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Extremely thermophilic energy metabolisms: biotechnological prospects

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New strategies for metabolic engineering of extremely thermophilic microorganisms to produce bio-based fuels and chemicals could leverage pathways and physiological features resident in extreme thermophiles for improved outcomes. Furthermore, very recent advances in genetic tools for these microorganisms make it possible for them to serve as metabolic engineering hosts. Beyond providing a higher temperature alternative to mesophilic platforms, exploitation of strategic metabolic characteristics of high temperature microorganisms grants new opportunities for biotechnological products. This review considers recent developments in extreme thermophile biology as they relate to new horizons for energy biotechnology.

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Introduction

By the dawn of the 21st century, the metabolic basis for microbial life could be probed through the strategic application of modern (molecular genetics, functional genomics) and classical (laboratory pure culture) methods. Such endeavors were largely focused on what are now referred to as 'model organisms'. *Escherichia coli* (gram-negative), *Bacillus subtilis* (gram-positive) and *Saccharomyces cerevisiae* (eukaryote), among other mesophilic microorganisms, were examined with increasingly sophisticated experimental and computational tools to the point that prospects for synthetic life forms and minimal genomes could be considered [1]. While studies of model microorganisms laid the foundation for understanding the complex machinery and mechanisms of life, they only hinted at the diversity and unique niches within which life exists and develops. Now, as the circle of 'model' microorganisms expands with molecular genetic tools becoming more widespread [2], so too does the potential of microbial biotechnology. Extremophiles epitomize this vast potential, particularly those prospering at the upper thermal limits for life. The lens of modern biology facilitates a more discerning look at these microorganisms for new metabolic pathways and physiological features that can be exploited for technological benefits, especially as this relates to reducing the reliance upon fossil fuels to meet the energy demands of modern society. In this review, we consider recent developments in the biology and biotechnology of extreme thermophiles, microorganisms that grow optimally (T_{opt}) at and above 70°C, as these relate to bioenergy.

Genetics

To capture the full potential of extremely thermophilic microorganisms for technologically relevant applications, molecular genetic tools are needed. These are important not only for understanding the intrinsic basis of their metabolism and physiology, but to establish them as metabolic engineering platforms [3]. To this end, over the past decade, genetic systems for several extreme thermophiles have been developed and utilized in multiple laboratories, including *Sulfolobus* sp. (T_{opt} = ~80°C) [4–6], *Thermococcus kodakarensis* (T_{opt} = 85°C) [7], *Pyrococcus furiosus* (T_{opt} = 100°C) [8,9], *Thermus thermophilus* (T_{opt} = 72°C) [10], *Thermoanaerobacter mathranii* (T_{opt} = 75°C) [11] and *Caldicellulosiruptor bescii* (T_{opt} = 78°C) [12]. The COM1 strain of the hyperthermophilic archaeon *Pyrococcus furiosus*, which was isolated in the laboratory, exhibits natural competence with chromosomal insertion achieved for gene clusters as large as 17 kb [13•]. Recently, a high temperature kanamycin resistance-based selection system (originally developed in *Thermus thermophilus* [14]) was adapted for use in the extremely thermophilic bacterium *Caldicellulosiruptor bescii*, significantly improving genetic manipulations with this bacterium and opening the door for metabolic engineering efforts [15•]. Genetic tools for the extremely thermoacidophilic archaeon *Metallosphaera sedula* (T_{opt} = 75°C) [16] and the hyperthermophilic bacterium *Thermotoga* sp. (T_{opt} = ~80°C) [17,18] have also been reported but are in their early stages and not yet widely utilized. As genetic tools for extreme thermophiles become more versatile and reliable, new methods for genome editing (CRISPR) and promoter

design can be incorporated into metabolic engineering efforts, thereby accelerating development of these microorganisms for industrial biotechnology.

