

Fuelling the future: microbial engineering for the production of sustainable biofuels

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Abstract | Global climate change linked to the accumulation of greenhouse gases has caused concerns regarding the use of fossil fuels as the major energy source. To mitigate climate change while keeping energy supply sustainable, one solution is to rely on the ability of microorganisms to use renewable resources for biofuel synthesis. In this Review, we discuss how microorganisms can be explored for the production of next-generation biofuels, based on the ability of bacteria and fungi to use lignocellulose; through direct CO₂ conversion by microalgae; using lithoautotrophs driven by solar electricity; or through the capacity of microorganisms to use methane generated from landfill. Furthermore, we discuss how to direct these substrates to the biosynthetic pathways of various fuel compounds and how to optimize biofuel production by engineering fuel pathways and central metabolism.

Thermohaline circulation

Large-scale ocean currents that result from water density gradients, which are under the control of temperature (thermo) and salinity (haline).

Over the past few decades, increasing atmospheric greenhouse gasses (GHGs), including CO₂, methane (CH₄) and nitrous oxide (N₂O), have been linked to global climate and environmental changes^{1,2}, such as rising sea levels, weakening of thermohaline circulation and eradication of coral reef. The global CO₂ release from fossil fuel usage is now approximately 7 Gt of carbon per year^{3,4}, and the atmospheric CO₂ concentration is 400 parts per million (ppm). If the current upward trend continues, the CO₂ release rate is likely to double by 2050 (REF. 3). Doubling the CO₂ emission rate without remediation is expected to increase the CO₂ concentration to 500 ppm³, which would lead to a global warming of approximately 2 °C above the level in 1900 (REF. 2). This level of temperature rise would increase the risk of disintegration of the West Antarctic Ice Sheet (WAIS)², as well as other negative effects. Complete disintegration of the WAIS, even if it were to occur gradually, would cause a disruptive rise of sea level by 4–6 metres (REF. 2). Although these scenarios and the quantitative relationship between CO₂ emission, atmospheric CO₂ concentration, global temperature and effect are subject to uncertainty, the potential risk of adverse effects on society, economy and the environment is too large to ignore. If we are to avoid reaching the 2 °C threshold, there would need to be a substantial decrease in carbon emission from fossil fuel usage, the scale-up of renewable energy supplies and a decrease in energy demand, all within a timescale of decades.

Compared with other renewable energy forms, liquid biofuels are more compatible with current infrastructure and have a higher energy density (BOX 1), which provides a basis for their long-term roles in the future energy mix. Traditionally, biofuels are defined as fuels that are produced from biomass by either biological or non-biological processes, but this definition can be broadened to include fuels produced from other renewable resources, such as landfill gas and CO₂, by biological processes. The most common biofuel today is ethanol that is derived from corn starch⁵ or cane sugar^{6,7}. To generate bioethanol, atmospheric CO₂ is fixed by plants to form carbohydrates, which are subsequently fermented by microorganisms, such as yeast cells. As the generation of bioethanol uses cane sugar or corn starch as substrates, it does not require an expensive biomass deconstruction step. However, to increase the effect of biofuels, the range of raw materials utilized for next-generation biofuels should be expanded, and biofuel production should ultimately utilize either substrates that result from a net increase in global CO₂ fixation or carbon that is released as greenhouse gases. Furthermore, replacing fossil fuels with biofuels does not necessarily reduce CO₂ emission, unless all collateral emissions are considered⁸. Therefore, the production of next-generation biofuels should also account for the emission caused by the use of land to grow and harvest biomass⁹, the effect of that land use on biodiversity¹⁰ and the additional manufacturing costs¹¹.

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Lignocellulose

The major component of the plant cell wall, which consists of cellulose, hemicellulose and lignin.

Cellulolytic organisms

Organisms that can digest cellulose.

Lithoautotrophic

Capable of using CO₂ as the carbon source and inorganic compounds as reducing equivalents.

Methylootrophs

Capable of utilizing reduced C₁ compounds.

One advantage of using microorganisms for the production of next-generation biofuels is the metabolic diversity of bacteria, fungi and microalgae, which enables the use of different substrates as the starting point for biofuel generation. This includes the ability of bacteria and fungi to utilize lignocellulose from wastes and energy crops, the ability of bacteria and microalgae to fix CO₂ and the capacity of microorganisms to use methane from landfill or natural gas wells that would otherwise be flared. For example, plant-derived lignocellulose can be utilized by cellulolytic organisms, such as *Clostridium thermocellum*, or hydrolysed by fungal cellulases into substrates for microbial fermentation (FIG. 1a). As an alternative to lignocellulose, CO₂ can be directly utilized by photosynthetic organisms, such as microalgae and cyanobacteria, or by lithoautotrophic organisms that can use energy that is derived from renewable sources (FIG. 1b,c). Finally, methane, which is a more

potent greenhouse gas than CO₂ (REF. 12), can be utilized by methanotrophs or, after activation to methanol, by methylotrophs (FIG. 1d).

The microbiological challenges in all of these processes are to improve the carbon assimilation pathways in microorganisms and then channel the metabolic flux of these pathways to produce a desirable product through various natural or designed pathways. In addition to ethanol, many other longer-chain alcohols, alkanes and fatty acid esters are desirable fuels because of their favourable properties for specific applications. However, although some of these compounds or their precursors can be produced from various metabolic pathways that naturally exist in microorganisms, these pathways often need to be optimized or redesigned to improve efficiency. Furthermore, the practical or theoretical yields (gram of product per gram of raw material) based on current pathways and the productivity (production rate per

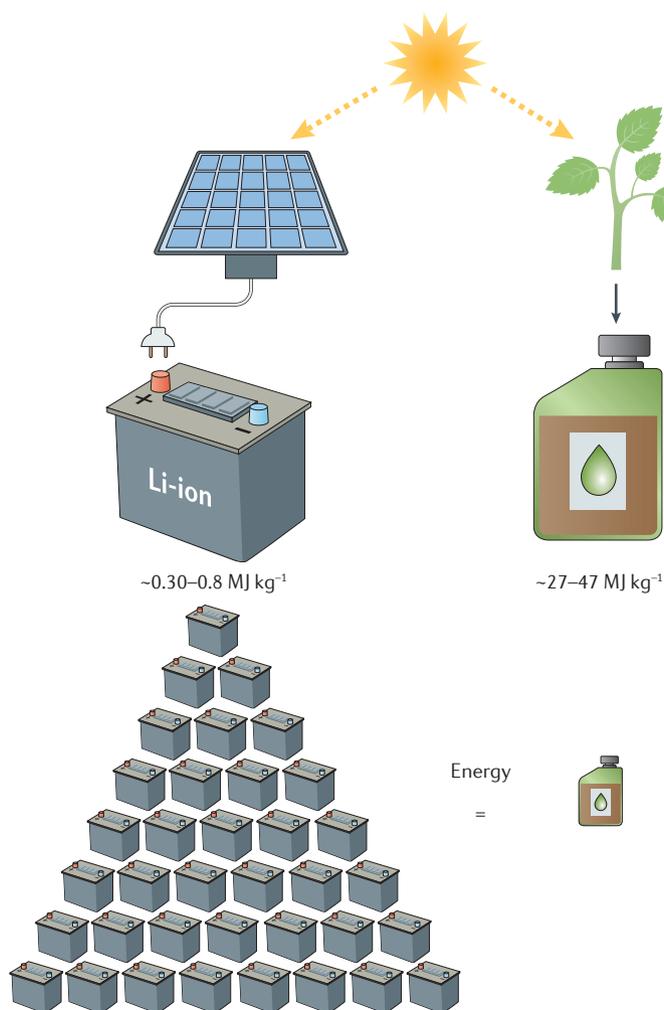
Box 1 | Justification for biofuel

To keep energy supply sustainable while mitigating the CO₂ problem, the ultimate energy source must come from the sun, which radiates 120,000 TW of energy to Earth. This is much larger than the total energy demand of humans, which is 13 TW currently¹⁶¹. Direct harvesting of solar energy using photovoltaic cells is one approach to harness solar energy and to avoid the accumulation of CO₂. Another approach is to rely on photosynthetic CO₂ fixation to produce biomass, which is then converted to liquid fuel. These two platforms are both clean and sustainable in principle, but both face substantial practical challenges. Although solar electricity is carbon free and efficient, electricity storage is a major hurdle to the widespread use of this approach. The energy density for common rechargeable lithium-ion batteries is approximately 1 MJ kg⁻¹ (REF. 162) (with a theoretical limit around 2 MJ kg⁻¹)^{163,164}, which is more than one order of magnitude lower than that of liquid fuel (typically 27–47 MJ kg⁻¹) (see the figure). The energy density consideration limits the use of solar electricity in transportation and other large-scale applications, and is a fundamental factor in favour of liquid biofuel that is derived from carbon recycled from the atmosphere (as opposed to fossil carbon). In addition, the manufacturing of batteries for electric vehicles needs to be considered when calculating CO₂ emission over the battery life cycle, and in some cases this may be comparable to, or greater than, burning fossil fuel in vehicles with internal combustion engines¹⁶⁵, even though electric vehicles have no tailpipe emissions.

Another major factor in favour of biomass-derived fuel is the abundance of biomass. Approximately 123 billion metric tons of CO₂ are fixed annually by terrestrial plants through photosynthesis¹⁶⁶. Of the biomass generated in the United States, approximately 500 million dry tons of forest and agricultural biomass is potentially available at a low price (US\$60 per dry ton or less¹⁶⁷ in 2012). This estimate includes forest and agricultural-land lignocellulosic waste resources that are not currently being used. If large-scale cultivation of energy crops is implemented, it is estimated that the biomass available at this price will increase to 1.1–1.5 billion dry tons by 2030 (REF. 167). 1 billion dry tons of biomass produced annually in a sustainable manner is estimated to be sufficient to displace 30% of the present US petroleum consumption.

Given its abundance and regeneration rate, biomass is considered to be the only foreseeable sustainable source of liquid fuel available to humanity. However, to harness this chemical energy and to convert plant biomass into readily useable fuel molecules, we must overcome several challenges, including land, water and fertilizer usage, the life cycle carbon saving and competition with

non-renewable fuels. Because of low yield in every step, even at \$60 per dry ton of biomass, the raw material cost for lignocellulosic biofuel can be more than \$1 per gallon, which is a substantial portion of the final fuel cost. Thus, the efficient utilization of the raw material in every step is crucial.



