Madison, Madison, Wisconsin, United States of America

2. DOE Center Advanced Bioenergy and Bioproducts Innovation, University of Wisconsin-Madison, Madison, WI, 53706, USA

3. DOE Great Lakes Bioenergy Research Center, University of Wisconsin-Madison, Madison, WI, 53706, USA

4. Institute of Biochemical Engineering, University of Stuttgart, Stuttgart, 70569 Germany

* Corresponding Authors

Brian.pfleger@wisc.edu takors@ibvt.uni-stuttgart.de

+1 608-630-7883 +49 711 685 64535

Abstract

Energy is one of the most complex fields of study and an issue that influences nearly every aspect of modern life. Over the past century, combustion of fossil fuels, particularly in the transportation sector, has been the dominant form of energy release. Refining of petroleum and natural gas into liquid transportation fuels is also the centerpiece of the modern chemical industry used to produce materials, solvents, and other consumer goods. In the face of global climate change, the world is searching for alternative, sustainable means of producing energy carriers and chemical building blocks. The use of biofuels in engines predates modern refinery optimization and today represents a small but significant fraction of liquid transportation fuels burnt each year. Similarly, white biotechnology has been used to produce many natural products through fermentation. The evolution of recombinant DNA technology into modern synthetic biology has expanded the scope of biofuels and bioproducts that can be made by biocatalysts. This opinion examines the current trends in this research space, highlighting the substantial growth in computational tools and the growing influence of renewable electricity in the design of metabolic engineering strategies. In short, advanced biofuel and bioproduct synthesis remains a vibrant and critically important field of study whose focus is shifting away from the conversion of lignocellulosic biomass towards a broader consideration of how to reduce carbon dioxide to fuels and chemical products.

Why Biofuels and Bioproducts Remain Critical to Society

The development of inexpensive liquid transportation fuels from petroleum [1] (**Figure 1a**, left) in the early 1900's is a clear marker on the timeline of human history. Modern lifestyles and the economic growth in the twentieth century would not be possible without the ability to cheaply move people and goods around the globe. Unfortunately, society is now seeing the cost of this advancement. Fossil fuel supplies are not universally distributed (**Figure 1b**), a fact that has led to economic inequities, price instability (**Figure 1c**), security concerns, and military conflict. Worse, the combustion of fossil fuels has deposited enormous volumes of carbon dioxide (**Figure 1d**, 100 ppm rise in less than 75 years) [2] and other greenhouse gases into the atmosphere leading to global climate change and associated natural and economic disasters [3–5]. The need to slow or reverse the rate of CO_2 accumulation in the atmosphere provides a clear motivation for the development of alternative energy carriers that can be produced in a sustainable if not carbon-negative way [6] (**Figure 1a**, right). In addition to providing inexpensive energy sources, fossil fuel refining provides the source material for the petrochemical industry – an interconnected network of chemical reactions that produce materials, solvents, pharmaceuticals, and other desired products [7]. If fossil fuel refining is curtailed due the use of renewable energy sources, alternative means of supplying the chemical industry with functional raw materials must also be developed. In addition to technical challenges, a web of interconnected economic, political, and regulatory conflicts of interest also needs to be navigated to ensure that technologies that benefit society in the long run are allowed to come into being. The topic of energy supply is one of the most complex in academia and a comprehensive discussion is far beyond the scope of any *Current Opinion*. Therefore, this opinion will focus primarily on biotechnology advances and trends over the last five years related to production of advanced biofuels and bioproducts.

