Production of lignofuels and electrofuels by extremely thermophilic microbes

Biofuels (2014) 5(5), 499–515

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Extreme thermophiles are microorganisms that grow optimally at elevated temperatures (≥ 70°C). They could play an important role in the emerging renewable energy landscape by exploiting thermophily to produce liquid transportation fuels. For example, *Caldicellulosiruptor* species can grow on unpretreated plant biomass near 80°C utilizing novel multi-domain glycoside hydrolases. Through metabolic engineering, advanced biofuels compatible with existing infrastructure liquid biofuels, so-called *lignofuels*, could be produced to establish consolidated bioprocessing at high temperatures. In another case, a new paradigm, *electrofuels*, addresses the inefficiency of biofuel production through the direct synthesis of advanced fuels from carbon dioxide using hydrogen gas as the electron carrier. This requires coupling of biological electron utilization to carbon dioxide fixation and ultimately to fuel synthesis. Using a hyperthermophilic host *Pyrococcus furiosus* and synthetic metabolic pathways comprised of genes from less thermophilic sources, temperature-regulated biosynthesis of industrial organic chemicals and liquid fuel molecules are possible. Herein, we review recent progress towards the synthesis of *lignofuels* and *electrofuels* by extremely thermophilic microorganisms.

Keywords: extreme thermophiles ■ *Pyrococcus furiosus* ■ *Metallosphaera sedula* ■ *Caldicellulosiruptor* ■ biofuels ■ electrofuels ■ CO_2 fixation ■ lignocellulose

Introduction

For the past century, the unabashed utilization of fossil fuels has resulted in an explosion in both the population and standard of living across much of the planet. The result has been the most rapid advancement humanity has seen [1]. However, this has been accompanied by an insatiable demand for energy that in the very near future will not be met through the use of fossil fuels [2]. Their finite nature will reach a critical point at which the rate of extraction will irreversibly begin to decrease and asymptotically approach zero. For the production of crude oil, this phenomenon is widely known as 'peak oil' [201]. Peak oil is believed to have occurred in the United Kingdom in 1999, while in the United States it is predicted to occur in 2020 when production reaches 13 million barrels per day [202]. Although the timing of peak oil on a global scale is controversial, even the most optimistic estimates place it within the lifespan of the current generation and their immediate offspring [201]. In addition, the use of fossil fuels has been accompanied by a dramatic increase (40%) in atmospheric CO_2 (from 280 to 400 ppm) [3]. Transportation related to fossil fuel use is estimated to account for 30–40% of anthropogenic CO_2 emissions [203]. Even more controversial than projections for fossil fuel consumption is the relationship between atmospheric CO_2 concentrations and climate change. Nevertheless, while the extent to which atmospheric CO_2 and climate change are anthropogenic can be debated [4], without a renewable energy supply, humanity will not survive regardless of CO_2 and climate.

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Developing renewable technologies capable of supporting future civilization is clearly of paramount importance. To this end, biofuels offer important opportunities. Of the renewable liquid transportation fuels aimed at replacing the current need for gasoline, diesel and aviation fuel, ethanol currently constitutes 94% of the world's biofuel production [204]. The highly developed bioethanol industry [5] is led by the United States and Brazil, using corn starch and sugar cane as primary feedstocks, respectively [205]. Bioethanol in the United States is ubiquitously found as a 10% blend in gasoline (E10) and, to a lesser extent, as an 85% blend (E85) that requires engine modification [206]. As a result, approximately 9% of gasoline used in the United States contains bioethanol [207]. However, the use of ethanol as a fuel faces serious limitations. In addition to the federally mandated blend wall at 10%, vehicles utilizing E85 will experience a 25–30% reduction in fuel economy, due to the lower energy density of ethanol compared with gasoline [206]. Moreover, ethanol is not compatible with the current liquid fuel infrastructure, particularly pipelines, because of its hygroscopic and corrosive nature [6,7]. The result of these limitations is that the US ethanol market is currently saturated and production has reached a plateau [208]. Consequently, efforts in this area are now turning to so-called 'advanced biofuels' that have superior fuel characteristics compared with ethanol. Advanced biofuels include higher alcohols, terpenoids and alkanes, all of which have higher energy density, lower hygroscopicity, and greater infrastructure compatibly [8–11]. Despite these advantages, current strategies for advanced biofuel production are still largely based on sugar from corn starch and sugar cane. This creates an inevitable conflict between energy and food production [12], in addition to an extremely low carbon efficiency since the majority of the carbon is re-released as CO_2 during the production of the fuel [13]. Resolving the issues of food vs fuel, infrastructure compatible fuels, as well as the efficiency of vehicles and buildings is a primary goal of US and European policymakers, as outlined in the Energy Independence and Security Act of 2007 (EISA 2007) [209] and the European Parliament directive 2009/28/EC on the promotion of the use of energy from renewable sources [210].

■ Biomass as a source of carbon for biofuels

The strategy outlined in EISA 2007 calls for the production of cellulosic ethanol, which in comparison to current starch-based methods, relies on utilizing the substantial, but not easily accessible, cellulosic- and hemicellulosic-based carbohydrate components of plants [14]. Cellulosic biofuels have, however, proven to be difficult to produce, as evidenced by the fact that current production is far below the EISA 2007 targets [204]. The primary hurdle to producing cellulosic biofuels is that plant biomass is extremely resistant to degradation. This property, referred to as 'recalcitrance', inhibits access to the fermentable sugars comprising lignocellulose [15–17].

Nevertheless, conversion of lignocellulosic biomass to liquid fuels is a promising alternative to fossil fuels in the decades to come. The global net primary production of land plants is estimated to be ~65 Gt carbon per year [18]. Although net primary production and energy consumption are not equally distributed around the planet, the light energy annually, if converted to chemical energy by land plants, exceeds the world's energy demand by a factor of three to four [19]. While it is not realistic to completely exploit this energy store, these estimates nonetheless demonstrate the enormous potential of lignocellulosic biofuels, or what will be termed here 'lignofuels'.

The major constituents of plant biomass are cellulose (40–50%), a glucose (C_6) polymer), hemicellulose (23–38%), a polymer of xylose (C_5) and a variety of other sugars, and lignin (19–38%), a complex polymer of aromatic units [20], with carbohydrates comprising about ~60% of the chemical energy stored in the plant. The conversion of lignocellulose to lignofuels involves fermentation of sugars, requiring oxygen-free (anaerobic) conditions. An anaerobic biochemical process for lignin degradation has not been reported, nevertheless, microbial fermentations could convert the chemical energy stored in the cellulose and hemicellulose into fuels. Hence, the conversion of lignocellulose would still be about three to four times more energy efficient than the conversion of corn starch to ethanol [20].

Despite their promise for addressing the 'energy gap' anticipated in the coming decades, there are significant challenges that face biofuels if they are to become a significant part of the overall liquid transportation fuel budget. As mentioned above, they should be produced from non-food, plant biomass derived materials (cellulose and hemicellulose). Furthermore, to improve process economics, these plant biomass feedstocks should require minimal (or preferably no) chemical/physical pretreatment, prior to the fermentation step. Yet another possibility for future biofuels production is to forego the use of plant biomass altogether and directly convert point sources of CO_2 directly into liquid transportation fuels. Direct biological conversion of unpretreated plant biomass and direct conversion of CO_2 to liquid fuels are attractive possibilities that have recently been gaining attention. In particular, approaches for converting unpretreated plant biomass and CO_2 directly to biofuels are being considered using extremely thermophilic microorganisms, which grow optimally (T_{opt}) above

70°C. Here, we explore the very recent progress that has been made using extreme thermophiles to address current and future biofuels needs.

■ Biotechnological application of thermophiles

Growth temperature is a canonical characteristic in the classification of microbes. While the exact nomenclature varies, 'psychrophilic' usually refers to an organism with a T_{opt} below 15°C; 'mesophilic' organisms have a T_{opt} between 15 and 45°C; and 'thermophilic' organisms grow optimally above 45°C. However, the temperature range for thermophily is very broad, extending to 122°C, the current upper temperature limit of life [21]. Therefore, thermophiles have been further divided into moderate thermophiles (T_{opt} = 45–69°C) and extreme thermophiles (T_{opt} ≥ 70°C, e.g. *Caldicellulosiruptor bescii*). Those extreme thermophiles growing with a T_{opt} ≥ 80°C (e.g. *Pyrococcus furiosus*) are referred to as hyperthermophiles [22, 23].