Impact of environmental factors on host metabolism

Microbial metabolic activity can be significantly influenced by environmental factors, creating a strategic opportunity for metabolic engineering. Extreme thermophiles could function as hosts for more moderately thermophilic enzymes and pathways, with the host metabolism operating in the 'background' while the heterologous pathways proceed. *P. furiosus* typically produces CO_2, acetate, and hydrogen from hexoses. Yet, insertion of a lactate dehydrogenase from *C. bescii*, an organism with optimal growth temperatures some 20°C below that of *P. furiosus*, enabled temperature-dependent gene regulation by insertion of the *ldh* gene downstream of the cold shock promoter. Lactate was not detected near the optimal temperature for *P. furiosus* (98°C), yet lactate (0.3 g/L) was produced near the optimal temperature of the heterologous enzyme (72°C) [19]. Efforts with *P. furiosus* to produce 3-hydroxypropionate (3HP), temperatures nearly 30°C below its optimal growth temperature were used in the production phase to minimize the background metabolism of the host [20]. In another study, metabolic profiling of *P. furiosus* revealed acetoin production (0.6 g/L) during growth at suboptimal temperatures (70–80°C), yet this compound was absent at its optimal growth temperature of 100°C. [21]. These results with *P. furiosus* illustrate the potential for temperature-based gene regulation, a metabolic engineering strategy closely aligned with extreme thermophily.

In addition to thermal gene regulation demonstrated in *P. furiosus*, other environmental changes can impact core metabolism, as was demonstrated through growth substrate changes in the metabolically versatile *Sulfolobus solfataricus* [22]. Toward that end, a genome-based metabolic network was constructed for *S. solfataricus* [23••], which was expanded and validated by comparing metabolite, transcript, and enzyme activity levels during growth on glucose and fucose [24]. Significant differences were noted between archaeal and bacterial core metabolism. A reversal of the ribulose-monophosphate pathway (involved in formaldehyde uptake in methanotrophs) was already known as an alternative to the pentose phosphate pathway in *S. solfataricus*, and in many other archaea [25]. This latest effort suggests that *S. solfataricus* uses a portion of the 3HP/4HB cycle in a manner similar to the glyoxylate shunt. *S. solfataricus* is able to obtain less biomass from growth on fucose than on glucose. To make up for this, it appears cells growing on fucose up-regulate enzymes of the 3HP/4HB cycle (resulting in a concomitant increase in chemical intermediates) in an attempt to replenish TCA cycle intermediates while minimizing loss of carbon during growth on this less nutritive sugar. These differences between archaea and bacteria will be important to understand, as archaea are considered further as metabolic engineering hosts.

Lignocellullose and biomass degradation

Efforts to transform native biomass, such as grasses and trees, into fuels and chemicals are hindered by the complex nature of lignocellulose and the plant cell wall structure. Most efforts to transform these feedstocks into commercial products rely upon chemical and thermal pretreatment, followed by addition of exogenous enzyme cocktails to produce fermentable hexoses and pentoses. As a further complication, many fermentative organisms, such as the moderate thermophile *Clostridium thermocellum*, utilize only hexose sugars [26].

Several species in the genus *Caldicellulosiruptor* not only degrade lignocellulosic biomass as a sole carbon source and access its carbohydrate content but also co-ferment hexoses and pentoses from complex polysaccharides [26]. Unique to the genus are a suite of glycoside hydrolases, polysaccharide lyases, and carbohydrate esterases, many of which are modular, multi-domain enzymes [27]. A number of these enzymes have been characterized in detail to determine substrate specificity, kinetics, and importance to the overall complex process of accessing the carbohydrate content of lignocellulosic feedstocks [28]. One such multi-domain enzyme has been demonstrated as the most potent cellulolytic enzyme identified to date, with seven times higher activity compared to the standard exo- and endo-cellullase mixture [29••]. These features provided the basis a system in which biomass is directly converted to products of interest without the expense of significant pre-treatment and exogenous enzyme additions that adversely impact the economics of lignocellulosic feedstock processes.