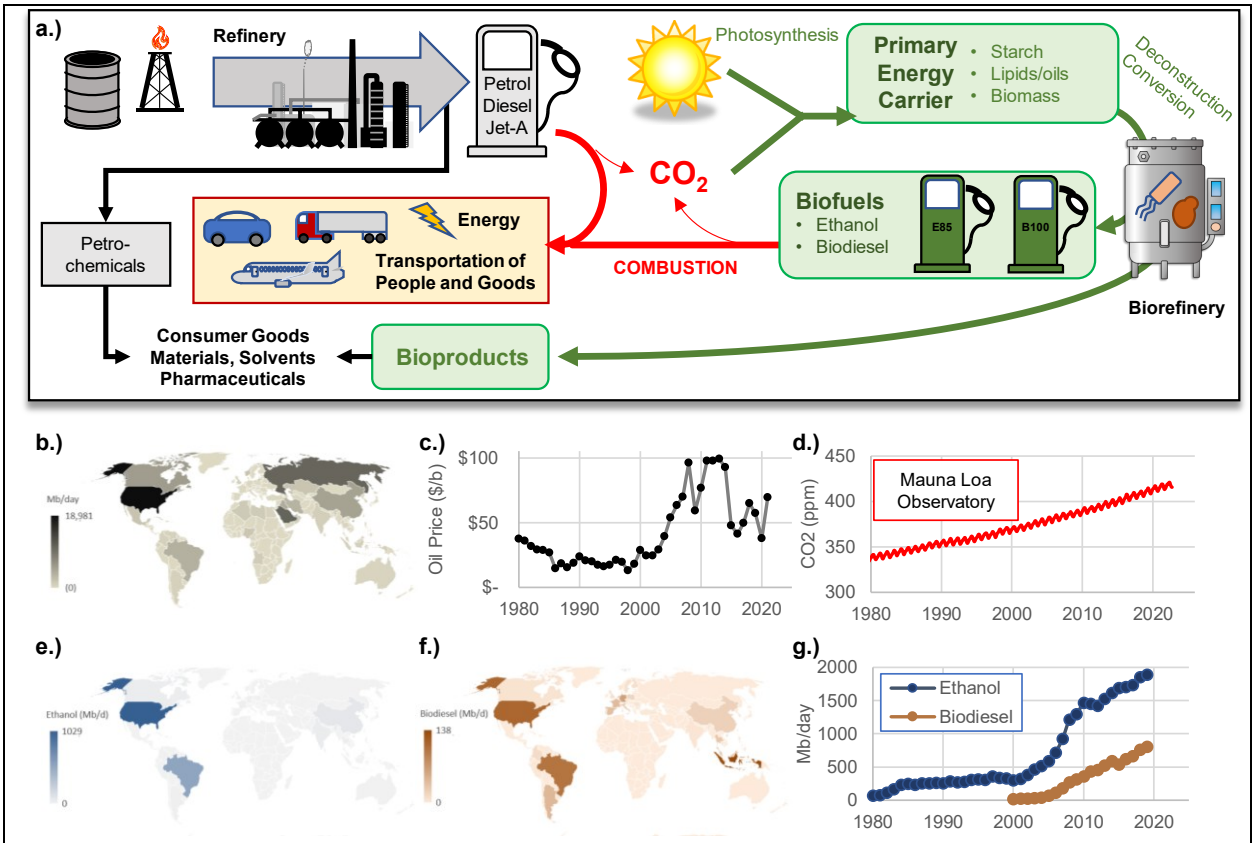

Figure 1. Motivation for producing biofuels. a.) Compare (left) the linear flow of carbon from petroleum and natural gas through refineries to gasoline, diesel, and jet fuel to the circular (right) flow from CO_2, through photosynthesis to primary energy carriers that can be deconstructed and converted in biorefineries to biofuels. The same comparison can be made for petrochemicals derived from oil and natural gas to bioproducts made from refining of energy carriers. b.) Petroleum and liquid hydrocarbons production is not universally distributed across the world (source: EIA). c.) The average annual price of petroleum has fluctuated widely over the last 20 years (source: EIA). d.) CO_2 is accumulating in the atmosphere as measured at the Mauna Loa Observatory (Source: Scripps Institution of Oceanography at UC San Diego, NOAA). Production of current biofuels e.) ethanol and f.) biodiesel occurs in only a few countries but has grown steadily over the last 20 years. Ethanol has grown in part due to a policy shift in the USA enacted in 2005 that required the use of 10% ethanol in gasoline as an oxygenate.