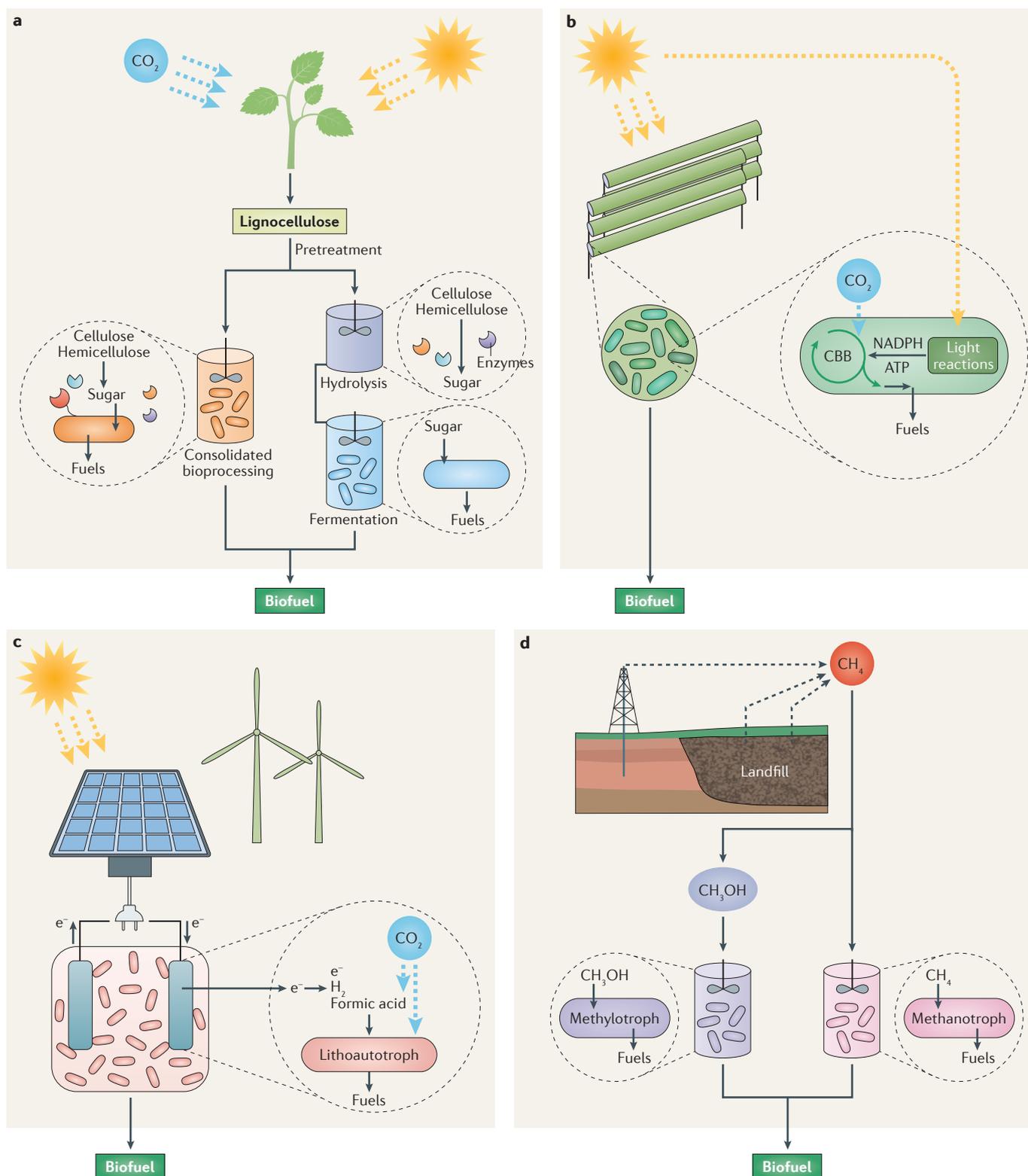


Figure 1 | **Overview of biofuel production from sunlight and atmospheric carbon.** **a** | The lignocellulose of plant biomass can be converted to fuels through hydrolysis followed by fermentation, or through consolidated bioprocessing, which combines the two processes in one reactor. **b** | Photosynthetic organisms, such as microalgae and cyanobacteria, can harness energy from sunlight to reduce CO₂ and convert it to liquid fuels. **c** | A broad range of lithoautotrophs can fix CO₂ to produce fuels with reducing power from electrons or electrochemically generated electron shuttles, such as H₂ and formic acid. **d** | Low-throughput methane from landfill or natural gas wells that is otherwise flared can be used directly by methanotrophs to produce fuels, or it can be converted to methanol (CH₃OH) and can then be utilized by methylotrophs for fuel production. CBB, Calvin–Benson–Bassham cycle.

Alkanes
Organic molecules with the general formula C_nH_{2n+2}.

volume) of bioprocesses are often too low to justify industrial applications. Coupled with the need for extremely high volumes and a low cost of biofuels, this means that a high yield and a high productivity are required for every step of biofuel generation. Many of these problems can be addressed by engineering microorganisms with redesigned metabolic systems to improve yield and productivity, such that the raw material requirement, manufacturing plant scale, capital intensity and operating cost are minimized. Notably, success in microbial engineering can also affect land use and biodiversity. For example, a 10% increase in biofuel yield from lignocellulose translates to a 10% saving in land usage because less raw material is required. Similarly, a 10% increase in microalga productivity results in a 10% reduction in the footprint of microalga cultivation. Engineering microorganisms to utilize diverse raw materials efficiently can also preserve natural biodiversity by reducing the need for importing non-native plants.

This Review focuses specifically on problems that are relevant to microbiology, rather than on other aspects of biofuels. We first describe the status of, and challenges in, the utilization of different substrates, including lignocellulose, CO₂ and methane, followed by a discussion of the major pathways that lead to the production of key fuel compounds. We focus on the design and optimization of microorganisms beyond their natural capabilities.

Substrates for biofuel production

To reduce or avoid collateral emissions, raw materials for sustainable biofuel production should eventually come from atmospheric CO₂ by net increased global carbon fixation or from carbon that is otherwise released as GHGs without being efficiently utilized. These materials must be available in sufficient quantities to displace a meaningful portion of the fossil fuels that are currently used. We do not discuss how these raw materials are derived in a sustainable way, but focus on how they can be converted to biofuels.

Biomass utilization. Plant biomass is the most abundant renewable resource on Earth (BOX 1). Typical plant biomass contains approximately 40–50% cellulose, 20–40% hemicellulose and 20–35% lignin, bound together in a complex structure. Because of the encapsulation of cellulose by lignin and hemicellulose, the hydrophobicity of lignin and the crystallinity of cellulose, the complex plant material has to be deconstructed to simpler chemical forms before further processing. These intermediate forms include sugar¹³, synthetic gas (syngas)¹⁴, organic acids^{15,16} and methane¹⁷. Utilization of lignocellulose through syngas can be accomplished using thermochemical processes¹⁴, followed by the Fischer–Tropsch reaction¹⁸ or microbial conversion¹⁹ through the CO₂-fixing Wood–Ljungdahl pathway²⁰. The conversion of lignocellulose to organic acids or methane is achieved using mixed anaerobic cultures similar to those used in rumen fermentation²¹. All of these processes have been explored for biofuel generation, but more research at the microbial, molecular and biochemical levels is needed to achieve their full potential as strategies to replace fossil fuels.

Currently, lignocellulose deconstruction to sugars generally starts with a pretreatment process followed by enzymatic hydrolysis. The pretreatment process can be physical, chemical, biological or a combination of these^{22,23}. The pretreated biomass is then hydrolysed by either non-complexed cellulase enzyme cocktails or by cellulolytic microorganisms¹³. Industrial production of non-complexed cellulase cocktails predominantly relies on the mesophilic wood soft rot fungus *Trichoderma reesei* (teleomorph *Hypocrea jecorina*), because of the high secreted protein yield of this species in comparison with other microorganisms^{24,25}. These cocktails typically contain at least three types of enzymes: cellobiohydrolases, endoglucanases and β -glucosidases (FIG. 2a). Cellobiohydrolases hydrolyse $\beta(1-4)$ -D-glucosidic linkages in cellulose and cello-dextrins, generating cellobiose from either the reducing (type I cellobiohydrolases) or the non-reducing (type II cellobiohydrolases) ends of the polymeric chains^{26,27}. Endoglucanases cut internal bonds randomly in the amorphous regions within a cellulose polymer, freeing more cellulose ends for cellobiohydrolases to act on. Indeed, synergy between cellobiohydrolases and endoglucanases has been reported²⁸. β -glucosidases then hydrolyse the disaccharide cellobiose to glucose. Notably, both type I²⁹ and type II³⁰ cellobiohydrolases and endoglucanases³⁰ from *H. jecorina* have been engineered to increase their optimal operating temperature from 60 °C to 70 °C by directed evolution. These engineered enzymes performed better than their wild-type counterparts, which demonstrates the benefit of using higher temperatures in cellulose hydrolysis. Furthermore, as the enzyme cost could account for up to 48% of the minimum ethanol selling price^{31,32}, cellulases from other organisms, such as fungi belonging to the genera *Penicillium*, *Acremonium* and *Chrysosporium*, have been characterized³³, but the cost of their production needs to be lower than that of the *T. reesei* enzymes to be industrially attractive.

In contrast to the secreted fungal proteins, cellulases and hemicellulases from anaerobic bacteria (such as *C. thermocellum*, *Clostridium cellulolyticum* and *Clostridium cellulovorans*) self-assemble on the cell surface to form microscopically visible protuberances called cellulosomes^{34,35} (FIG. 2b). The cellulosomes in *C. thermocellum* are the best studied^{35,36}. At the core of the *C. thermocellum* cellulosome is the scaffoldin protein CipA³⁷. This scaffoldin has nine type I cohesin modules that bind strongly to the type I dockerin modules on various secreted *C. thermocellum* cellulases, thus acting as the key assembly site for cellulosome super structure. CipA also has type II dockerin modules, which bind to the type II cohesin module on the cell surface, effectively anchoring the cellulosome onto the cell. The various enzymes that constitute the *C. thermocellum* cellulosome include endoglucanases (CelA and CelB), exoglucanases (CelS and CbhA), xylanases (XynA and XynC), mannanase (ManA) and lichenase (LicB)³⁸. *C. thermocellum* cellulosomes break down cellulose into a mixture of cello-dextrins with an average length of four sugar units³⁹, and these are taken up by cells through an adenosine-binding cassette system, followed by phosphorolytic

Cellulose

A long-chain linear polysaccharide of $\beta(1-4)$ -linked D-glucose; this polysaccharide is a major energy and carbon storage material in plants.

Hemicellulose

A group of heteropolymers in plant cell walls, commonly consisting of mainly pentoses (such as xylose, arabinose and mannose) and some hexoses (such as glucose).

Lignin

A group of heterogeneous crosslinked polymers that are rich in phenolic moieties and provide structural support in plant cell walls.

Synthetic gas

(Syngas). A mixture of CO and H₂.