Due to their inherent stability against denaturation, thermophilic enzymes have been utilized in many biotechnological applications, such as polymerases for DNA manipulation, dehydrogenases and esterases for chemical synthesis, and amylases for starch liquefaction [24,25]. A case in point is a thermostable DNA polymerase used in the Polymerase Chain Reaction (PCR) for DNA amplification, a technique essential for the development of modern biotechnology. PCR requires a DNA polymerase capable of surviving repeated cycles of heating to temperatures approaching the normal boiling point of water. Thermostability of proteins also correlates positively with their resistance to other denaturing conditions, such as detergents and organic solvents [26]. These properties allow thermophilic enzymes to play a role in various chemical processes under conditions that otherwise would be too harsh for conventional enzymes from more conventional mesophilic microbes. While thermophiles have been considered for biofuel production for some time, it is only very recently with the emergence of molecular genetics tools that efforts with these microbes have greatly intensified. As described in the following, bioenergy applications that exploit thermophily have now been envisioned and demonstrated, and hold great promise in addressing current energy supply challenges.

Lignofuels: utilizing recalcitrant plant biomass for fuel production

Recalcitrance of plant biomass is the major obstacle for their efficient and economic conversion to fuels. It arises from both the crystalline nature of the cellulose microfibrils in lignocellulose and the inert polyaromatic nature of lignin. This problem has been traditionally addressed by harsh thermochemical and physical pre-treatments of plant biomass, followed by enzymatic digestion of the liberated cellulose and hemicellulose polymers [27,28], ultimately yielding oligomeric sugars available for ethanol fermentation by yeast and bacteria. Consolidated bioprocessing (CBP) has been suggested as an alternative, more economical process [29]. CBP involves the use of cellulolytic microorganisms as comprehensive biocatalysts, combining the steps of enzyme production, enzymatic hydrolysis and fermentation. However, at present, no single microorganism or consortium of microorganisms can produce ethanol or another biofuel from unpretreated lignocellulose at commercially relevant yields and titers. Indeed, the ability to degrade crystalline cellulose is a relatively rare ability among microorganisms in general.

Thermophilic microbes able to breakdown and utilize lignocellulosic substrates and convert them to other products belong to a phylogenetic group of microbes known as the Firmicutes, mostly from the genera *Clostridium* (such as *Cl. thermocellum*, T_{opt} = 55–60°C), *Thermoanaerobacter* (T_{opt} = 60–75°C), *Thermoanaerobacterium* (T_{opt} = 55–70°C), and *Caldicellulosiruptor* (T_{opt} = 65–78°C). Among these naturally cellulolytic species, *Cl. thermocellum* is the most studied. In particular, it contains a large membrane-bound multienzyme complex known as the cellulosome that functions to attach to and degrade crystalline cellulose [30]. However, while *Cl. thermocellum* efficiently degrades cellulose at ~60°C, it does not utilize C5 sugars derived from hemicellulose nor does it produce a sufficient amount or yield of ethanol, despite extensive efforts to genetically engineer its fermentation pathways [31]. A co-culture of a genetically engineered strain of *Cl. thermocellum* and a strain of the ethanologenic *Thermoanaerobacterium saccharolyticum*, however, produced almost 40 g L^{-1} ethanol from 92 g L^{-1} crystalline cellulose [32]. While this is a promising step towards CBP, it remains to be seen if the use of co-cultures of microbes is effective on lignocellulosic substrates (rather than crystalline cellulose).

The genus *Caldicellulosiruptor*, a clade of extremely thermophilic, gram positive, anaerobic, and asporogenous bacteria, contains species that grow on a broad range of lignocellulosic materials as growth substrates. The first known *Caldicellulosiruptor* species was isolated from a terrestrial hot spring in the Rotorua region of New Zealand's North Island in 1987 and named *Caldocellum saccharolyticum* [33]. This bacterium was of great interest since it was able to produce an array of glycoside hydrolases (GHs) capable of degrading β-linked complex polysaccharides at temperatures at or above its optimal growth temperature of approximately 75°C [34–37]. Despite the fact that several other related species were isolated from terrestrial hot springs around the

world over the subsequent two decades, there was limited interest in their applications towards the production of renewable chemicals [38,39]. However, the recent push for biofuels from lignocellulosic substrates has heightened interest in *Caldicellulosiruptor* species [40,41]. These bacteria thrive in terrestrial hot springs by using plant biomass as growth substrates in the form of fallen trees and branches or from runoff from adjacent grassy areas. *Caldicellulosiruptor* species are prolific cellulose degraders, and they do so at the highest temperatures known for this process [40,42,43].

Diversity of Caldicellulosiruptor species

Caldicellulosiruptor species are globally distributed throughout North America, Iceland, Russia, Japan, and New Zealand and have optimum growth temperatures between 70°C and 78°C [44]. Not only are these species able to grow on pure crystalline cellulose and hemicellulose, but also on industrially-relevant loadings of unpretreated biomass (200 g L^{-1}), digesting up to 85% of insoluble unpretreated switchgrass, as in the case of *C. bescii* [16]. *Caldicellulosiruptor* species ferment the sugars liberated from the biomass substrate to molecular hydrogen, lactate, acetate, and small amounts of ethanol [45]. These characteristics make *Caldicellulosiruptor* species attractive candidates for development into CBP microorganisms [38] (see Figure 1). To date, eight genomes of *Caldicellulosiruptor* species have been sequenced, revealing a core genome comprised of 1543 genes shared by all eight species that is non-cellulolytic [46]. The 'pan-genome', which is the collection of all genes in all eight species (4009 genes), was found to be open, indicating that sequencing of new species should reveal novel genes [46]. Our recent review [41] catalogs the biochemically characterized carbohydrate-metabolizing enzymes (CAZymes) from *Caldicellulosiruptor* species. Within the genus, cellulolytic capability varies widely, with five species, namely *C. bescii*, *C. kronotskyensis*, *C. obsidiansis*, *C. lactoaceticus*, and *C. saccharolyticus* having higher cellulolytic ability than the other species with sequenced genomes [46]. Because of their high cellulolytic activity, the focus here is on this core group of five species, in particular the more extensively studied *C. bescii*, *C. obsidiansis* and *C. saccharolyticus*.

Carbohydrate-Active enZymes (CAZymes) in *Caldicellusiruptor*

The ability of lignocellulolytic organisms to effectively breakdown plant biomass is conferred by the diversity and specificity of carbohydrate-active enzymes (CAZymes)