What molecules can we make via biotechnology

Biofuels and bioproducts can be defined functionally as molecules that are either made from a biological source or produced with a biotechnology. Biofuels are combustible molecules used primarily as liquid transportation fuels. Bioproducts are petrochemical replacements used throughout the chemical industry. Exemplary molecules from both classifications, e.g. ethanol and acetone, have been produced for centuries, and now advances in synthetic biology allow metabolic engineers to develop strategies for producing a much wider portfolio of compounds [8] including natural products [9,10], terpenoids [11,12], amino acids [13], fusel alcohols [14], flavonoids [15], oleochemicals [16], alkaloids [17], and bioplastics [18–20]. Programs like DARPA's 1000 Molecule program demonstrated that the field is capable of designing, building, and testing first generation biocatalysts that produce lab-scale quantities of nearly any molecule [21]. Unfortunately, translating these initial successes to industrial scale has not been common because industrial scale cultures are challenged with technical mixing limits, the need to achieve high-yield, environmental heterogeneity, and additional stresses not encountered in the laboratory. The white biotechnology industry has commercialized many bioproducts including amino acids, lactic acid, and 1,3-propanediol, but many other ventures have failed to gain market share (e.g. succinic acid, 3-hydroxypropionic acid) [22]. The enormous transportation fuel market motivated analogous commercial forays into advanced biofuel synthesis (e.g. isobutanol [23] and farnescene [24]), particularly as production of the first widely-available biofuels, ethanol and biodiesel, continued to grow (**Figure 1e-g**). Advanced biofuels are broadly defined as organic molecules, beyond ethanol that are capable of being blended with traditional transportation fuels or burned directly in engines. Advanced biofuels are promoted because of properties that overcome the many disadvantages of ethanol (high hygroscopicity, low energy density, costly purification) and plant-based biodiesel (high cloud point, tropical deforestation). Advanced biofuels can be produced by microorganisms [25], or by chemical upgrading of biologically produced intermediates e.g., catalytic upgrading of terpenoids to jet fuel [26]. The latter processing can overcome inherent toxicity of advanced biofuels [27] as well as difficult terminal reactions [28–31] required for producing hydrocarbons. Interest in processes that integrate the strengths of biological and thermal catalysis [32–34] are displacing the historical competition between the fields and motivating searches for *bio-privileged molecules* [35], i.e., platform compounds produced biologically in high yield that can be catalytically upgraded to a range of higher-value products. While the concept is attractive, technical hurdles, such as catalyst poisoning, intermediate purification, and heat integration must be overcome to pass intermediates between each type of catalysis [36,37].

Most of the enzymes and metabolic pathways needed to produce advanced biofuels were discovered and first engineered in the last two decades. Early efforts developed platform organisms for producing oleochemicals, terpenes, fusel alcohols, and natural products by optimizing flux to core intermediates in each pathway and expressing terminal enzymes capable of generating desired chemical products. The discovery trend has slowed as the field exhausts known natural compounds [38], but recent exploration has yielded exciting new compounds such as polycyclopropanated polyketides [39] that could serve as jet fuels. The discovery trend may soon be reversed as the boundaries of known biochemical space are expanded with the design and/or engineering/evolution of enzymes capable of catalyzing unnatural biochemistries [40,41]. Initial demonstrations of new enzymatic conversions almost always possess poor catalytic performance that researchers hope to improve with protein engineering and directed evolution [42]. These approaches have been augmented with artificial intelligence and machine learning methods [43–47] to increase the frequency of beneficial mutations tested. Similarly, structure-guided mutagenesis methods have improved [48–51] and are likely become more broadly useful with the increased availability of accurate structure predictions coming from AI-tools such as AlphaFold [52] and RoseTTAFold [53]. Success in protein engineering will open similar opportunities to develop novel pathways [54] to produce advanced biofuels and bioproducts from feedstocks not considered today. Tools for broadly mapping