Fischer–Tropsch reaction

A process that converts a mixture of H₂ and CO to liquid fuels using cobalt, iron, or ruthenium as catalysts at 150–350 °C.

Wood–Ljungdahl pathway

A CO₂-fixing pathway that is found in certain bacterial and archaeal species and that synthesizes acetyl-CoA; also known as the reductive acetyl-CoA pathway.

Cellulosomes

Cell surface-attached multi-enzyme complexes that are produced by several cellulolytic bacteria for lignocellulose degradation.

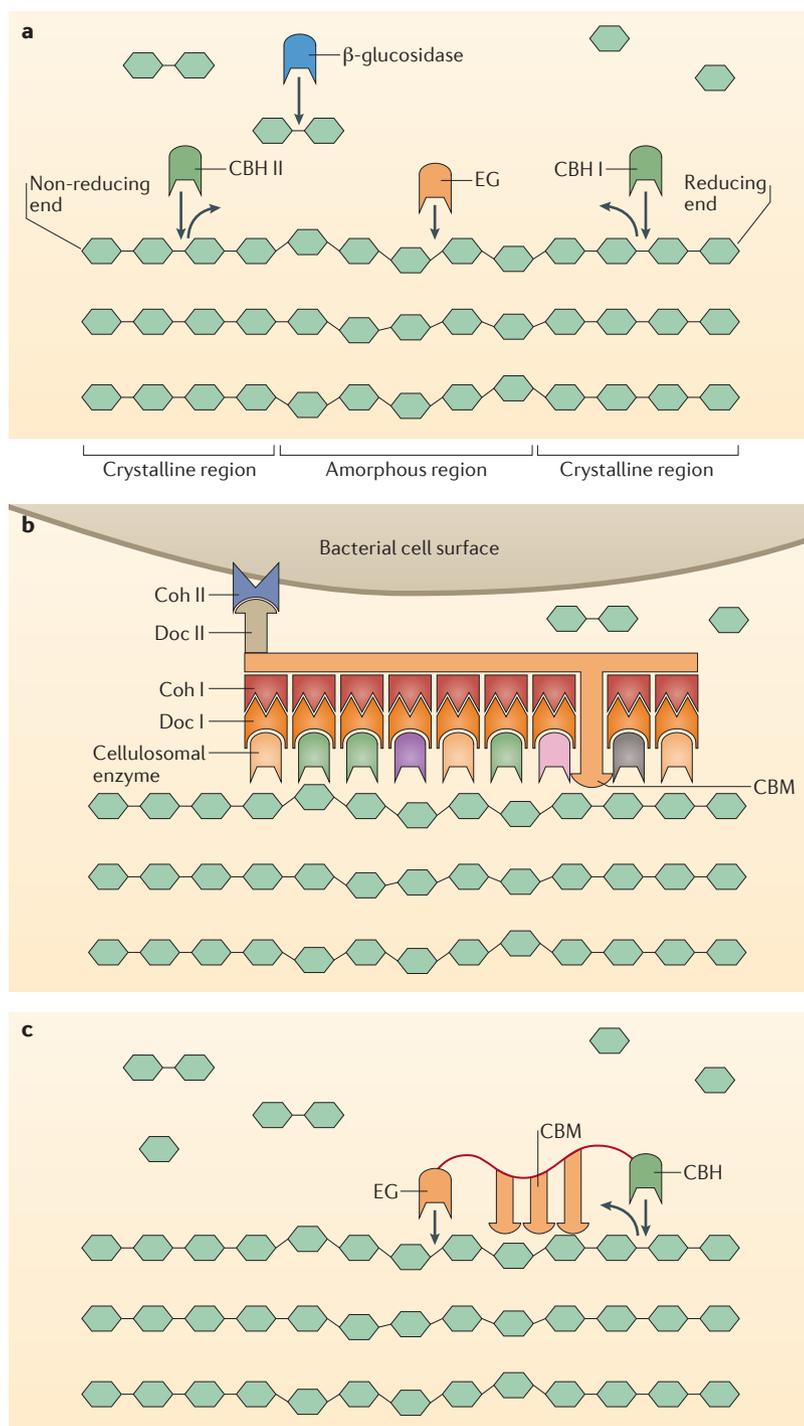


Figure 2 | Three modes of cellulose degradation in different organisms.

a | A simplified mechanism of action for fungal non-complexed cellulases. Cellobiohydrolases (CBHs) are exoglucanases that cleave disaccharide units from the reducing or non-reducing end (type I or type II enzymes, respectively) of the cellulose molecule. β -glucosidase cleaves the disaccharides into glucose monomers. Endoglucanases (EGs) cleave bonds in the amorphous region of cellulose, generating ends that act as CBH substrates. **b** | The interaction of the *Clostridium thermocellum* cellulosome with cellulose polymers. Cohesin modules (Coh) in the cellulosomal scaffoldin (CipA) and on the bacterial cell surface bind to dockerin modules (Doc) in the scaffoldin and the cellulases, forming a large enzymatic complex that binds to cellulose through the scaffoldin carbohydrate-binding module (CBM). **c** | The mechanism of action for the bifunctional CelA protein of *Caldicellulosiruptor bescii*, which contains both EG and CBH modules as well as three CBMs.

cleavage to save energy⁴⁰. When used in combination, cellulosomes and non-complexed cellulases exhibit synergistic effects⁴¹ because they act through different mechanisms: non-complexed enzymes penetrate into cell walls and hydrolyse exposed microfibrils, whereas complexed enzymes have been reported to act by a peeling action⁴², exposing new reactive surfaces as solubilization proceeds.

The digestion mechanism of the anaerobic cellulolytic bacterium *Caldicellulosiruptor bescii* represents an interesting intermediate strategy between fungal non-complexed cellulases and clostridial cellulosomes (FIG. 2c). The most crucial and abundant enzyme for *C. bescii* cellulose degradation is CelA⁴³. This enzyme has three carbohydrate-binding modules and two catalytic domains⁴⁴, one with endoglucanase activity and the other with exoglucanase (cellobiohydrolase) activity. It has been demonstrated that the single enzyme CelA outperforms a mixture of fungal endoglucanase and exoglucanase⁴⁴, which suggests that there is a benefit to having complementary catalytic units within the same enzyme molecule. However, the potential advantages of bacterial cellulose deconstruction remain to be realized industrially, and the current cellulosic biofuel plants all use fungal cellulases.

In addition to cellulose, lignocellulose also includes hemicellulose and lignin. Unlike the ordered structure of cellulose, the structures of hemicellulose are much more variable. Despite this structural diversity, the key catalytic modules of hemicellulases are either glycoside hydrolases, used for breaking glycosidic bonds, or carbohydrate esterases, used for side group hydrolysis⁴⁵. A portion of hemicellulose can be hydrolysed using pretreatment methods²², such as dilute acid pretreatment. Furthermore, most organisms that are capable of cellulose degradation also express hemicellulases (such as xylanases, xylosidases, arabinofuranosidases, mannanases, and so on). Among the cellulolytic organisms, *C. thermocellum* represents an interesting case: this species has several hemicellulases in its enzyme repertoire, but the organism itself is unable to metabolize the pentoses released from hemicellulose hydrolysis⁴⁶. Similarly, the main industrial ethanol producers, *Saccharomyces cerevisiae* and *Zymomonas mobilis*⁴⁷, cannot utilize xylose, the major pentose in plant biomass, as a carbon source for biofuel production. In an attempt to circumvent this problem, *S. cerevisiae*⁴⁸ and *Zymomonas* spp.⁴⁹ have been engineered to utilize xylose, with varying degrees of success. However, simultaneous, efficient and complete utilization of hexose (which results from cellulose degradation) and pentose (which results from hemicellulose degradation) remain a challenge because all known D-xylose transporters are competitively inhibited by D-glucose⁵⁰. Mutants that are insensitive to the inhibition⁵⁰ have been isolated and may help solve this problem.

The third main component of lignocellulose, lignin, constitutes 20–35% of plant biomass. Lignin can be combusted to generate heat and electricity or gasified to syngas for further use. Converting lignin to fuel⁵¹ or value-added products is challenging because of its structural diversity and product heterogeneity, which increase the cost of both conversion and product purification. Several chemical

approaches exist for lignin conversion⁵². Biological transformation depends on ligninolytic enzymes, including laccases and peroxidases⁵³, which are oxidative enzymes that are native to a large diversity of fungal hosts. Furthermore, several species of bacteria, such as *Pseudonocardia autotrophica* and *Comamonas testosteroni*, have been identified as producers of ligninolytic enzymes⁵⁴. In bacteria, the products of aromatic catabolic pathways typically form compounds, such as catechol or protocatechuate, as central intermediates⁵⁵. Therefore, improved utilization of lignin as a substrate in these pathways will probably stem from the ability to channel these catabolic intermediates into the specific metabolic pathways that are used for the production of chemicals⁵⁶. As the lignin conversion process is likely to be complex and challenging, the products that are generated by this process need to justify the cost to make economic sense.

Direct CO₂ utilization. An alternative to using biomass for biofuel production is to explore the ability of microorganisms to carry out the direct conversion of CO₂ into energy-dense liquid fuels. Phototrophic organisms, such as eukaryotic microalgae and prokaryotic cyanobacteria, can fix CO₂ with reducing power harnessed from sunlight, and these organisms can be metabolically engineered to produce fuel compounds directly (FIG. 1b).

The capacity of many microalgal species to produce large amounts of lipids (up to 70% lipid per dry weight)⁵⁷ as storage products makes microalgal biomass an attractive resource for the production of biodiesel. Bioprospecting for algal strains with high lipid accumulation as well as superior growth and harvesting characteristics can provide suitable candidates⁵⁸. Furthermore, modern approaches, such as next-generation sequencing⁵⁹ and RNAi⁶⁰, have provided insights into algal physiology and metabolism⁶¹ that can be explored for the production of biofuels. For example, RNA-sequencing technologies have helped to identify genes that are involved in the accumulation of triacylglycerol under conditions of nitrogen deprivation⁵⁸, thus providing potential targets for the engineering of strains with a high level of lipid production. Gene editing techniques have also enabled the engineering of cyanobacteria, which are genetically more tractable than eukaryotic microalgae and therefore more versatile for the direct production of fuels and chemicals. For example, introducing exogenous biosynthetic pathways into *Synechococcus elongatus* PCC 7942 or *Synechocystis* sp. PCC 6803 has led to the production of various compounds directly from CO₂ (REF. 62).