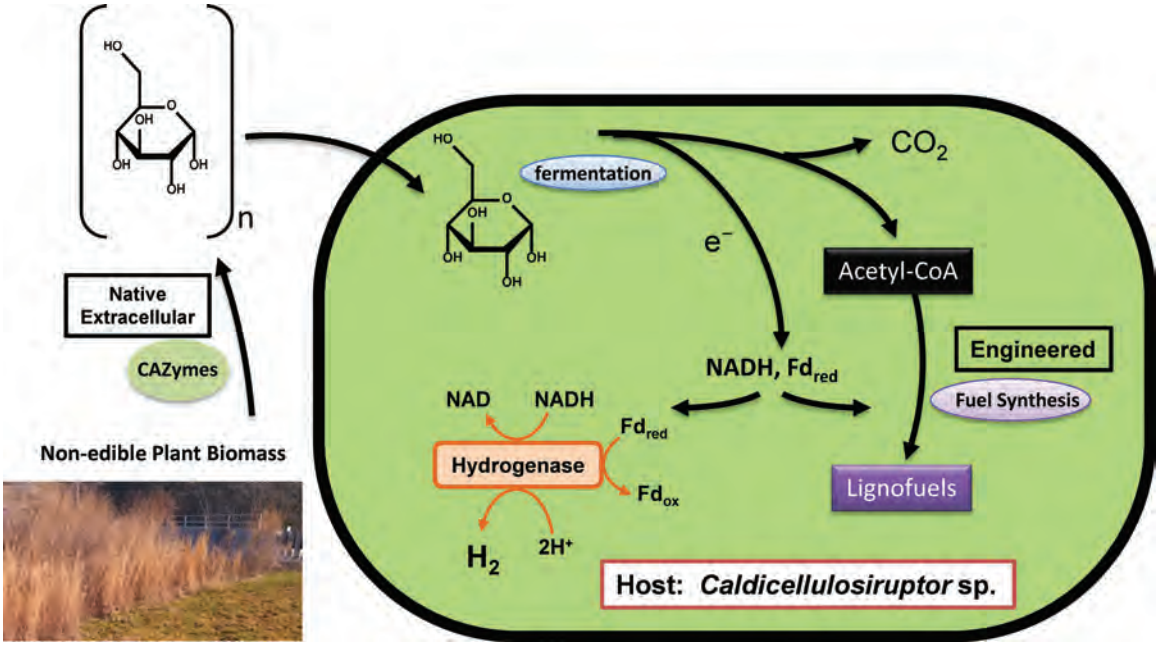

Figure 1. The proposed strategy for lignofuel production in *Caldicellulosiruptor* sp. Native extracellular and cell surface enzymes deconstruct untreated plant biomass at elevated temperature. They release oligomeric C_5 and C_6 sugars that are taken up and fermented to produce reductant (reduced ferredoxin and NAD(P)H) and acetyl-CoA. While in non-engineered strains, reductant oxidation is coupled to hydrogen production, reductant will be used in engineered strains to reduce acetyl-CoA to advanced biofuels or lignofuels.

they can produce. Of these, glycoside hydrolases (GHs) are of particular importance since they target the most challenging and recalcitrant portions of plant biomass, i.e. cellulose and hemicellulose. *Caldicellulosiruptor* species characteristically have a large inventory of GHs in their genomes that generate a variety of pentose and hexose monosaccharides from lignocellulose, which are metabolized simultaneously without regulation by carbon catabolite repression [35,38,39]. When looking at the five most highly cellulolytic *Caldicellulosiruptor* species, they all possess a combination of three extracellular GHs present across these genomes: GH9-CBM3-CBM3-CBM3-GH48, GH74-CBM3-CBM3-GH48, and GH9-CBM3-CBM3-CBM3-GH5, where CBM stands for Carbohydrate-Binding Module. A key features of these *Caldicellulosiruptor* CAZymes is not only that they are multi-modular, but also that they combine very different types of active sites, which also makes them multi-functional (Table 1). Comparisons with other *Caldicellulosiruptor* species as well as other genera [46,47] indicate that the prevalence of CAZymes with multi-modular architecture is unique among cellulolytic bacteria [41]. In addition, the presence of GH48 and CBM3 domains appear to be essential for crystalline cellulose degradation, compared with the weakly cellulolytic species where GH5 and GH9 enzymes are not associated with GH48 domains [46,48]. Most cellulolytic organisms contain only one GH48 enzyme, whereas *Caldicellulosiruptor* species contain multiple copies with different functionalities, conveyed by the other GH domains linked to them through multi-modular configurations. The only other instance for multiple GH48 domains is found in cellulolytic thermophiles (T_{opt} = 55–60°C), such as *Clostridium* (*Cl.*) *thermocellum*, *Cl. clariflavum* and *Cl. straminisolvens*, which possess two copies of GH48, one cellulosomal and one secreted as a free enzyme [49]. Secretome analyses of both *C. bescii* and *C. obsidiansis* have revealed that the most abundant multi-functional, multi-domain cellulases contained GH5, GH9, GH10, GH43, GH44, GH48, and GH74 domains, as well as highly conserved CBM3 domains [50]. Combinations of these 7 GH families are found throughout all the *Caldicellulosiruptor* genomes, but especially in the most cellulolytic species (Table 1). In addition to GH domains, multi-modular CAZymes in *Caldicellulosiruptor* species can also contain catalytic domains from the PL (polysaccharide lyase) and CE (carbohydrate esterase) families, allowing for a broader spectrum of catalytic synergism from individual enzymes.

CelA, a primary cellulase in *Caldicellulosiruptor* species

CelA is one of the largest, most thermostable, and most active GH enzymes yet identified for crystalline cellulose and it is ubiquitous in the most highly cellulolytic *Caldicellulosiruptor* species (see Table 1, GH9-CBM3-CBM3-CBM3-GH48). CelA is the most highly secreted cellulase when *C. bescii* grows on plant biomass [50]. Recently, it has been reported that this cellulase is capable of excavating through the middle of the cellulose microfibrils creating cavities approximately 15–20 nm wide and 15–30 nm long, whereas previously characterized cellulases degrade the polymer from the ends of crystalline cellulose microfibrils [51]. This hydrolysis mechanism promotes the formation of new cellulose fiber chain ends that can be synergistically digested by other cellulases produced by *Caldicellulosiruptor* species. Compared with thermophilic model organisms, such as *Clostridium thermocellum*, that rely on

Table 1. Distribution of multimodular enzymes containing multiple GH domains across the *Caldicellulosiruptor* genomes.

Multidomain architecture	Gene loci	Catalytic domain activity
GH43-CBM22-**GH43**-CBM6	Cbes_0182, Calkro_2388, Csac_2411, Calow_0121	xylanase, arabinanase
GH10-CBM3-CBM3-**GH48**	Cbes_1857, Calkro_0853	endo-1,4-β-xylanase, cellobiohydrolase
GH5-CBM3-CBM3-**GH44**	Cbes_1859, Calkro_0851, Csac_1077	mannanase, endoglucanase
GH74-CBM3-CBM3-(CBM3)-**GH48**	Cbes_1860, Calkro_0861, Csac_1085, COB47_1664, Calla_0015	xyloglucanase, cellobiohydrolase
GH9-CBM3-CBM3-CBM3-**GH5**	Cbes_1865, Calkro_0855, COB47_1669, Csac_1079	endoglucanase, mannanase
GH5-CBM3-CBM3-CBM3-**GH5**	Cbes_1866, Calkro_0854	mannanase, cellulase
GH9-CBM3-CBM3-CBM3-**GH48**	Cbes_1867, Calkro_0850, Csac_1076, COB47_1673	endoglucanase, cellobiohydrolase
GH10-CBM3-CBM3-**GH5**	COB47_1671	endo-1,4-β-xyalanse, endoglucanase
GH10-CBM3-**GH5**	Csac_1078	endo-1,4-β-xyalanse, endoglucanase
PL-CBM3-CBM3-CBM3-**GH44**	Calla_0017	rhamnogalacturonan lyase, endoglucanase
CBM22-CBM22-**GH10**-CBM3-CBM3-CBM3-**GH43**-CBM6	AAD30363	endo-1,4-β-xylanase, arabinanase

The abbreviations are GH, glycoside hydrolase; CBM, carbohydrate binding domain; PL, polysaccharide lyase. The gene loci are shown for *C. bescii* (Cbes), *C. kronotskyensis* (Calkro), *C. saccharolyticus* (Csac), *C. owensensis* (Calow), *C. obsidiansis* (COB47), *C. lactoaceticus* (Calla), and *Caldicellulosiruptor* sp. Tok7B.1 (accession number only)

the membrane-bound multi-complex cellulosome [30], *Caldicellulosiruptor* species, therefore, represent a new paradigm for microbial cellulose degradation by way of these complex, multi-domain secreted cellulases.

Mechanisms of adhesion to plant biomass

Compared with other thermophilic lignocellulosic organisms where the cellulosome plays a key role in the attachment to plant biomass, *Caldicellulosiruptor* species have a number of diverse mechanisms that allow cells to maintain proximity with the lignocellulose substrate. *Caldicellulosiruptor* species have a broad range of S-layer homology (SLH) proteins that contain domains that have affinity to the outer para-crystalline protein layer in these organisms, as well as complex arrangements of carbohydrate binding domains and glycoside hydrolases. One key enzyme in this category was found in the core pan-genome and contains GH5 and CBM28 domains. This enzyme plays a key role in adherence to plant biomass by C. *saccharolyticus* [52], *C. bescii* [47] and *C. obsidiansis* [53]. Beyond this SLH protein shared by the core genome, many others have been identified in the *Caldicellulosiruptor* species, with large multimodular domains. In addition to SLH domains, other classes of proteins have been associated with adherence to cellulose include a type 4 pilus (T4P) in *C. bescii* [47] and *C. obsidiansis* [53], and a two putative adhesins [46]. It, therefore, seems very clear that the breadth of mechanisms involved in surface-microbe interactions in the *Caldicellulosiruptor* species has only begun to be uncovered and merits further exploration.