Marine-based substrates for bio-based fuels and chemicals, such as algae and related seaweeds, are much less recalcitrant due to their lack of lignin and crystalline cellulose. The moderately thermophilic marine bacterium *Defluviitalea phaphyphila* (T_{opt} 60°C) produced ethanol (10 g/L) at yields approaching 50% of theoretical from brown algae [30•,31]. The ability to metabolize alginate, laminarin, and mannitol from the algae demonstrates that marine-based photosynthetic carbon sources could be renewable feedstocks, potentially avoiding land use conflicts.

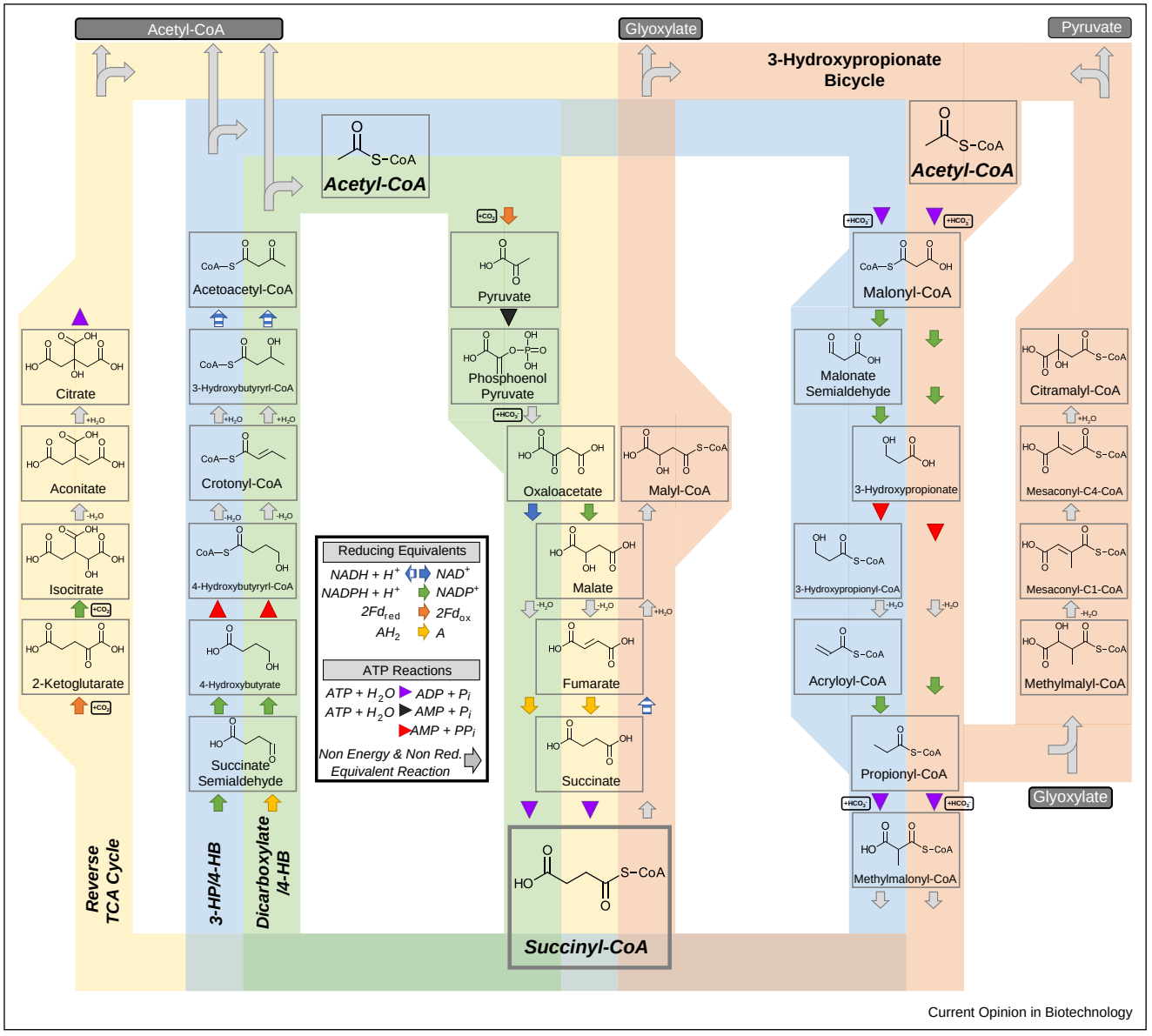
Carbon dioxide fixation

Two of the six presently known CO_2 fixation pathways are found exclusively in extremely thermoacidophilic archaea. *Metallosphaera sedula* utilizes the 3-hydroxypropionate/4-hydroxybutyrate (3HP/4HB) cycle [32], while *Ignicoccus hospitalis* (T_{opt} = 90°C) contains the closely related dicarboxylate/4-hydroxybutyrate cycle [33]. These cycles, related to both the reverse TCA cycle found in many thermophiles and the 3-hydroxypropionate cycle

first identified in the thermophilic green non-sulfur bacterium *Chloroflexus aurantiacus* (T_{opt} = 55°C), provide opportunities for products as pathway intermediates (see Figure 1) as well as the potential to incorporate CO_2. To this end, a reaction kinetic model was recently reported for the 3HB/4HB cycle as it appears in the Sulfolobales which was employed to evaluate metabolic engineering options for using all or parts of the cycle to produce bio-based chemicals [34].