One major challenge for producing photosynthesis-derived biofuels is to provide large light-exposing surfaces⁶³. Although open-pond cultivation systems are inexpensive, they need large areas of land and have problems with water loss, contamination and controlling the culture conditions^{64,65}. By contrast, closed photobioreactors are costly for biofuel production⁶⁴. In addition, the photosynthetic apparatus can use radiation within only a limited spectrum and intensity range, thus limiting photosynthetic microorganisms to only ~50% of the incident solar energy. Furthermore, the photosynthetic apparatus

is photo-inhibited above a certain light intensity⁶⁶, and high temperatures and salt concentrations also present problems. *Synechococcus* sp. PCC 7002 displays favourable properties in tolerating heat and salts⁶⁷. In general, biofuels that are derived directly from photosynthesis still need to compensate for many of the unique problems originating from light capturing and distribution to be economically viable.

Another option is to use electricity and lithoautotrophs to convert CO₂ into liquid 'electrofuels' (REF. 68) (FIG. 1c). Owing to the higher efficiency of photovoltaic technologies compared with biological photosynthesis⁶⁹, electrofuels could have better sun-to-fuel efficiency than photosynthesis-derived biofuels, in theory. Furthermore, the separation of light and dark reactions circumvents the expensive cultivation systems that are required for photosynthetic microorganisms. Moreover, as solar or wind-generated electricity is intermittent, the conversion of such electricity to liquid biofuel is the most energy-dense approach for long-term energy storage⁷⁰ (BOX 1).

A broad range of lithoautotrophic microorganisms are capable of fixing CO₂ with reducing power from electron shuttles, such as H₂ and formic acid. H₂ can be produced on cathodes from water splitting, whereas formic acid can be synthesized electrochemically using water and CO₂. Lithoautotrophic organisms, such as *Cupriavidus necator* (formerly known as *Ralstonia eutropha*), have been engineered to produce biofuels from CO₂ using H₂ (REF. 71,72) or formic acid⁷¹⁻⁷³. Similarly, the H₂-utilizing heterotroph *Pyrococcus furiosus* has been engineered⁷⁴ to incorporate CO₂ using a portion of the 3-hydroxypropionate/4-hydroxybutyrate CO₂ fixation pathway from *Metallospira sedula* to produce 3-hydroxypropionic acid. However, the low solubility of H₂ and low mass transfer rate in these processes may increase the cost of biofuel production.

Alternatively, some autotrophic microorganisms, such as *Geobacter* spp., can directly accept electrons from cathodes for anaerobic respiration⁷⁵. Recently, a wide range of acetogenic microorganisms, including *Sporomusa* spp., *Clostridium* spp. and *Moorella thermoacetica*, have been shown to be capable of accepting electrons from a cathode and fixing CO₂ into acetate and a small amount of 2-oxobutyrate^{76,77}, indicating their potential to produce biofuel from CO₂ with direct electron feeding. However, bioreactors with large electrode surfaces are required to establish this process, and this will inevitably increase the cost associated with producing biofuels using this strategy.

Methane utilization. Methane can be produced from landfill or by anaerobic digestion of various organic wastes and is also the major component in natural gas, the production of which has undergone a dramatic surge in the past decade. Unfortunately, a substantial number of natural gas wells do not generate a high enough output for use, and the gas product is flared to CO₂ or leaked out. As methane is a more potent GHG than CO₂ (REF. 12), there is increasing pressure to find ways of more efficiently using methane as a carbon source. Microbial methane oxidation acts as the only known biological sink of methane, as methanotrophs limit the release of methane from many methanogenic areas⁷⁸.

Microfibrils

Fine fibre-like strands formed of multiple cellulose polymers that are aligned in parallel via hydrogen bonding, creating structural crystallinity and mechanical strength.

Water splitting

A chemical reaction that separates water molecules into O₂ and H₂.

3-hydroxypropionate/ 4-hydroxybutyrate CO₂ fixation pathway

A cyclic pathway that was discovered in the archaeon *Metallospira sedula* and uses 3-hydroxypropionate and 4-hydroxybutyrate as key intermediates to convert CO₂ into acetyl-CoA.

Acetogenic

Able to produce acetate as a major metabolite in anaerobic respiration.

The conversion of methane into liquid biofuels, such as ethanol or 1-butanol, is thermodynamically favourable and can be accomplished through chemical or biological methods. Chemical methods, such as the Fischer–Tropsch process, have high capital costs, as they are energy intensive processes that utilize high temperatures and pressures⁷⁹. By contrast, biological methods (FIG. 1 d) first require the oxidation of methane to methanol under physiological conditions. In all methane-assimilating microorganisms, this oxidation step is carried out using methane monooxygenase (MMO). MMO exists in two forms: soluble MMO (sMMO) and membrane-bound particulate MMO (pMMO). Most methanotrophs accommodate pMMO by producing internal cytoplasmic membrane stacks⁸⁰. sMMO is a unique member of a family of bacterial monooxygenases that are able to oxidize methane, and the reaction mechanism of this enzyme has recently been elucidated⁸¹.

The application of methanotrophs as platforms for biofuel production requires three main obstacles to be overcome: the efficiency of methane activation must be improved, alternative pathways for the production of liquid fuels must be found and productivity within large-scale bioreactors must be increased⁷⁹. For example, MMO oxidizes methane to methanol in the first step of methane assimilation, but requires a high-energy electron donor, such as NADPH, as an energy input to functionalize the otherwise inert methane molecule. Although this step is thermodynamically favourable and highly exothermic, no energy output is generated, resulting in the loss of approximately 40% of the energy within the methane molecule⁷⁹. This large energy loss can, in principle, be addressed by the direct engineering of enzymes for alternative mechanisms of oxidizing methanol⁷⁹. These envisioned methane-activation strategies may use a dioxygenase-like enzyme that is capable of oxidizing two methane molecules simultaneously. However, such an enzyme has not been found or engineered successfully to date.

The oxidized product of methane, methanol, is used by methanotrophs and methylotrophs through the ribulose monophosphate (RuMP) pathway, the serine cycle or the assimilation of CO₂ through the Calvin cycle after its complete oxidation (BOX 2). The recently demonstrated methanol condensation cycle⁸² illustrates how pathways can be optimized for biofuel production from methanol.

Finally, finding appropriate conditions for cultivating methanotrophs is another challenge that has yet to be met. One intrinsic problem is the low solubility of methane, as well as poor kinetics of the enzymes that metabolize methane⁸³. These problems need to be solved to make biofuel production from methane viable in an industrial setting.

Biofuel production pathways

Following the saccharification of biomass or the assimilation of carbon from CO₂ or methane, these raw materials are metabolized to either pyruvate or acetyl-CoA. These are common starting metabolites from which the biofuel ethanol can be directly produced. Several pathways can increase the carbon chain length and convert

the metabolites to other fuel compounds, including the keto acid pathway, CoA-dependent reverse β -oxidation, fatty acid biosynthesis and the isoprenoid pathway (FIG. 3). Some longer-chain alcohols, esters and alkanes have higher energy densities and are more compatible with the existing infrastructure than ethanol. Very roughly, C₈ compounds are suitable for substituting gasoline, C₁₂ compounds for aviation fuel and C₁₆ compounds for diesel, but other fuel properties need to be considered.

Although parts of these metabolic pathways may exist in some microorganisms, few have a complete pathway or can synthesize the desired compound efficiently. In principle, any pathway can be engineered into any host to produce the desired fuel, but the challenge is to increase the titre, yield and productivity associated with biofuel production.

Ethanol pathways. In microorganisms, ethanol can be produced from pyruvate decarboxylation with or without going through acetyl-CoA. For example, *S. cerevisiae* and *Z. mobilis*, the two most productive ethanol producers, directly decarboxylate pyruvate, followed by reduction to ethanol, without using acetyl-CoA. By contrast, most other microorganisms, including *Escherichia coli* and *Clostridium* spp., use CoA to activate the acyl group during pyruvate decarboxylation, before reduction to ethanol. This difference implies that direct decarboxylation is a more efficient route, and this direct pathway has been engineered into many other organisms.

Importantly, the production strain must be robust against several challenges in industrial settings, such as high ethanol concentration⁸⁴, high temperature⁸⁵ and high acid levels⁸⁶, and must be resistant to contamination in minimally sterilized conditions. Compared with *S. cerevisiae*, *Z. mobilis* is more tolerant to ethanol⁸⁷. In addition, *Z. mobilis* can fix nitrogen⁸⁸, achieving higher specific ethanol production rates and lower cell mass yields when N₂ is used as the nitrogen source rather than ammonium⁸⁸. However, *S. cerevisiae* is more tolerant to acid, insensitive to phage infection and more robust against contamination; indeed, most industrial producers use *S. cerevisiae* because of its robustness under industrial conditions.

Another strategy to produce biofuel, termed consolidated bioprocessing⁸⁹, is to achieve cellulose hydrolysis and fuel production in one reactor without enzyme supplementation, using one or several organisms (FIG. 1 a). The cellulolytic thermophile *C. thermocellum* is one of the leading candidates for consolidated bioprocessing owing to its rapid cellulolytic function and growth rate. *C. thermocellum* has a non-conventional glycolytic pathway⁹⁰ with no identified pyruvate kinase. Instead, pyruvate is produced through a malate shunt, and *C. thermocellum* conserves energy using electron-bifurcating enzymes^{91,92}. A less characterized cellulolytic thermophile, *C. bescii*, has also emerged as a potential candidate for consolidated bioprocessing⁹³, owing to its impressive rate of biomass deconstruction. This organism is not naturally ethanogenic, but has been engineered to produce ethanol (yielding 700 mg ethanol l⁻¹); this was achieved by knocking out the competing lactate dehydrogenase and

Acetyl-CoA

An acetyl group linked to CoA by a thioester bond; this molecule acts as the acetyl group donor in cells.

Keto acid

An organic acid that contains a ketone group.

Malate shunt

A pathway that converts phosphoenolpyruvate to pyruvate through oxaloacetate and malate; in this pathway, the reducing equivalent NADH is converted to NADPH.

Electron-bifurcating enzymes

Multi-enzyme complexes that were recently discovered in anaerobic microorganisms and are capable of coupling a thermodynamically unfavourable redox reaction with a thermodynamically favourable one.

expressing a bifunctional acetylaldehyde–alcohol dehydrogenase from *C. thermocellum*⁹⁴. Similarly, *T. reesei* has also been engineered to produce ethanol (yielding about 10 g ethanol l⁻¹), through several rounds of genome shuffling and strain selection⁹⁵. Finally, instead of engineering native cellulolytic organisms to produce ethanol, another

approach is to engineer an ethanol producer to digest cellulose. Examples include engineering *S. cerevisiae*, *E. coli* or *Z. mobilis* to digest cellulose through the expression of heterologous cellulolytic enzymes^{96,97}. However, much work is still required to bring biofuel production by these engineered microorganisms to an industrial level.