Caldicellulosiruptor plant biomass conversion

Among the few microorganisms capable of deconstructing plant biomass, *Caldicellulosiruptor* species possess the highest temperature optima, approaching 80°C [54], and they exploit the heat for accelerated lignocellulose degradation [16]. Moreover, *C. bescii* degrades plant biomass without thermochemical pretreatment [16,55], an enormous technological and economic advantage that has not been demonstrated for other microorganisms. In addition, concentrations of acid-pretreated switchgrass higher than 20 g L^{-1} caused a significant growth inhibition, likely due to substances released by the pretreatment process [56]. After growth of three successive cultures of *C. bescii* on unpretreated switchgrass, where the residual plant biomass remaining after growth of one culture served as the carbon and energy source for the next, approximately 80% of the switchgrass was solubilized and able to pass through a 20 µm filter [16]. Surprisingly, the remaining 20% was not a 'ball of lignin', as might be expected. Rather, the cellulose/hemicellulose/lignin ratio was similar to that of the unprocessed biomass. These data suggest that *C. bescii* deconstructs switchgrass by an 'onion-peeling' mechanism. Since the amount of biomass carbohydrate that was degraded matched the carbon products that were generated by *C. bescii* [16], even using high loads [56], lignin is clearly not serving as a carbon or energy source for the organism. It would appear that degradation of the polymeric carbohydrate components by *C. bescii* also releases lignin complexes (< 20 µm), thereby exposing additional carbohydrate and enabling continued deconstruction of the biomass. Microbial enrichment cultures that degrade lignin under anaerobic conditions have been reported previously [57–60]. However, neither a biochemical pathway for the anaerobic degradation of lignin nor an enzyme involved in lignin activation without oxygen have been characterized. Such a process or enzyme would be of significant interest within the fields of biochemistry and biotechnology. It is not known if *C. bescii* produces enzymes that can degrade components of the lignin directly, or if lignin release is a consequence of the hydrolysis of plant carbohydrates.

For biomass to biofuel conversion on an industrial scale, a titer of at least 4% (40 g L^{-1}) ethanol has to be achieved [61,62], which is equivalent to about 110 g L^{-1} untreated switchgrass [16,56]. While *Caldicellulosiruptor* species do not yet generate ethanol using this substrate concentration, it is technologically promising that concentrations of switchgrass of more than 100 g L^{-1} are readily used as a carbon source and do not inhibit growth of *C. bescii*. This also suggests that acid-pretreatment is potentially dispensable and, perhaps, counterproductive for CBP based on this microbe [56]. In pH-controlled media-optimized fermentations using 50 g L^{-1} substrate loads, *C. bescii* completely degraded ~30 g L^{-1} crystalline cellulose and ~10 g L^{-1} of unpretreated switchgrass. All substrate carbon could be accounted for in the products, based on the amount of substrate degraded, with acetate and CO_2 being the major end products [56].

Unlike *Cl. thermocellum*, *Caldicellulosiruptor* species possess a complete pentose phosphate pathway, so that they can use C_5 sugars derived from hemicellulose in addition to C_6 sugars [46]. Major fermentation end products are acetate and hydrogen, with only traces of ethanol produced, although very recently a *Caldicellulosiruptor* species has been isolated that produced as much as 72 mM ethanol from cellulose although it has yet to be fully characterized [40]. Lactate production is also observed, but this can be avoided if the hydrogen partial pressure is kept low [56]. While *Caldicellulosiruptor* species are not yet optimized for the production of liquid biofuels, they are very effective in channeling reductant towards H_2. In fact, molecular hydrogen production in *C. saccharolyticus* has been shown to approach the Thauer limit of 4 moles H_2 per mol glucose [63]. In sugar fermentation, *Caldicellulosiruptor* species produce both

ferredoxin (by the pyruvate ferredoxin oxidoreductase reaction) and NAD(P)H (in glycolysis) [45,64], and both can obviously be re-oxidized by hydrogen production. *Caldicellulosiruptor* genomes encode for a bifurcating hydrogenase [65] that presumably accounts for the majority of hydrogen production.

■ Current limitations and perspectives

The recent development of a genetic system in *C. bescii* [66,67] now enables gene deletions [68] and potentially expression of foreign genes and pathways. These developing tools will be very helpful in elucidating the role of different CAZy proteins in biomass degradation, and also make possible metabolic engineering of *Caldicellulosiruptor* species for efficient degradation of lignocellulosic biomass for liquid biofuel production (Figure 1). However, pathways for the production of liquid biofuels are rare in thermophilic microbes. *Cl. thermocellum* (T_{opt} = 55°C) and different *Thermoanaerobacter* species (T_{opt} = 60–75°C) can produce ethanol as the major end product of sugar fermentation [64,69–72]. These obligately anaerobic and thermophilic ethanologens use a different pathway than that used in facultative anaerobic mesophilic bacteria and yeasts, wherein pyruvate decarboxylase produces acetaldehyde from pyruvate. The acetaldehyde is then further reduced to ethanol by a primary alcohol dehydrogenase. In contrast, thermophilic anaerobes oxidize pyruvate to acetyl-CoA, a reaction catalyzed by pyruvate ferredoxin oxidoreductase. A key enzyme for ethanol production is a bi-functional aldehyde/alcohol dehydrogenase, referred to as AdhE [70,73] that catalyzes a two-step reduction of acetyl-CoA to ethanol.

Since the growth temperatures of *Caldicellulosiruptor* and *Thermoanaerobacter* species are similar [64], expression of enzymes from ethanologenic *Thermoanaerobacter* species, in particular alcohol dehydrogenases, is a promising engineering strategy for ethanol production in *Caldicellulosiruptor* species. As an alternative strategy, *Thermoanaerobacter* species could be grown in co-culture with *Caldicellulosiruptor* species. In that scenario, the latter would break down the biomass and provide a certain fraction of the cellulose- and hemicellulose-derived sugars to the non-cellulolytic but ethanologenic *Thermoanaerobacter* species. This approach has been used with *Cl. thermocellum*, which cannot utilize C_5 sugars, growing together with non-cellulolytic *Thermoanaerobacterium* or *Thermoanaerobacter* species on defined C_6 and C_5 sugar substrates and resulting in 2- to 4-fold increases in ethanol titer [32,42,74]. Currently, there is only one example of a *Thermoanaerobacter-Caldicellulosiruptor* co-culture, which showed a higher ethanol yield from cellulose fermentation than the pure *Caldicellulosiruptor* culture, ranging between 175% to 210% higher ethanol yields on the mol % basis [40]. A possible third strategy is to identify all essential enzymes for plant biomass breakdown in *Caldicellulosiruptor*, and then genetically engineer an ethanologenic *Thermoanaerobacter* or *Thermoanaerobacterium* species to utilize cellulose and hemicellulose. Given the recent development of genetic tools in these organisms, this may be possible in the not too distant future [31,43]. An advantage is that the heterologous expression of secreted extracellular enzymes (e.g., CelA [75]) might be easier to achieve than heterologous production of complex cellulosomes [76,77].

Another obstacle to overcome is the efficient deconstruction of lignocellulose. *C. bescii* completely degrades up to 60% of 50 g L^{-1} crystalline cellulose in pH-controlled fermentations. However, conversion of soluble sugars (cellulose, glucose) is incomplete, with sugars accumulating in the medium [56]. This shows, on the one hand, that the glycoside hydrolases are relatively insensitive to inhibition by end products, but on the other hand, that *C. bescii* itself is sensitive. Specifically, the fermentation end products acetate and lactate accumulate in the medium to concentrations above 150 mM, inhibiting growth and further sugar fermentation [56,78]. This inhibition is mainly due to ionic strength rather than osmotic pressure [56] and, of course, is likely overcome by engineering a strain that produces a neutral end product, such as ethanol or butanol.