chemical transformations have been developed [55] and used to identify bio-privileged [56] molecules and novel biochemical routes between a desired feedstock and product [57]. Systematic searches can generate comprehensive lists of pathways and analytical tools can then rank them according to thermodynamics, availability of enzymes, number of (novel) steps, feasibility, efficiency, or any other desired metric; helping researchers prioritize efforts in the laboratory [58–61]. Novel pathways have been created to fix carbon [62,63], metabolize glucose to acetyl-CoA in 100% carbon yield [64,65], and produce novel chemical building blocks [66]. Regardless of whether a pathway is natural or unnatural, once it is established in a host, it must be engineered to efficiently convert a low-cost feedstock to a desired product. The low cost of fossil fuels and petrochemicals and relatively comparable costs of traditional biotechnology substrates leave little margin for loss. Maximizing yield in the laboratory remains a laborious endeavor, one that is only made more challenging when cells are asked to perform in the non-ideal environments of industrial scale bioreactors or use complex hydrolysates as feedstocks. The relative costs of feedstocks and products and the resulting requirement for high yields distinguish the biosynthesis of biofuels and bioproducts from the more lucrative synthesis of biopharmaceuticals and therapeutic proteins in the red biotechnology sector.

Biomass – a plentiful but still recalcitrant feedstock

The vast majority of biofuels and bioproducts are derived from sucrose, starch, and plant oils respectively – termed first generation biofuels. Second generation processes that leverage waste or lignocellulosic feedstocks and third generation fuels that directly reduce CO_2 remain in development despite substantial investment of academic and industrial resources. Lignocellulosic biomass contains three major fractions – cellulose, hemicellulose, and lignin. The first two are sugar polymers that can be degraded chemically or enzymatically to sugar hydrolysates that are feedstocks for biological or catalytic upgrading. Production of sugar hydrolysates has been viewed as the sustainable replacement of starch-based feedstocks, but widespread availability remains to be seen. Three US-based cellulosic ethanol plants were launched in the early 2010s but all have been closed, sold, and/or converted to other processes [67]. Many of the original barriers to 2nd generation biofuels, including recalcitrant breakdown of biomass polymers, generation of microbial inhibitors, and reduced conversion relative to model substrates, remain obstacles to implementing processes on large scale despite recent academic progress [68,69]. In addition, daily handling and processing of lignocellulosic materials collected from farmlands hampered its utilization, making 2nd generation procedures much more laborious than 1st generation approaches.

Lignin, the third biomass component, is a heterogenous polymer of aromatics that has received substantial attention as a feedstock in recent years. Lignin catabolism has been described as biological funneling [70], where the polymer is broken and the heterogenous mixture of resulting aromatics are enzymatically converted to a small subset of organic molecules that can serve as products or feedstocks for further upgrading. Recent studies have demonstrated conversion of lignin streams to molecules such as β-ketoadipate [71], PHA [72], mucconate [73], itaconate [74], and 2-pyrone-4,6-dicarboxylic acid (PDCA) [75,76], using engineered microbes. *Pseudomonas putida* has emerged as a favorite microorganism for this metabolism due to its native repertoire of aromatic catabolism pathways and its tolerance to the aromatic feedstocks and related toxins [77]. A wide range of genetic tools have been developed for *P. putida* including genome engineering techniques [78], promoters [79], CRISPRi [80,81], reduced genome strains, metabolic models [82] and genome-wide fitness association datasets [83]. Many of the approaches for lignin processing are also now being applied to up-cycling of aromatic components of plastic waste [84].