Box 2 | Biological methane and methanol conversion

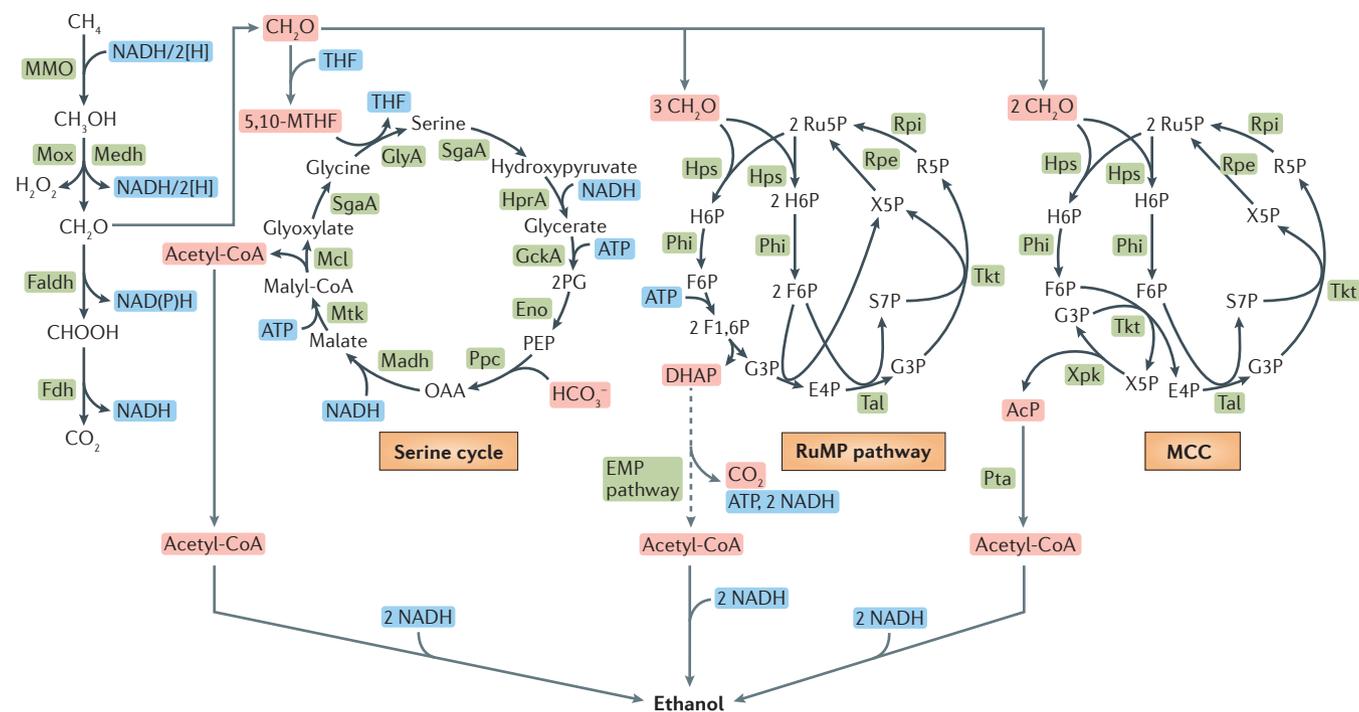
In aerobic methane-assimilating microorganisms, methane oxidation is carried out using methane monoxygenase (MMO) (see the figure). The product, methanol (CH₃OH), is further oxidized to formaldehyde (CH₂O) using pyrroloquinoline quinone (PQQ)-dependent¹⁶⁸ or NAD-dependent¹⁶⁹ methanol dehydrogenase (Medh), or methanol oxidase (Mox)¹⁷⁰. Methanotrophs are able to assimilate formaldehyde by C₁ assimilation pathways, including the serine cycle¹⁷¹, the ribulose monophosphate (RuMP) pathway¹⁷² and the recently engineered methanol condensation cycle (MCC)⁸² (see the figure). Alternatively, formaldehyde can be oxidized completely to CO₂ then assimilated through the Calvin cycle¹⁷³.

The serine cycle¹⁷¹ assimilates formaldehyde using 5,10-methylene tetrahydrofolate (5,10-MTHF) as an intermediate to condense with glycine and generate serine using a serine hydroxymethyltransferase (SHMT), GlyA. Conversion of serine to phosphoenolpyruvate (PEP) and the subsequent carboxylation of PEP generates the C₄ compound oxaloacetate (OAA). OAA can then be converted into malyl-CoA and split to ultimately yield glycine, which is used to regenerate the cycle, and acetyl-CoA, which is used for cell growth¹⁷¹. This cycle requires both ATP and NADH, which must come from additional oxidation of reduced carbon to CO₂.

In the RuMP pathway¹⁷², formaldehyde is condensed with ribulose-5-phosphate (Ru5P) to generate fructose-6-phosphate (F6P) using hexulose-6-phosphate (H6P) as an intermediate. This F6P is then

converted into the glycolytic intermediate, dihydroxyacetone phosphate (DHAP), which is then converted to acetyl-CoA through the Embden–Meyerhof–Parnas (EMP) pathway or Entner–Doudoroff (ED) pathway. This cycle is useful for growth, but it requires ATP and loses one carbon in the decarboxylation of pyruvate to acetyl-CoA.

The recently engineered MCC⁸² addresses the issue of carbon loss by using the key enzyme X5P phosphoketolase (Xpk), which splits xylulose-5-phosphate (X5P) into acetyl-phosphate and glyceraldehyde-3-phosphate (G3P). MCC functions as a combination of the RuMP cycle and non-oxidative glycolysis (NOG)¹⁵⁹ (see BOX 3), but avoids the use of ATP⁸². Similarly to the RuMP cycle, it also uses Ru5P to assimilate formaldehyde, but the G3P generated by Xpk is carbon rearranged to regenerate Ru5P. The intermediate acetyl-phosphate (AcP) can be converted to acetyl-CoA and then to ethanol or 1-butanol. The stoichiometric amount of NADH gained from methanol oxidation by Medh balances the reducing power required to reduce acetyl-CoA to ethanol or butanol. The net reaction is the condensation of two methanol molecules to one ethanol molecule, or four methanol molecules to one 1-butanol molecule, independently of ATP or reducing power. This pathway avoids the decarboxylation of pyruvate and achieves complete carbon conservation in converting methanol to ethanol or 1-butanol with the loss of only water. Complete carbon conservation from feedstock to biofuel is important both because it decreases the raw material cost and reduces the emission of greenhouse gas.



E4P, erythrose-4-phosphate; Eno, enolase; F1,6P, fructose-1,6-bisphosphate; Faldh, formaldehyde dehydrogenase; Fdh, formate dehydrogenase; GckA, glycerate-2-phosphate kinase; HprA, hydroxypyruvate reductase; Hps, H6P synthase; Madh, malate dehydrogenase; Mcl, malyl-CoA lyase; Mtk, malate thiokinase; PG, phosphoglycerate; Phi, phosphohexulose isomerase; Ppc, PEP carboxylase; Pta, phosphate acetyltransferase; R5P, ribose-5-phosphate; Rpe, R5P epimerase; Rpi, R5P isomerase; S7P, sedoheptulose-7-phosphate; SgaA, serine–glyoxylate aminotransferase; Tal, transaldolase; Tkt, transketolase.

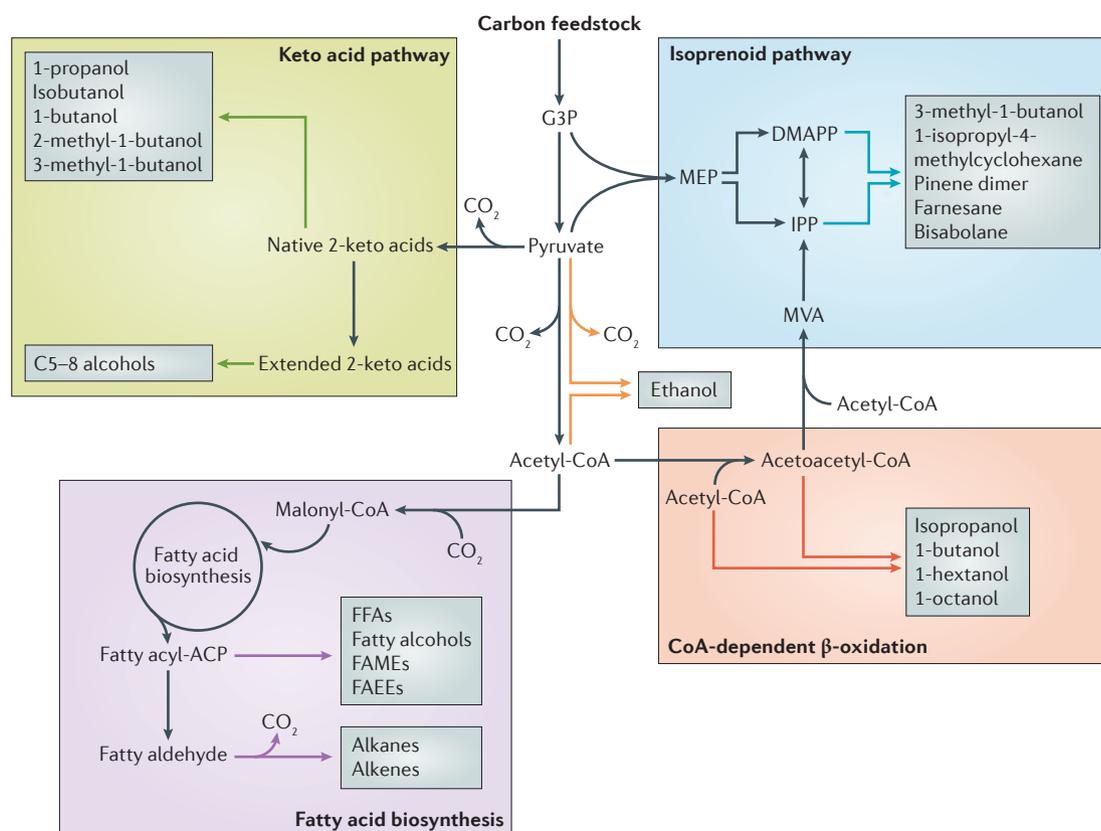


Figure 3 | **Biosynthetic pathways of biofuels.** Ethanol is produced from either pyruvate or acetyl-CoA (orange arrows), with acetaldehyde as a common intermediate. The keto acid pathway (green arrows) can be used to produce both branched and straight-chain alcohols. It uses parts of amino acid biosynthesis pathways for keto acid chain elongation. This is followed by decarboxylation and reduction of the keto acid, analogous to the conversion of pyruvate to ethanol. Fatty acid synthesis (purple arrows) extends acyl-acyl carrier proteins (ACPs) in a cyclical manner, using malonyl-CoA as a precursor. Fatty acyl-ACPs may be converted into free fatty acids (FFAs) with acyl-ACP thioesterase. FFAs can be esterified to esters, such as fatty acid methyl esters (FAMEs) or fatty acid ethyl esters (FAEEs), reduced to fatty alcohols, or reduced to fatty aldehydes followed by decarbonylation to alkanes and alkenes. The CoA-dependent pathway (red arrows) uses reverse β -oxidation chemistry for the production of higher alcohols or decarboxylation of the precursor acetoacetyl-CoA for the production of isopropanol. Isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), the universal precursors of isoprenoid biofuel biosynthesis (blue arrows), may be produced either through the mevalonate (MVA) or methylerythritol-4-phosphate (MEP) pathway. G3P, glyceraldehyde-3-phosphate.