Less is known about why *Caldicellulosiruptor* species stop degrading unpretreated switchgrass. Interestingly, independent of the initial switchgrass concentration, the same percentage (~30%) of unpretreated switchgrass is degraded by *C. bescii* [16,56]. The spent media from pH-controlled fermentations contain a significant fraction of unidentified sugars that represent ~20% of the product carbon. *C. bescii* did not grow on the so-called 'spent medium', which is that remaining at the end of a switchgrass fermentation, while a related microorganism, *Thermoanaerobacter mathranii,* did grow on the same spent medium. This suggests a species-specific inhibitor may be released into the medium during growth on switchgrass. While it has been shown that *C. bescii* tolerates high concentrations of unpretreated plant biomass, it will be crucial to elucidate and ameliorate the inhibitory mechanism to efficiently convert the lignocellulosic biomass. A likely source for inhibitory compounds is phenolic lignin monomers that are released during lignocellulose deconstruction by *C. bescii* [16,79]. However, it remains unclear why the inhibition is concentration-independent.

To date, research into the production of fuels in thermophiles has focused on ethanol, just as it has in mesophiles. Much has been done with thermophilic bacteria to increase ethanol productivity, particularly

in *Thermoanaerobacter*, *Caldicellulosiruptor*, and *Clostridium* species [15,80,81]. However, there is little research thus far on engineering thermophiles to produce advanced biofuels, and there are no reports using extreme thermophiles or hyperthermophiles. There is one report of advanced biofuel production in a moderate thermophile (55°C), in which *Thermoanaerobacterium saccharolyticum* was engineered to produce *n*-butanol from xylose at 1 g L^{-1} titers and 26% theoretical yield [82]. The reason for the dearth of advanced biofuels produced by thermophiles is the lack of metabolic pathways to produce such molecules in these organisms. Discovering or engineering these pathways merits further investigation and may require environmental sampling and sequencing of hot springs and/or combining known pathways into new configurations for fuel synthesis.

It is clear that *Caldicellulosiruptor* species are promising candidate organisms for CBP, particularly as they deconstruct high concentrations of unpretreated plant biomass at temperatures above 70°C. They take advantage of an arsenal of extracellular and cell surface attached CAZymes [46], some of which are among the most efficient cellulolytic enzymes known [51]. The recent development of a genetic system [66–68] will lead to a better understanding of essential enzymes for plant biomass breakdown, and how these bacteria could be engineered to efficiently convert high concentrations of plant biomass to biofuels.

Electrofuels: using CO_2 directly as a carbon source for fuel production

The use of plant biomass for biofuel production depends on the photosynthetic capture of solar energy, which operates at approximately 1% energy efficiency on an annual basis (Figure 2). The resulting low efficiency of this process poses a significant problem in moving to the necessary industrial scales [83,84]. A new non-photosynthetic paradigm for renewable fuels, 'electrofuels', exploits a variety of autotrophic pathways for the direct conversion of fully oxidized inorganic carbon into infrastructure-compatible fuels. This occurs via the incorporation of low potential electrons from inorganic energy forms such as hydrogen gas or an electrical current. This strategy was designed explicitly to avoid the inefficient photosynthetic production of carbohydrate intermediates in favor of a more direct and efficient approach [83,85–87]. In the electrofuels strategy, the energy supply, such as hydrogen gas, is an energy carrier and not the actual energy source. At present, the ultimate energy sources for these electrofuels are still fossil fuels, but in the not-too-distant future electrofuel production would be driven by solar energy. Hence, rather than using the solar energy to store electrons in carbohydrates via plant-based photosynthesis, the electrofuels would be generated using a variety of recent technological advances to capture solar energy. For example, photovoltaic-driven electrolysis of water to hydrogen (with 10% energy efficiency) has been shown to be roughly 10-fold more efficient than plant-based photosynthesis and 3-fold more efficient than microalgae-based photosynthesis (3% energy efficiency) [84]. Additionally, wireless systems are available, such as the 'artificial leaf', that catalyze the direct solar-driven splitting of water using earth-abundant materials and near-neutral conditions, and do so at 2.5% energy efficiency [88]. In addition to hydrogen gas, formate is an efficient energy carrier electrochemically produced from carbon dioxide with high efficiency [89]. Moreover, it is highly soluble, which is critical for efficient mass transfer in an aqueous reaction, and its electrons are readily accessible via biological means via formate dehydrogenase-type enzymes. Formate and hydrogen have similar energy contents. For example, the hydrogen electrode and the formate/CO_2 redox couples have the same reduction potential (E_o' = –420 mV, pH 7.0).

Biological CO_2 fixation

Assimilation of inorganic carbon in the form of CO_2 is key to the concept of electrofuels. There are currently six pathways of CO_2 fixation known to occur in nature, four of which are found in extreme thermophiles: the reductive tricarboxylic acid (rTCA) cycle, the reductive acetyl-CoA or Wood-Ljungdahl (W/L) pathway, the 3-hydroxypropionate/4-hydroxybutyrate (3-HP/4-HB) cycle, and the dicarboxylate/4-hydroxybutyrate (DC/4-HB) cycle. The reductive pentose phosphate cycle and 3-hydroxypropionate bicycle occur in moderate thermophiles, but not in extreme thermophiles growing above 70°C. The nature of all six carbon-fixation pathways has recently been reviewed extensively [90–94] and so the focus herein is on the impact of pathway features for electrofuels production.

Each of the CO_2 fixation pathways found in extreme thermophiles produces acetyl-CoA as its product, but they differ in the identity of electron donors, oxygen tolerance and energetics. The rTCA, W/L, and DC/4-HB cycles use NAD(P)H and ferredoxin as electron donors, while the 3-HP/4-HB cycle uses only NADPH [85]. The rTCA and 3-HP/4-HB cycles are oxygen-tolerant and known to operate in aerobes, despite the use of oxygen-sensitive ferredoxin in the rTCA cycle [81,90]. In contrast, the W/L and DC/4-HB pathways are found only in anaerobes, likely due to the oxygen sensitivity of the key enzymes CO dehydrogenase/acetyl-CoA synthase and pyruvate synthase, respectively. The thermodynamic and kinetic characteristics of the carbon-fixation cycles have been evaluated due to their impact on electrofuel and biofuel production and on global