The promise of biofuels comes from the ability to harvest solar energy to reduce carbon dioxide and produce energy carriers, usually through a photosynthetically synthesized carbohydrate or lipid intermediate. Conversely, third generation biofuels are molecules produced directly from CO_2 using

renewable electrons, often by photosynthetic organisms or chemoautotrophs. Direct photosynthetic production of biofuels and bioproducts remains an active area of research [85]. Genetic engineering tools for algae and cyanobacteria have grown substantially in the last decade [86,87]. These tools have been used to engineer production of novel biofuels and bioproducts in several fast-growing species [88–90] and enhance the titer of natural lipids. That said, many commercial efforts to grow algae for oils that can be converted to biodiesel have come and gone. Unfortunately, algae cultivation is plagued by volumetric light limitations, slow growth, contamination (pond crash), low biomass and oil titers, and the challenge of co-locating the light, water, carbon dioxide and nutrient sources needed for maximizing production[91]. While directly producing biofuels and bioproducts in phototrophs remains challenged, the algal biomass itself could alternatively be used as a feedstock for heterotrophic biofuel and bioproduct synthesis [92,93] – taking advantage of the organism's large areal productivity relative to common terrestrial plants. A similar areal argument can be made for using solar cells to convert photons into electricity, a process that captures a larger fraction of the energy in a photon than photosynthesis [94]. The growing Interest in using electricity as an intermediate in producing third generation biofuels and bioproducts is one example of how energy research is shifting.

The energy landscape is changing

Since 2000, when first-generation production accelerated, biofuels have been promoted as the future for all forms of travel – personal, long-haul freight, and flight. Ethanol and biodiesel are available in the marketplace for personal travel and trucking but make up only a small fraction of the fuels used in this industry. The first demonstrations of transcontinental flight and naval operations (i.e. great green fleet) using biofuels have been made, but at costs substantially above petroleum-derived alternatives. Here, biofuels were produced by gasification of biogenic material followed by Fischer-Tropsch synthesis. In the same period, renewable sources of electricity, such as wind and solar, have become more prominent, offering a low-cost source of sustainable electrons. The low cost provides the opportunity to have electric vehicles deliver local transportation for a large fraction of people and goods, assuming battery technologies become sufficiently inexpensive and widely available. Inexpensive electricity (typically below 5 cent/KWh [95]) also creates opportunities to reduce CO_2 and generate energy carriers (**Figure 2**), with hydrogen being one of the most attractive. Hydrogen can be used by many microbes as an electron donor to respiration or to reduce C_1-gases (carbon dioxide, carbon monoxide) through the Wood-Ljungdahl pathway into acetate, ethanol, and other small molecules, that could be used as biofuels or building blocks for other bioproducts [96,97]. In one noteworthy example, a *Clostridium* species was engineered to convert Wood-Ljungdahl derived acetyl-CoA into hexanol via a reversed β-oxidation pathway [98]. Upgrading C_1 gases is attractive because the carbon could be derived from gasification of waste materials (e.g. biogas), stranded natural gas (via steam reforming and water gas shift), waste gases (e.g. steel mill, fermentation off-gas), or potentially from atmospheric capture if economically viable technologies become available. Beyond hydrogen, electrochemistry offers the chance to provide other electron carriers to cells such as formate [62,63,99–102], methanol [103], ammonia, urea, reduced metals [104], and other organic electron shuttles [105]. These carriers can be used to enhance yields, balance redox, and generate energy in strains engineered to produce advanced biofuels and bioproducts. The availability of these feedstocks is creating a paradigm shift away from single, polysaccharide dominant feedstocks, to a strategy where carbon, electrons, and energy can potentially come from different molecules. This paradigm shift, sometimes referred to as electrofuels, provides a unique opportunity for metabolic engineers to design sustainable pathways for producing bioproducts in high yields.