Keto acid pathways. Analogous to the decarboxylation of pyruvate (a keto acid) for ethanol production in *S. cerevisiae* and *Z. mobilis*, any longer-chain keto acid can be decarboxylated and reduced to longer-chain alcohols^{98,99} (FIG. 3). This strategy involves chain elongation at the level of 2-keto acids, which are used in branched chain amino acid biosynthesis. Keto acid chain elongation starts through acetohydroxyacid synthase in the valine biosynthesis pathway, or isopropylmalate synthase in the leucine biosynthesis pathway, followed by isomerization, reduction (or oxidation) and dehydration to regenerate the 2-keto acid functional group. For example, alcohols, such as isobutanol, 1-butanol, 2-methyl-1-butanol, 3-methyl-1-butanol and 2-phenylethanol, can be produced in *E. coli* after expressing keto acid elongation enzymes and keto acid decarboxylation enzymes⁹⁸. In particular, an *E. coli* strain (Δadh , Δldh , Δfrd , Δfnr , Δpta , $\Delta pflB$) was engineered to produce isobutanol at a high titre (22 g isobutanol l⁻¹, which represents 86% of the theoretical yield)⁹⁸ by overexpressing several enzymes:

2-hydroxy-3-ketol-acid reductoisomerase (encoded by *ilvC*), dihydroxy-acid dehydratase (encoded by *ilvD*) and alcohol dehydrogenase (encoded by *yqhD*), which are native to *E. coli*, and two heterologous enzymes, catabolic acetolactate synthase (encoded by *alsS* from *Bacillus subtilis*) and ketoisovalerate decarboxylase (encoded by *kivD* from *Lactococcus lactis*). Isobutanol is currently being commercialized as a biofuel using this pathway.

Additional efforts can further increase the yield of isobutanol production under anaerobic conditions, including the engineering of ketol-acid reductoisomerase and alcohol dehydrogenase¹⁰⁰. Isobutanol has also been produced from sugar in other engineered organisms, such as yeast (yielding 0.23 g l⁻¹)¹⁰¹ and *B. subtilis* (yielding 2.62 g l⁻¹)¹⁰², from cellulose using *C. thermocellum* (yielding 5.4 g l⁻¹)¹⁰³ and from cellobiose using *E. coli* (yielding 8 g l⁻¹)¹⁰⁴. The advantage of these keto acid pathways is their compatibility with many organisms, as they build on the amino acid biosynthesis pathways already present and can be expanded in several ways¹⁰⁵. However, because

Acyl carrier protein
A protein molecule that transfers the acyl group during fatty acid biosynthesis.

these pathways have been developed only recently, there are less established markets for the commercialization of their products.

CoA-dependent reverse β -oxidation. CoA-dependent reverse β -oxidation is a pathway that uses the same chemistry as β -oxidation for fatty acid degradation, but in the reverse direction. In contrast to fatty acid biosynthesis, this pathway uses CoA as opposed to acyl carrier protein (ACP) to activate the acyl group. The pathway is used to produce primary alcohols, such as 1-butanol and 1-hexanol, or the secondary alcohol isopropanol in many *Clostridium* spp. In this pathway, two acetyl-CoA molecules are condensed to produce acetoacetyl-CoA, which then reverse β -oxidizes^{106,107} to produce 1-butyryl-CoA. This acyl-CoA can then be reduced to produce 1-butanol¹⁰⁶, or go through the cycle again to increase the chain length by two carbon atoms in each cycle before reduction to 1-hexanol, 1-octanol or even longer-chain alcohols¹⁰⁷. Acetoacetyl-CoA can be converted to isopropanol after decarboxylation¹⁰⁸.

CoA-dependent reverse β -oxidation has also been engineered into several organisms that do not possess this pathway, such as *E. coli* (yielding 15 g butanol l⁻¹)¹⁰⁶, *S. cerevisiae* (yielding 0.0025 g butanol l⁻¹)¹⁰⁹, *B. subtilis* (yielding 0.024 g butanol l⁻¹)¹¹⁰, *Pseudomonas putida* (yielding 0.05 g butanol l⁻¹)¹¹⁰ and *S. elongatus* (yielding 0.3 g butanol l⁻¹)¹¹¹.

Notably, CoA-dependent reverse β -oxidation also enables the production of odd-chain alcohols, as acetyl-CoA can be condensed with propionyl-CoA if a source of propionate is available¹¹². Propionyl-CoA can be synthesized through ketobutyrate^{113,114}, which is a metabolite downstream of the threonine biosynthesis pathway, or through the 1,2-propanediol pathway¹¹⁵. However, the production of odd-chain alcohols loses redox potential, which results in lower yields, unless properly balanced by pairing a redox-deficient pathway with a redox-surplus pathway¹¹⁴, whereas the production of even-chain alcohols from carbohydrates is redox balanced. Therefore, in general, the production of even-chain alcohols from carbohydrates is more efficient than the production of odd-chain alcohols because it does not waste redox equivalents.

The production of acetone, 1-butanol and ethanol (ABE fermentation) by *Clostridium acetobutylicum*^{116,117} was one of the largest industrial fermentation processes in the early twentieth century. However, when the 1-butanol pathway was introduced into *E. coli*, the butyryl-CoA dehydrogenase–electron-transferring flavoprotein (Bcd–Etf) complex (a bifurcating enzyme⁹¹ involving both ferredoxin and NADH as electron mediators) was poorly expressed. To overcome this problem, an NADH-dependent enzyme, *trans*-enoyl-CoA reductase (Ter), was used^{106,118}. Further engineering to achieve accumulation of the precursor acetyl-CoA and the cofactor NADH provided more driving force¹⁰⁶ and achieved a high-titre production of 1-butanol (15 g l⁻¹, or 30 g l⁻¹ with *in situ* product removal)¹⁰⁶. Similarly, engineering *E. coli* to express *thl* (which encodes an acetyl-CoA acetyltransferase from *C. acetobutylicum*), *atoAD*

(which encodes an acetoacetyl-CoA transferase from *E. coli*), *adc* (which encodes an acetoacetate decarboxylase from *C. acetobutylicum*) and *adh* (which encodes a secondary-alcohol dehydrogenase from *Clostridium beijerinckii*) resulted in the production of isopropanol (143 g l⁻¹)¹⁰⁸ in a gas-stripping bioreactor. Furthermore, the ABE fermentation products were also upgraded by chemical catalysis¹¹⁷ to longer-chain alkanes that can be used for gasoline, jet fuel and diesel.

Finally, the CoA-dependent pathway can also be used to produce acids by hydrolysis of the CoA-acylated intermediates, generating butyric acid¹¹⁹ and hexanoic acid¹²⁰. These acids can be transformed to fuel compounds by hydrogenation if the cost can be justified. The CoA-acylated compounds in this pathway may also be condensed with alcohols produced from various pathways to form esters, through the expression of alcohol O-acyltransferase¹⁰⁵.

Fatty acid biosynthesis. Fatty acid biosynthesis uses similar chemistry to β -oxidation for fatty acid degradation, but uses ACP for acyl group activation. In mammals and yeast, cytosolic fatty acid synthesis is mediated through one or two multidomain, multifunctional proteins, termed type I fatty acid synthases (FASs). By contrast, in most bacteria and in the mitochondria and chloroplasts of eukaryotic cells, fatty acid synthesis is catalysed by dissociated, individual gene products, termed type II FASs. Fatty acid synthesis is typically initiated by malonyl-CoA, which is transacylated to malonyl-ACP. The fatty acid chains are extended in a cyclical manner using ACP-activated intermediates. In mammals, free fatty acids (FFAs) are formed from fatty acyl-ACPs using fatty acyl-ACP thioesterases. In yeast, the final acyl-ACP product (usually C16:0) of cytosolic FAS is transferred to CoA by an intrinsic transferase reaction centre and released as palmitoyl-CoA¹²¹. In bacteria, acyl-ACP is directly transferred to lipids by an acyl-transferase.

The use of acyl compounds as biofuels requires that they be esterified to esters, decarboxylated to alkanes or alkenes, or reduced to fatty alcohols¹²² (FIG. 3). The synthesis and degradation of fatty acids are tightly regulated^{123–125} and represent key targets for metabolic engineering. For example, *E. coli* fatty acid biosynthesis is subject to feedback inhibition by acyl-ACP, but this inhibition can be released by the overexpression of thioesterase¹²⁶, which results in the production of FFAs. These FFAs can then be converted to acyl-CoA by acyl-CoA ligase. Acyl-CoA can be converted to fatty alcohols by fatty acyl-CoA reductases, or to fatty acid ethyl esters (FAEEs) by ester synthase, using endogenously synthesized ethanol¹²².

FAEEs are a desirable product because they can act as potential diesel substitutes. Furthermore, the chain length of FAEEs can be controlled by expressing different thioesterases with different substrate specificities¹²². *E. coli* has been engineered to produce FAEE (yielding 680 mg l⁻¹, which represents 9.4% of the theoretical yield) with an organic phase overlay to prevent evaporation¹²². Fatty acyl-ACP or FFAs can be further converted to alkenes and alkanes. For example, an *E. coli* strain was engineered to produce alkanes and alkenes with length

C_{13-17} (yielding approximately 80 mg l^{-1})¹²⁷ from acyl-ACP. This strain uses heterologous expression of a cyanobacteria acyl-ACP reductase, which reduces a saturated or unsaturated fatty acyl-ACP to the corresponding aldehyde, and an aldehyde decarbonylase, which splits the aldehyde to alkane (or alkene) and CO ¹²⁷. Alternatively, alkanes have been produced from FFAs in *E. coli* (yielding 6 mg l^{-1}) using the fatty acid reductase (FAR) complex, encoded by the genes *luxC*, *luxD* and *luxE* from the bioluminescent bacterium *Photobacterium luminescens*, followed by decarbonylation of the resultant fatty aldehydes using aldehyde decarbonylase (NpAD) cloned from *Nostoc punctiforme*¹²⁸.