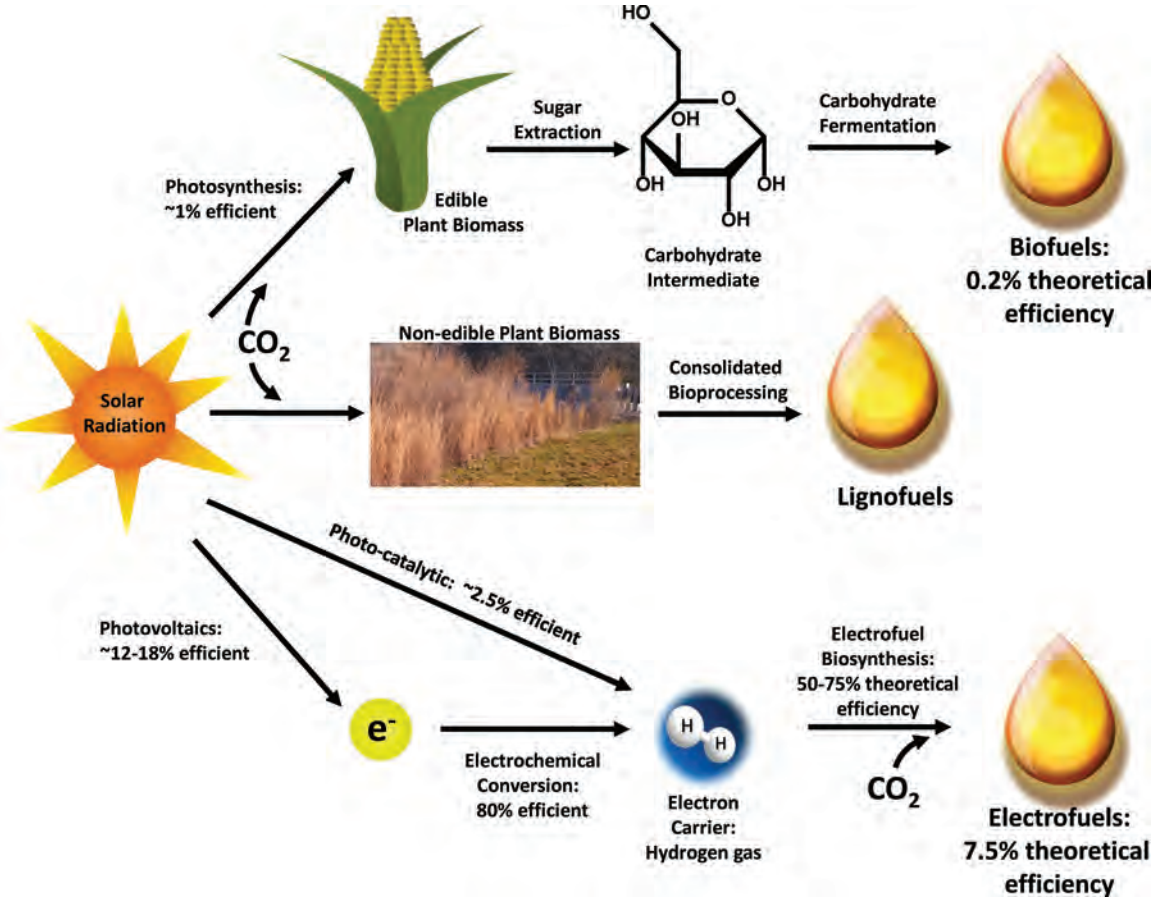

Figure 2. The current biofuel strategy depends on the plant-based capture of solar energy in the form of carbohydrate intermediates. This process has a measured overall energy efficiency of 0.2% in the conversion of photons to fuel. Lignofuels will utilize non-edible plant biomass that doesn't compete with food production and greatly increases the carbon efficiency. Electrofuels are based on the photovoltaic capture of solar energy. This energy is coupled to a carbon fixation and fuel synthesis pathway via an inorganic electron carrier such as hydrogen gas. This process has a potential energy efficiency of up to 7.5%, depending on the strategy for fuel synthesis.

carbon cycling. However, different conclusions can be reached as to which one is "best", depending on the parameter being considered. For example, maximizing biomass production in the context of photorespiration results in the rTCA cycle having the highest energetic efficiency [95]. A recent review [96] suggests that the W/L pathway is the best option when viewed from the perspective of energy efficiency in utilizing CO_2 and H_2. However, as concentrations of gaseous substrates decrease with increasing process temperature, the faster kinetics exhibited by the 3-HP/4-HB and DC/4-HB cycles may become important [93]. For biotechnological applications, the 3-HP/4-HB cycle would be more desirable than the DC/4-HB cycle since, in contrast to the 3-HP/HB cycle [97–100], most but not all of the genes for the DC/4-HB cycle have been identified [97].

The characteristics of carbon fixation pathways obviously impact their suitability for use in electrofuels production. While there are a variety of options for mesophilic microbes, robust genetics tools are not available for thermophilic autotrophs that naturally fix CO_2 directly. Hence, rather than engineering into a CO_2-fixing, autotrophic thermophile a pathway to produce a biofuel, efforts have focused on the recombinant expression of a CO_2-fixation pathway in a thermophilic heterotrophic host. However, given the complexity of the CO_2-fixing pathways, this presents a major challenge for electrofuels production at high temperature. In addition, the pathway must also integrate within and be compatible with the host's metabolism. This includes providing the appropriate electron donor(s) to the pathway, accounting for oxygen tolerance, and

supplying the appropriate amount of ATP. This compatibility, while necessary, may also pose an issue as the host's metabolism can also interfere with the recombinant pathway leading to lowered efficiency. For this reason, extreme thermophiles are an attractive option since decreasing the operating temperature could allow for the optimal operation of a recombinant pathway in a background of minimized host metabolism [85–87]. In fact, as described below, this strategy has already been employed in which a metabolic pathway from a thermophile has been expressed in a hyperthermophilic host. Upon a temperature shift from the temperature of the hyperthermophilic host (90–100°C) to the recombinant thermophilic pathway (60–80°C), the metabolic activity and potential interference of the host decreases, while the activity of the recombinant pathway becomes optimal.

Progress towards the production of electrofuels in a hyperthermophilic host

The hyperthermophile *P. furiosus* is an attractive option for metabolic engineering via genetic modification. It is a marine anaerobe that is capable of growing between 70 and 103°C (T_{opt} = 100°C) by fermenting carbohydrates to hydrogen gas, carbon dioxide, and acetate [101]. A naturally competent variant was discovered during attempts to introduce DNA in *P. furiosus* [102]. This variant was used to construct a markerless deletion of the gene *pyrF* for uracil biosynthesis, and the resulting strain, designated COM1, became the basis for the development of an extremely robust genetic system [102]. The wide temperature range of *P. furiosus* and the availability of a genetic system have enabled the temperature-dependent production in the organism of the enzyme lactate dehydrogenase from *Caldicellulosiruptor bescii* (T_{opt} = 78°C). The gene encoding this NADH-dependent enzyme was expressed in *P. furiosus* and this resulted in a temperature dependent shift of the metabolic end product. At a high growth temperature (98°C), the strain produced the wild-type end products carbon dioxide and acetate; however, upon a temperature shift to 72°C, there was a shift in the metabolism toward lactate production [103].

One of the first genetic manipulations of *P. furiosus* resulted in the homologous overexpression of its soluble NADP-dependent hydrogenase I (SHI), resulting in a 10-fold increase over that normally produced [104]. This enzyme is of particular biotechnological interest as it catalyzes the reversible oxidation of hydrogen gas and reduction of NADP to NADPH [105,106], a reaction that could be used to drive an NADPH-dependent biosynthetic pathway with reductant from hydrogen gas. Hydrogen can be added to the electrofuel-producing system in a variety of ways. The simplest method, i.e. gas-sparging, faces the challenge of low solubility and mass transfer. An alternative method would be to supply a highly soluble electron carrier, such as formate, and produce the substrate hydrogen gas within the cells themselves. This has also been demonstrated in *P. furiosus* by the heterologous expression of an 18-subunit membrane-bound formate-hydrogen lyase (FHL) from an organism with a T_{opt} of 80°C [107,108]. The resulting *P. furiosus* strain is capable of converting high concentrations of formate to hydrogen and carbon dioxide, which can potentially be used directly as substrates for electrofuels synthesis [108].

As previously discussed, the electrofuels strategy is defined by directly coupling the use of low potential reductant to the assimilation of fully oxidized inorganic carbon (CO_2). In *P. furiosus*, the native hydrogen uptake system (SHI) can be coupled to the 3-HP/4-HB cycle for carbon fixation via the compatible electron carrier NADPH [97–100,105,106]. The strategy for electrofuels production in *P. furiosus* is based on the heterologous expression of the 3-HP/4-HB carbon fixation pathway from the archaeon *Metallosphaera sedula,* which grows optimally at 73°C (Figure 3). To demonstrate this as proof of principle, the five genes encoding the first three enzymes of the pathway were expressed in *P. furiosus*. In combination, these catalyze the ATP-dependent carboxylation of acetyl-CoA to malonyl-CoA and subsequent two reductive steps to the cycle-intermediate 3-hydroxypropionate. The result was hydrogen- and carbon dioxide-dependent formation of 3-hydroxypropionate and validation of the electrofuels concept in *P. furiosus* [87]. This process was further improved upon by studying strategic deletions aimed at directing the flow of acetyl-CoA toward the heterologous pathway and away from the metabolism of the host [109]. The host normally converts acetyl-CoA to acetate while simultaneously producing ATP via the enzyme acetyl coenzyme A synthase (ACS) [110,111]. A single deletion in this pathway resulted in a 3-fold increase in 3-hydroxyproprionate production in comparison to the original strain [109].