Fixation of CO_2 (either from the atmosphere or from industrial emissions) to create renewable chemicals is a global, aspirational goal to address and reduce greenhouse gas emissions. Plants and algae as renewable feedstocks rely upon the Calvin–Benson–Bassham (CBB) carbon fixation cycle. However, other fixation pathways present opportunities to incorporate CO_2 directly into fuels and chemicals, either bypassing carbohydrates as intermediates or supplementing the carbohydrates for improved mass yield. Incorporation and reduction of CO_2 requires significant energy input, which is often provided through chemolithoautotrophic growth in certain extreme thermophiles. Utilizing a CO_2 incorporation step in a metabolically engineered pathway can be most advantageous

Figure 1



Four carbon fixation cycles found in thermophilic organisms: reverse TCA cycle (yellow), 3-hydroxypropionate/4-hydroxbutyrate (blue), dicarboxylate/4-hydroxybutyrate (green), and 3-hydroxypropionate bicycle (orange).
*AH_2 indicates electron carrier (unknown or varies by organism). A represents its oxidized counterpart.
**CoA inclusion and dissociation reactions not explicitly shown.

for products containing highly-oxidized, functional groups (*e.g.*, succinic acid) that maintain carbon in a highly oxidized state. This approach minimizes energy and redox inputs that would otherwise be required to incorporate carbon in a more reduced state. Along these lines, recombinant *P. furiosus* strains have been created that express five genes encoding three enzymes from the *M. sedula* 3HP/4HB cycle to produce 3-hydroxypropionate from CO_2 and maltose [35,36]. While the incorporation of CO_2 occurs at the expense of ATP and is followed by two NADPH-dependent steps, traditional Embden–Meyerhof–Parnas glycolysis generates two ATP and four reducing equivalents from glucose, thus creating an energy and redox neutral pathway if reductases are present to transfer electrons to NADPH.