Appropriate gasoline substitutes include medium-chain hydrocarbons (C_{4-12}), but microbial fatty acid production typically generates longer (C_{14-18}) fatty acids. Two strategies have been reported for reducing hydrocarbon chain length in *E. coli*: inducible degradation of an essential ketoacyl synthase¹²⁹, and the use of a *tesA* mutant¹³⁰ that has shifted activity toward medium-acyl-chain substrates. In the first strategy¹²⁹, an *E. coli* ketoacyl synthase (encoded by *fabF*) that is responsible for chain elongation was mutated to limit its activity range to make C_8 acyl-ACP. Simultaneously, another ketoacyl synthase (encoded by *fabB*) was engineered to be degraded on the addition of an inducer. This strategy produced approximately 260 mg l^{-1} of total FFAs in *E. coli*¹²⁹. In the second strategy¹³⁰, a mutated fatty acyl-ACP thioesterase (encoded by *tesA*) was expressed to favour the conversion of C_{4-12} fatty acyl-ACPs to FFAs, which were converted to alkanes by the sequential action of *E. coli* fatty acyl-CoA synthetase, *C. acetobutylicum* fatty acyl-CoA reductase and *Arabidopsis thaliana* fatty aldehyde decarbonylase. The final engineered *E. coli* strain¹³⁰ produced up to 580.8 mg l^{-1} of medium-chain alkanes (C_{9-14}). Importantly, the production of alkanes or alkenes from highly oxygenated raw materials, such as carbohydrates, suffers from the intrinsic loss of molecular mass, which results in low theoretical yields.

Certain species of yeast, such as *Yarrowia lipolytica*^{131,132}, are capable of accumulating lipids to levels in excess of 70% of their biomass. As biosynthetic enzymes that are involved in the synthesis of saturated fatty acids are subjected to feedback inhibition, one strategy to increase yields is to convert saturated fatty acids into monounsaturated fatty acids¹³³ while sequestering pathway products in a fatty acid sink. Overexpression of acetyl-CoA carboxylase (ACC1), diacylglyceride acyl-transferase (DGA1) and $\Delta 9$ stearoyl-CoA desaturase (SCD; a regulatory enzyme that catalyses the desaturation of palmitoyl-CoA and stearoyl-CoA to palmitoleoyl-CoA and oleoyl-CoA, respectively), resulted in high lipid accumulation. The engineered strain achieved lipid titres of 55 g l^{-1} , which is 84.7% of the theoretical maximum yields, with a high productivity ($1 \text{ g l}^{-1} \text{ h}^{-1}$ during the stationary phase). Similarly, a strain of *Y. lipolytica* has been engineered for fast growth and lipid overproduction by reverse engineering, based on mammalian cellular obese phenotypes¹³³. In short, stearoyl-CoA desaturase was hypothesized to have a key regulatory role in fat accumulation, on the basis of an analysis of published

mammalian transcriptome data, and the corresponding gene was overexpressed in *Y. lipolytica* to create the obese phenotype in yeast. In general, overcoming intricate cellular regulation has proved to be one of the major challenges in the overproduction of fatty acids¹²⁵.

Isoprenoid pathways. Another route for carbon chain elongation is through the isoprenoid pathways, which increase the chain length by multiples of five through addition of the pyrophosphate-activated isopentenyl pyrophosphate (IPP) to dimethylallyl pyrophosphate (DMAPP) (FIG. 3). After chain elongation to geranyl pyrophosphate (GPP) or farnesyl pyrophosphate (FPP), the isoprenoid compounds are then modified by various terpene synthases or other enzymes to generate the final compound. IPP is produced either through the mevalonate (MVA) pathway, which exists in mammals, yeasts and plant cytosol, or the methylerythritol-4-phosphate (MEP) pathway, which is found in bacteria and plant plastids¹³⁴. The MVA pathway initiates from three acetyl-CoA molecules, whereas the MEP pathway begins with pyruvate and glyceraldehyde-3-phosphate. As acetyl-CoA is derived from pyruvate with a carbon loss, the MVA pathway is intrinsically less efficient than the MEP pathway in terms of carbon yield. Nonetheless, the MVA pathway from *S. cerevisiae* has been engineered in *E. coli*^{135,136} to provide dual routes to the same products. Furthermore, the overexpression of *nudB* (encoding a Nudix hydrolase) and *nemA* (encoding a reductase in the old yellow enzyme family)¹³⁷ were used to convert the C_5 intermediates IPP and DMAPP to isopentenols and isopentanol. The production of these alcohols in *E. coli* has been accomplished using either the MVA pathway¹³⁸ or the MEP pathway¹³⁹. The MVA pathway achieved yields of 2.23 g l^{-1} of 3-methyl-3-buten-1-ol (which corresponds to approximately 70% of pathway-dependent theoretical yield), 150 mg l^{-1} of 3-methyl-2-buten-1-ol and 300 mg l^{-1} of 3-methyl-1-butanol, whereas the MEP pathway achieved approximately 60 mg l^{-1} of isopentanol. By contrast, 3-methyl-1-butanol was produced using the keto acid pathway in *E. coli*, yielding 4.4 g l^{-1} . A related compound, isoprene, was also produced from either cyanobacteria¹⁴⁰ or *B. subtilis*¹⁴¹ by expressing the isoprene synthase gene (*IspS*) from *Pueraria montana*.

Pinene ($C_{10}H_{16}$)¹⁴² has been produced in *E. coli* to 28 mg l^{-1} using GPP as an intermediate, by combinatorially expressing three pinene synthases and three GPP synthases (GPPSs). Pinene is of interest because pinene dimers have been shown to have a volumetric heating value similar to the jet fuel JP10 (REF. 143). Limonene ($C_{10}H_{16}$) has been produced in *E. coli* to 400 mg l^{-1} from glucose using a heterologous MVA pathway and limonene synthase¹⁴⁴. Limonene has also been produced in *Synechococcus* sp. PCC 7002 (REF. 145) to 4 mg l^{-1} .

Using FPP as a starting substrate, various sesquiterpenes can be produced by expressing the corresponding sesquiterpene synthases, including amorphadiene¹⁴⁶, bisabolene¹⁴⁷ and farnesene¹⁴⁸. Bisabolene is a desirable product because its hydrogenated product, bisabolane, has properties similar to D2 diesel fuel¹⁴⁷.

Sesquiterpenes

A class of C_{15} isoprenoid compounds described by the general chemical formula $C_{15}H_{24}$.

Bisabolene was produced in both *E. coli* and *S. cerevisiae* to titres more than 900 mg l⁻¹ by screening for an appropriate bisabolene synthase¹⁴⁷. This compound was also produced in *Streptomyces venezuelae*¹⁴⁹ and *Synechococcus* sp. PCC 7002 (REF. 145) to widen the host range.

Farnesene is being commercialized as a biofuel and can also be used in other applications, such as cosmetics, perfumes, detergents and industrial lubricants. Farnesene has been produced by *S. cerevisiae* after expressing the farnesene synthase from the plant *Artemisia annua* and overexpression of the native yeast enzymes of the MVA pathway¹⁴⁸. As farnesene has a low water solubility, high-level production of this compound is well tolerated by *S. cerevisiae*. Subsequent chemical hydrogenation yields farnesane, which can act as a biodiesel¹⁴⁸. Other attempts to increase the production of sesquiterpene include overexpression of foreign MVA pathway genes in *E. coli*¹⁵⁰, and overexpression of the catalytic domain of 3-hydroxy-3-methylglutaryl-CoA (HMG CoA) reductase in yeast¹⁵¹.

Optimizing biofuel production

Because of the low unit price and extremely large quantity required for biofuels, the production of biofuel needs to be thoroughly efficient. Every opportunity to increase yield, titre and productivity needs to be explored. Almost all metabolic engineering strategies start from overexpressing the desired pathway genes, followed by knocking out competing pathways that drain precursors, products or cofactors. Creating a new pathway for precursor supply has also been shown to be useful¹³⁵. Circumventing or deleting the native regulatory loops^{110,130} or constructing an artificial regulatory circuit^{152,153} can also be beneficial, as can global mutation and selection strategies^{85,154}.

The use of electron carriers (NADH, NADPH or reduced ferredoxin) needs to be properly balanced to streamline cofactor regeneration. For example, the change of enzyme cofactor from NADPH to NADH by an engineered enzyme enabled the anaerobic production of isobutanol at 100% theoretical yield in *E. coli*¹⁰⁰. The production of 1-butanol and 1-propanol also benefited from cofactor balancing^{106,114}, in which an NADH-producing or NADPH-producing pathway is matched by a consumption pathway using the same redox cofactor. Furthermore, in anaerobes, electron flux is often complex and undercharacterized, so understanding the electron flux in the organism in question is often the key to high-yield production^{155,156}.

Identifying the driving force for pathway flux often leads to useful strategies for increasing production^{62,106}. Efficient metabolic pathways need to be driven by both kinetic and thermodynamic driving forces, which can be manipulated through the size of the metabolite or cofactor pool. For example, in the CoA-dependent 1-butanol synthesis pathway, the thiolase reaction that condenses two moles of acetyl-CoA is thermodynamically unfavourable. In *S. elongatus* PCC 7942, this step was re-routed through malonyl-CoA¹⁵⁷ by a decarboxylative condensation to form acetoacetyl-CoA, by overexpressing *nphT7* (encoding a newly identified acetoacetyl-CoA

synthase) from *Streptomyces* sp. In this way, one mole of ATP was invested to drive the reaction, as well as the irreversible decarboxylation.

Prolonging the production phase without cell growth is a common hallmark of successful industrial biofuel production⁶. Extending the working life of cells would reduce the carbon loss for cell mass production, increase the product titre and reduce the frequency of down-time between batches of cell growth. However, because of the increased titres, product toxicity becomes a determining factor of the process¹⁵⁸. Importantly, toxicity here is defined not as toxicity to growth, but rather as toxicity to production; there are many examples of systems in which product continues to accumulate in the stationary phase⁹⁸ to a level that is much higher than that seen during cell growth, which suggests that toxicity to production (which occurs mostly during stationary phase) would be a major problem in such systems.

Most compounds of interest start from the C₂ unit acetyl-CoA in biosynthesis, with exceptions, such as isobutanol and MEP-derived isoprenoids. In most organisms, acetyl-CoA is synthesized from the C₃ metabolite pyruvate through a decarboxylation step which loses one-third of the carbon. Unless the organism can fix CO₂ through pathways such as the Wood–Ljungdahl pathway (BOX 3), this decarboxylation step limits the maximum theoretical carbon yield to 66.6% for any products that are derived from acetyl-CoA. The loss of carbon is a necessary result from glycolytic pathways, such as the Embden–Meyerhof–Parnas pathway (EMP pathway), which involve pyruvate as an intermediate in the partial oxidation of carbohydrates to gain reducing power and ATP. This loss of carbon substantially decreases the theoretical yield of desired metabolic products and simultaneously releases GHGs into the environment. In principle, the Wood–Ljungdahl pathway can be introduced into the producing organisms of interest to recapture the lost carbon, if additional reducing power can be supplied through either external electron sources or photosynthesis. However, technical difficulties remain to be solved before this approach can be realized. Alternatively, a non-oxidative glycolysis pathway (NOG pathway) or a reverse glyoxylate shunt (rGS) can also be used to conserve carbon^{159,160} (BOX 3). Indeed, the NOG pathway was engineered into *E. coli* and demonstrated the generation of acetyl-CoA through redox-neutral carbon rearrangement from sugars, thus boosting the theoretical yield of acetyl-CoA from two to three molecules per glucose¹⁵⁹.