This strategy for the recombinant expression of the *M. sedula* 3-HP/4-HB pathway in *P. furiosus* involved dividing the cycle into three sub-pathways and transferring them one at a time. The initial strain expressed the first three enzymes and produced the key intermediate 3-hydroxypropionate [87]. While this is a small step towards making a fuel molecule, 3-hydroxypropionate itself is a molecule of significant biotechnological interest. In addition to being a commercial chemical produced at commodity-scale itself, 3-hydroxypropionate can readily be converted to the commodity chemicals 1,3-propanediol, acrylate, methyl acrylate, and acrylamide. This progress represents a biological and

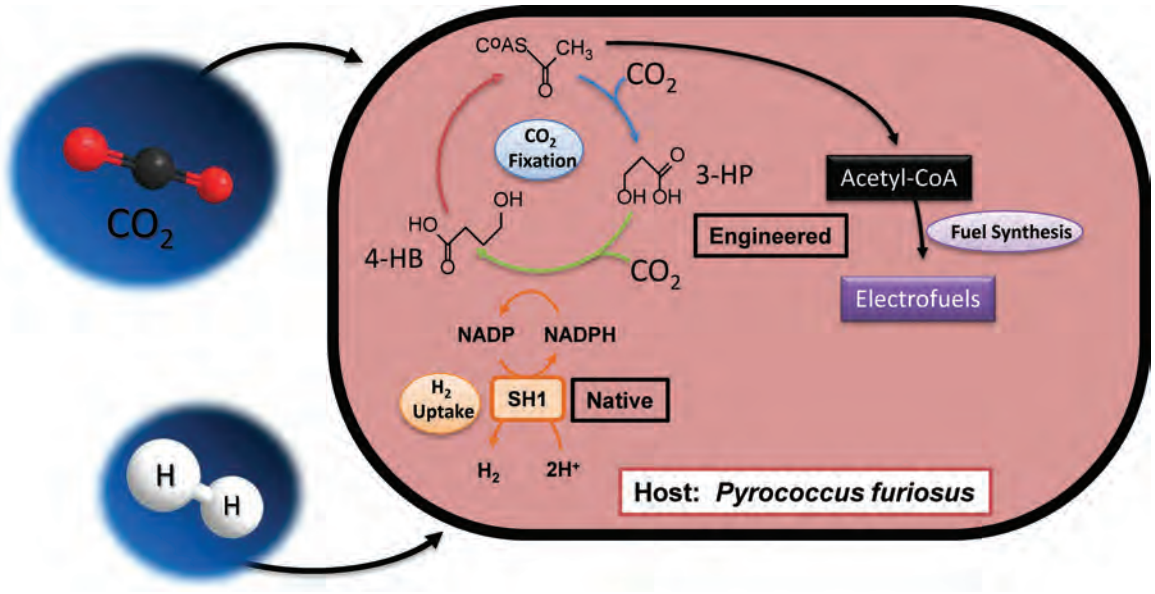

Figure 3. The proposed strategy for electrofuel production in *Pyrococcus furiosus*. The native hydrogen uptake system, SH1, will be coupled to the engineered 3-HP/4-HB carbon fixation pathway from *M. sedula* via the compatible electron carrier NADPH. The generated acetyl-CoA will then be fed into an advanced biofuel synthesis pathway. Key intermediates for the carbon fixation pathway are: acetyl-CoA, 3-hydroxypropionate (3-HP), and 4-hydroxybutyrate (4-HB).

renewable means to generate a molecule (3-hydroxypropionate) that constitutes a large and petroleum dependent chemical market. The resulting 3-hydroxypropionate is listed among the Department of Energy's list of top value added chemicals from biomass [112]. Significant progress along these lines illustrates the potential for commercial bioprocesses based on the electrofuels paradigm.

Large-scale bioprocessing at elevated temperatures

Utilizing a hyperthermophile at a large-scale raises the question of how to provide sufficient energy to heat a microbial fermentation. However, this may prove to be a significant advantage of a hyperthermophilic platform organism. Large-scale fermentations of mesophilic organisms require a substantial and constant cooling effort due to the energy released from active metabolism [113]. This is typically carried out by circulating refrigerated glycol and represents the major energy consuming process for a large scale fermentation [114]. On the other hand, for a hyperthermophile at the appropriately large scale (hundreds of thousands to millions of liters), this energy can serve to maintain the temperature. This would result in the required heat exchange being limited to a one-time heating expenditure rather than a constant cooling requirement for the entire duration of the process. Moreover, a hyperthermophilic process requiring a temperature drop of 30°C or more will be more easily accomplished owing to the large temperature differential between the fermentation and the ultimate heat acceptor, the ambient air. This is due to the rate of heat flow being directly proportional to the respective temperature difference. In the case of a hyperthermophilic fermentation versus one at ambient temperature, heat will flow very quickly from the 80–100°C fermenter to the ~25°C air. For a mesophilic process at 37°C, the smaller temperature differential is insufficient for rapid heat transfer, and refrigerated glycol is required for effective cooling. The temperature control of a hyperthermophilic process could therefore represent an enormous benefit, with refrigerated glycol replaced with active air circulation as the primary means of cooling.

The use of metabolic activity for heating and circulated air for cooling would allow for a strategy that cycles at different temperatures (Figure 4) between that of a hyperthermophilic host (90–100°C) and a recombinant thermophilic pathway (60–80°C). The first stage of such a process would be an initial heating of the growth medium from approximately room temperature to the growth temperature of the hyperthermophilic host (90–100°C). This represents a single large energy investment, but also an opportunity to lower the risk of contamination. Mesophilic fermentations typically require

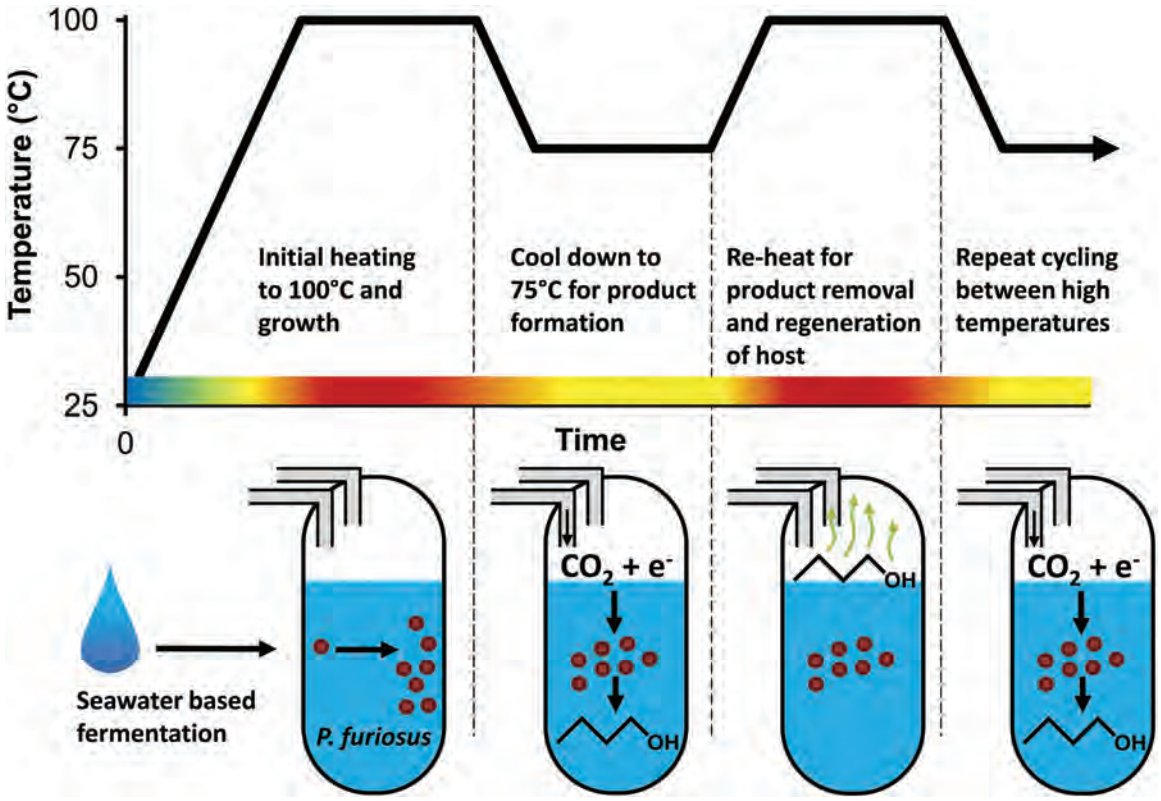

Figure 4. Cycling between temperature of a hyperthermophilic host (90–100°C) and a recombinant thermophilic pathway (60–80°C). The first heating stage would allow for the initial growth of the host followed by a temperature shift for product formation. Afterwards, the temperature would be raised again for product removal and the regeneration of the host. This cycling would be repeated as long as product formation is occurring.