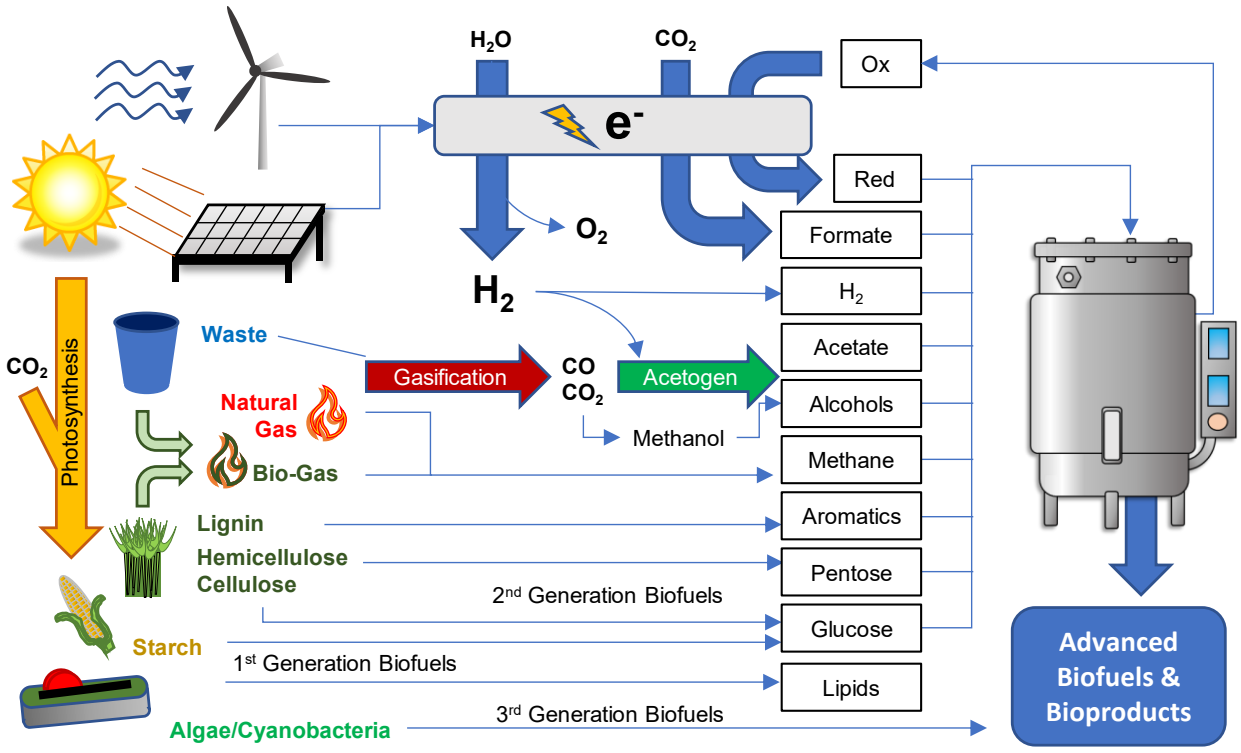

Figure 2. The landscape of technologies for producing biofuels and bioproducts is far more complex today. The presence of renewable electricity from wind and solar provides routes to produce hydrogen and other reduced energy carriers that can be fed to bioprocesses, so called electrofuels (blue arrows at top). Starch and lignocellulosic biomass in all its forms remain a large reserve of fixed carbon that could be used to displace petroleum as a source of fuels and chemical products either through traditional deconstruction to sugar hydrolysates and lignin derived aromatics or via gasification and upgrading of synthesis gas. Municipal wastes and stranded natural gases could be processed in the same way. Biological conversion of C_1 gases, CO, CO_2, CH_4, offers the ability to directly produce biofuels and bioproducts or building blocks (e.g. acetate) that can be fed to downstream bioprocesses with additional energy carriers. Third generation biofuels and bioproducts are produced by directly reducing CO_2. Historically, this research focused on photoautotrophs grown in raceway ponds and photobioreactors, but now chemoautotrophic and electrofuel strategies should also be included.

Where do we go from here?