Outlook

Although not all biofuels are sustainable, replacing fossil fuels with truly sustainable biofuels is a desirable approach to ameliorating the climate change problem, but it presents daunting challenges on multiple fronts^{9,11}. These include the efficient generation and collection of renewable resources followed by high-yield microbial conversion to desirable fuels. Every step needs to be improved to increase efficiency and reduce the direct or indirect costs to the environment and the manufacturer. These challenges are not limited to the optimization of existing technologies, but in many cases call for

Embden–Meyerhof–Parnas pathway

(EMP pathway). The most common native glycolytic pathway for converting glucose to pyruvate and thereby generating reducing equivalents and ATP; the resultant pyruvate is then converted to acetyl-CoA.

Non-oxidative glycolysis pathway

(NOG pathway). A pathway that can be constructed, through metabolic engineering, to enable the digestion of glucose or other sugars in a redox-neutral manner into acetyl-phosphate or acetyl-CoA without losing carbon to CO₂. This is in contrast to native glycolysis, which is oxidative and loses carbon to CO₂ when producing acetyl-CoA.

Reverse glyoxylate shunt

(rGS). A synthetic pathway that is designed to convert malate to acetyl-coA without carbon loss, using glyoxylate as a key pathway intermediate.

new engineering design and scientific discovery. In the short term, plant biomass seems to be the most abundant raw material and has a minimal technological barrier for commercialization. All of the processes that are required for the conversion of plant biomass are technologically feasible, albeit economically challenging. Indeed, a handful of plants producing lignocellulosic ethanol have been in operation for the past few years. Another possible raw material is methane, either from landfill or from otherwise flared natural gas. Ultimately, atmospheric CO₂ should be the carbon source for fuel production. Methane or CO₂ utilization by microorganisms still requires substantial scientific and technological improvement.

However, in many cases, a combination of chemical and microbial processes may present synergistic advantages, such as the production of electrofuels, or the chemical oxidation of methane to methanol followed by microbial methanol utilization.

In terms of a fuel compound, ethanol will still be the front runner in the near future, and possibly longer, because of both the ease of its production and the existing market. However, longer-chain fuels, such as isobutanol and fatty acid-derived and isoprenoid-derived fuels, are promising because of technological advances and better fuel properties. Some longer-chain biofuels can be used as, or converted to, aviation fuels (~C₁₂) or diesel (~C₁₆),

Box 3 | Rewiring central metabolism to achieve total carbon conservation

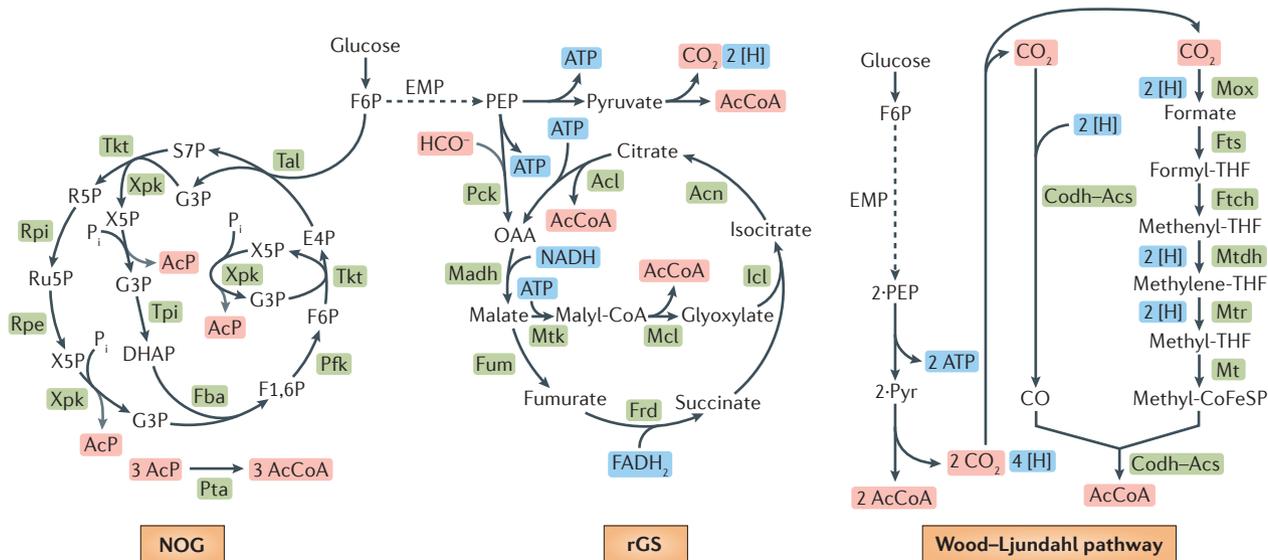
Glycolysis splits sugar to pyruvate and then to acetyl-CoA as the starting metabolite for almost all fuel compounds of interest. The conversion of pyruvate to acetyl-CoA involves decarboxylation and carbon loss in the form of CO₂ or formate. This loss of carbon substantially decreases the theoretical yield of desired fuel products. To achieve total carbon conservation between glucose and acetyl-CoA molecules, three alternatives exist: the natural Wood–Ljungdahl pathway²⁰; and two synthetic pathways, non-oxidative glycolysis (NOG)¹⁵⁹ and the reverse glyoxylate shunt (rGS)¹⁶⁰ (see the figure).

Nature's way of conserving carbon is through fixing the CO₂ released in glycolysis by the Wood–Ljungdahl pathway²⁰, which is found in acetogens and methanogens. This pathway directly fixes two CO₂ molecules to synthesize acetyl-CoA. However, the pathway involves several complex enzymes that are oxygen sensitive. Therefore, engineering this pathway remains a challenge.

NOG can occur in several modes, but each mode starts from a sugar phosphate, such as fructose-6-phosphate (F6P). The input F6P is first recombined with erythrose-4-phosphate (E4P) through the action of transaldolase (Tal) and transketolase (Tkt) to generate xylulose-5-phosphate (X5P), which is then cleaved by phosphoketolase (Xpk) to

generate acetyl-phosphate (AcP) and glyceraldehyde-3-phosphate (G3P). The irreversible Xpk-mediated reaction also acts as a driving force for the entire pathway. The sugar phosphates in the cycle go through several steps of carbon rearrangement, similar to that found in the pentose phosphate pathway and the Calvin cycle, eventually regenerating E4P ready for the next round of conversion. The end product, AcP, can be converted to ethanol or acetate. A near stoichiometric conversion from carbohydrate to acetate through NOG has been experimentally demonstrated *in vivo*¹⁵⁹.

rGS¹⁶⁰ provides a different approach to achieve the complete carbon conservation. The overall scheme of rGS starts by converting phosphoenolpyruvate (PEP) to oxaloacetate (OAA) using PEP carboxykinase (Pck) while fixing one molecule of CO₂. The resulting C₄ metabolite, OAA, can be converted to two acetyl-CoA molecules through rGS and the associated reactions. Reversal of the glyoxylate shunt is made possible through an ATP-dependent malate thiokinase (Mtk) and malyl-CoA lyase (Mcl)¹⁶⁰. The other PEP generated from glucose goes through the lower part of typical glycolysis to generate another acetyl-CoA. The two parallel pathways combined can reach reducing-power neutrality with a net output of three acetyl-CoA molecules, the same as NOG.



AcCoA, acetyl-CoA; Acl, ATP-citrate lyase; Acn, aconitase; Codh-Acs, carbon monoxide dehydrogenase–acetyl-CoA synthase; CoFeSP, corrinoid iron–sulfur protein; DHAP, dihydroxyacetone phosphate; EMP, Embden–Meyerhof–Parnas pathway; F1,6P, fructose-1,6-bisphosphate; Fba, fructose 1,6-bisphosphate (FBP) aldolase; Frd, fumarate reductase; Fch, formyl-THF cyclohydrolase; Fts, formyl-THF synthase; Fum, fumarase; Icl, isocitrate lyase; Madh, malate dehydrogenase; Mox, methanol oxidase; Mt, methyltransferase; Mtdh, methylene-THF dehydrogenase; Mtr, methylene-THF reductase; Pfk, phosphofructokinase; P_i, inorganic phosphate; Pta, phosphate acetyltransferase; R5P, ribose-5-phosphate; Rpe, R5P epimerase; Rpi, R5P isomerase; Ru5P, ribulose-5-phosphate; S7P, sedoheptulose-7-phosphate; THF, tetrahydrofolate; Tpi, triose phosphate isomerase.

both of which have growing markets. Other fuel compounds, such as farnesene, that can act as precursors for higher-value products are also being actively pursued.

Because of advances in molecular genetics and genome-editing techniques, resource utilization and product formation can, in principle, be implemented in any microbial host of interest. When choosing the production organism, one should consider the potential to achieve the highest yield and productivity within the time frame of interest, and the robustness of the organism against environmental challenges. Although native producers typically give a better outlook initially, non-native producers often produce surprisingly well, presumably because of the lack of native regulation that restrains the pathway flux. Another factor to take into account when selecting a producing organism is that anaerobic production may preserve electrons for fuel production and save the cost of aeration, but it may also reduce the rate of production. Therefore, the cost of aeration needs to be offset by a productivity increase if aeration is needed.

Robustness against contamination by other microorganisms and phages should also be considered, as should tolerance to extreme conditions, such as high or low pH and temperature.

At present, it is easier to modify organisms that natively contain resource utilization pathways, such as cellulose degradation, CO₂ fixation or methane oxidation, than it is to engineer production strains to carry these resource utilization pathways. However, when setting long-term goals, one should consider the theoretical limitation of the pathway design, as advances in genetic tools are likely to overcome practical difficulties and enable production to achieve the theoretical limit. Substantial room exists to improve the yield and productivity beyond the scope of natural metabolic networks. From a microbiology standpoint, the discovery and design of novel enzymes, pathways or organisms, coupled with innovative, efficient and inexpensive processing technologies, will contribute significantly to the ultimate success of sustainable biofuels.

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Competing interests statement

The authors declare [competing interests](#): see Web version for details.