sterilization at 121°C prior to inoculation and subsequent sterilization cycles to minimize contamination, a large energy investment given the scale of operation. In addition to cooling, losses due to contamination represent a substantial cost to current industrial fermentations [115]. This is typically due to wild-type mesophilic organisms naturally inhabiting the area where the fermentation plant is located. Such mesophilic contaminants would be eradicated during an initial growth stage at 90–100°C. Thermophiles are still prevalent in the environment, so a complete lack of sterile technique might still lead to contamination. Utilizing a marine hyperthermophile, such as *P. furiosus*, affords the additional advantage of not requiring fresh water. Utilizing primarily sea water would alleviate serious concerns over the limited nature of fresh water [116] and would geographically localize the process to the coast, which is where human population density is greatest [117].

Once the hyperthermophilic host has grown to a sufficient density, the fermentation would then be cooled to a temperature optimal for the heterologous pathway (60–80°C) via active air circulation. During this phase, the genes encoding the thermophilic enzymes would be expressed and the liquid biofuel would be produced. This shift to a lower temperature has the key advantage of reducing the metabolism of the host. This is critical to an industrial process as it reduces the host maintenance energy and interference from the host's metabolism. These advantages are analogous to the goals of the emerging field of synthetic biology [118–120]. Rather than creating artificial organisms, this goal can be accomplished by a simple shift in temperature of a hyperthermophilic host containing a designed thermophilic process. The advantages of this temperature shift would be difficult to exploit in a mesophilic host, as it would require psychrophilic gene donors and would be limited by the freezing temperature of the growth medium. Finally, the fermentation process would be cycled back to a high temperature (90–100°C) for regeneration of the catalyst (re-growth of the strain) and simultaneous distillation of volatile fuel molecules [211] in preparation for another cycle of production formation (60–80°C) (Figure 4). These heating and cooling cycles would be accomplished using metabolically generated heat and

active air circulation, potentially requiring only small additional energy inputs.

Initial pilot projects for large-scale hyperthermophilic fuel production would be of similar size to current cellulosic ethanol pilot plants, which produce up to 25 × 10^6 gal $year^{-1}$[212] and require fermentation volumes on the order of 10^5–10^6 L. This scale can take advantage of heating through active metabolism as discussed above. Second-generation plants would likely be even larger, in order to take further advantage of economies of scale.

Future perspectives

While still in early stages, there are a number of promising directions for the use of thermophiles in solving problems with producing liquid transportation fuels and industrial chemicals. Certainly, other developments will positively impact this situation. Microbially-enabled consolidated bioprocessing of recalcitrant plant biomass for lignofuel production is an important opportunity, but other strategies are being investigated to make lignofuels relevant to a renewable future. For example, the engineering of plant biomass to reduce recalcitrance and advanced strategies for pre-treatment and carbohydrate extraction may prove vital to the production of lignocellulose-derived fuels. By improving carbon efficiencies and utilizing non-competing crops such as switchgrass or food crop waste such as corn stover, lignofuels are sure to play a role in the near and distant future of sustainable energy.

The development of electrofuels as an advanced strategy for biofuels has been a part of the expanding diversity of biotechnological research of carbon fixation pathways. Where plant-based biofuels rely only on the Calvin-Benson cycle and the enzyme Rubisco, the research on electrofuels has explored a variety of alternate carbon fixation pathways that nature has to offer. For example the 3-HP bicycle, the 3-HP/4-HB cycle, and the W/L pathway have also been exploited in electrofuels strategies. These strategies have seen varying degrees of success. Some, such as efforts by OPX Biotechnologies focusing on engineering bacteria for efficient fuel production (www.opxbio.com/), have attracted private sector investment. Others on liquid fuel from renewable electricity and bacteria have led to the formation of new companies. These success stories are part of the electrofuels program, which is funded by the Advanced Research Project Agency – Energy (ARPA-E: http://arpa-e.energy.gov/). ARPA-E is aimed at advancing high-risk and high-reward technologies to improve the US economic prosperity, national security, and environmental wellbeing. This strategy continues to be a success as ARPA-E projects have attracted more than $625 million in private funding to date. This agency will continue to drive advancements such as metabolic engineering and photovoltaics as a part of the upcoming revolution of renewable energy.

Acknowledgements

This work described here was supported in part by the US National Science Foundation (CBET-1264052 and CBET-1264053) and by the US Department of Energy (DOE) through the ARPA-E Electrofuels Program (DE-AR0000081), the Division of Chemical Sciences, Geosciences and Biosciences of the Office of Basic Energy Sciences (DE-FG05-95ER20175), and the BioEnergy Science Center (DE-PS02-06ER64304), a Bioenergy Research Center supported by the Office of Biological and Environmental Research in the DOE Office of Science. Oak Ridge National Laboratory is managed by UT-Battelle, LLC, for the DOE under Contract DE-AC05-00OR22725.

Executive summary

Background

- The need for renewable energy is paramount for sustaining our society.
- Current biofuel strategies utilize readily accessible sugars from food crops but have low carbon and energy efficiency as only a small portion of the plant can be utilized.

Lignofuels: utilizing recalcitrant plant biomass for biofuel production

- Utilizing recalcitrant lignocellulosic biomass for biofuels can eliminate the competition with food crops and improve the carbon efficiency of the process.
- Microbial fermentations could be used to convert ~60% of chemical energy of the plant, which is stored in the cellulose and hemicelluloses, into fuels.
- The genus *Caldicellulosiruptor*, a clade of extremely thermophilic, anaerobic bacteria, contains species that grow on unpretreated lignocellulosic materials.
- *Caldicellulosiruptor* sp. could be potentially metabolically engineered to produce advanced biofuels compatible with existing infrastructure, so-called lignofuels.

Electrofuels: using CO_2 directly as a carbon source for biofuel production

- Plant based biofuels depend on the photosynthetic capture of solar energy, which operates at approximately 1% energy efficiency on an annual basis, and poses a significant problem in scale up.
- Electrofuels exploit the direct conversion of fully oxidized inorganic carbon into advanced biofuels via the incorporation of low potential electrons from inorganic energy forms such as hydrogen gas or an electrical current.

(Continued)

Biological CO_2 fixation

- There are currently six pathways of CO_2 fixation known to occur in nature.
- Four are found in extreme thermophiles (T_{opt} ≥70°C) and produce acetyl-CoA as its product: the rTCA, W/L, DC/4-HB, and 3-HP/4-HB pathways.
- The pathways vary greatly in energy use and kinetics and the 3-HP/4-HB cycle requires only NADPH as an electron donor.

Progress toward the production of electrofuels in a hyperthermophile

- The hyperthermophile *Pyrococcus furiosus* is an attractive option for metabolic engineering via genetic modification.
- Its native hydrogen gas uptake system can be coupled to the 3-HP/4-HB cycle for carbon fixation via the compatible electron carrier NADPH.
- The first sub-pathway of the 3-HP/4-HB cycle has been expressed in *P. furiosus* and the key intermediate 3-HP has been produced.

Large-scale bioprocessing and elevated temperatures

- Metabolic energy can be harnessed to heat a hyperthermophilic process and air can be used for cooling due to the large temperature differential.
- Product formation will occur during a cooling phase where the host metabolic activity is reduced. This results in lower interference from the host's metabolism and decreased maintenance energy.
- Temperature cycling can simultaneously regenerate the host and potentially distill a volatile fuel product.

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