The *role of traditional biofuels* and the *structure of the chemical industry* are topics that deserve closer inspection now that competing forms of renewable transportation are emerging. From the perspective of climate protection, the reduction of greenhouse gas emissions from fossil resources is a *conditio sine qua non*. In this sense, (bio)processes utilizing renewable resources should replace fossil production routes for commodities and fine chemicals on large production scale to maximize climate benefits. Not all existing fuels and chemical products need “bio”-replacements, so perhaps it is time to focus research on the biomanufacturing of fuels that lack viable alternatives, e.g., commercial and military jet fuel, and products that displace the largest volumes of fossil resources. Establishment of such a circular bioeconomy is a large, disruptive change where the chemical industry will become based on oxidized instead of reduced carbon. The change is essential to save living standards for future generations but will not happen overnight. Instead, there will be a long-lasting competition guided by technical, economic, environmental, and geopolitical constraints. For example, ethanol fermentation has satisfied the technical and

environmental demand for oxygenated gasoline, but not displaced gasoline as the primary personal transportation fuel in the United States. This failure is in part due to the fact that 1st and 2nd generation resources are insufficient to meet demand for all feed, food, fuel, and materials needed today, let alone the growing population of developed nations. That said, sugars can be used to produce a subset of perhaps the most critical chemical products, if high carbon-yields can be achieved. High yields are essential because novel bio-production routes must immediately compete with optimally-networked, fully-depreciated industrial sites that are the successful result of a century of optimization. Steady research has managed to push microbial producers of some compounds to their theoretical limits and industrial viability, but many more are produced at levels far from a viable carbon economy. Furthermore, biological production of an individual chemical is decoupled from all others, meaning companies have a choice to enter into any market. This contrasts a petroleum refinery where fuel production is subsidized by the synthesis of high-value petrochemicals because all molecules in a barrel of crude must be processed. For this reason, feedstocks and products need to be matched to ensure the maximum carbon efficiency. For example, converting lignin to aromatics and related diacids is more carbon efficient than breaking down biomass to fermentable substrates and respiring some of the resulting sugars to produce the energy needed to synthesize the same aromatics. The availability of low-cost electricity and corresponding energy carriers will provide opportunities to find novel feedstock-product pairs that maximize carbon and energy yield and support a new bioeconomy.

Traditional biofuels, bioproducts, and their feedstocks will have a role in the new chemical industry, but to meet all demand, other carbon sources, such as CO_2, will need to be used to produce the products of tomorrow. As discussed above, CO_2 can be incorporated directly into biosynthesis pathways or reduced to other energy carriers that can be fed to industrial biotechnologies. In the short term, high concentration CO_2 sources such as cement production, waste combustion, biogenic syngas formation, fermentation, etc. are available as starting points. In the long-run, carbon capture technologies will be needed to off-set the historical CO_2 release from combustion of fossil fuels. The success of such chemistry heavily relies on the availability of vast amounts of green electricity and hydrogen. For instance, zero CO_2 emissions of the entire German chemical industry will require more than 600 TWh/a by 2050 [106]. Hence, novel (bio)chemical production sites are likely to be built around the globe making use of locally produced green energy from wind, solar power, and geothermal sources to keep electricity cost within the economically attractive range below 3-5 cent/KWh [95]. If successful, the net incorporation of CO_2 into chemical products will be of dual benefit: not only will greenhouse gas emissions be reduced but also extra costs for releasing CO_2 into the atmosphere will be prevented (e.g. 55 €/ton_{CO2} in Germany in 2025). This advantage is all the better as the fossil production will not vanish from one day to the next.

Conclusions

In summary, advanced biofuel and bioproduct synthesis remains a vibrant and critically-important field of study. However, the landscape is shifting with expansion of available feedstocks, energy sources, biochemistries, synthetic biology tools, modeling approaches, and competing technologies. Irrespective of whether pathways are engineered in mono-cultures or microbial consortia [107], it is the task of systems metabolic engineering to provide strains and sustainable processes that efficiently produce fuels and chemical products from renewable feedstocks. Production of highly functionalized molecules with small production volumes and high EBIT margin will likely remain based on traditional sugars to leverage existing technologies for strain engineering and fermentation. For biotechnologies to contribute to a new circular chemical economy, the field must now identify efficient pathways for incorporating CO_2 and other waste carbon into feedstocks industrial microbes can efficiently use to produce advanced biofuels and bioproducts. Renewed investment in classical biochemical engineering research and training [108] will then bring these processes to market with the goals of saving the planet and maintaining modern lifestyles.

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