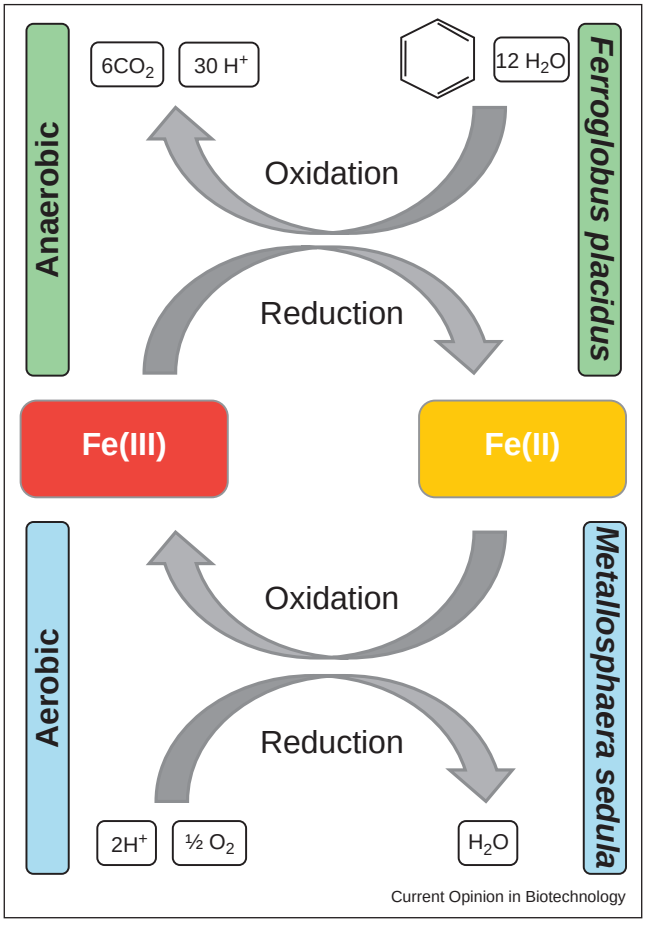
Iron and sulfur oxidation for chemolithoautotrophy

Most metabolic engineering efforts are centered upon the fermentation of saccharides, such that energy and reducing equivalents are obtained through central metabolism and pyruvate is converted to a desired metabolite. However, there exist other options for cellular bioenergetics. Thermoacidophiles inhabit environments that are often devoid of organic carbon and oxidize metals and sulfur to provide the reducing potential and energy for autotrophy. By oxidizing Fe(II) to Fe(III), organisms such as *Metallosphaera sedula*, can generate energy and reducing equivalents required to grow autotrophically. As the thermodynamics of carbon fixation require many Fe(II) atoms in order to fix a single carbon, relying solely upon Fe(II) oxidation presents a challenge to conceiving a viable biotechnological process with reduced metal as the energy source.

Metal species can also act as important electron sinks in certain environments where electron acceptors, such as oxygen and sulfur, are absent. The thermoacidophilic archaeon, *Ferroglobus placidus* (T_{opt} 85°C), has been shown to utilize benzene as the electron donor with its oxidation coupled to the reduction of Fe(III) to Fe(II) [37]. While the pathways of aromatic degradation were not completely elucidated, evidence was provided to confirm benzene consumption and reduction of Fe(III) as the sole means of energy metabolism. By combining both iron oxidation and reduction in a cycle (Figure 2), the potential exists to utilize complex aromatic substances, such as lignin, as a substrate for production of chemicals [38].

In addition to iron oxidation, many extreme thermoacidophiles grow by the oxidation of metal sulfides (*i.e.*, pyrite and chalcopyrite) [39,40] and elemental sulfur [41]. Growth on metallic ores is strategic for acidophiles, as the low pH solubilizes the metal substrate. On the other hand, elemental sulfur is sparingly soluble, and low pH prevents abiotic formation of more soluble sulfur compounds, such as thiosulfate and polysulfides [42]. An acidophilic solution to sulfur oxidation, first identified in thermoacidophilic archaea [43], is cytoplasmic sulfur oxygenase reductase (SOR), which has also been reported recently in acidophilic bacteria [44]. In archaea, SOR seems to be essential for growth on elemental sulfur, although species that lack SOR may be able to grow on other sulfur compounds.

Figure 2



Iron as an electron donor and acceptor by acidothermophiles. Aerobic oxidation of Fe^{2+} by *M. sedula* allows the fixation of carbon dioxide. Anaerobic reduction of Fe^{3+} by *F. placidus* permits oxidation of reduced carbon in benzene [39].

Carbon monoxide as source of reducing power

The utilization of CO (typically from a syngas stream) by microorganisms has been considered for some time as a route to bio-based products. While certain anaerobic microorganisms can utilize CO via the Wood–Ljungdahl pathway (reductive acetyl-CoA), the ability to engineer in cofactors necessary for this pathway adds complexity to the metabolic engineering effort. However, other options exist to utilize CO. *P. furiosus* has recently been

engineered with a 16-gene cluster encoding CO dehydrogenase/hydrogenase from *Thermococcus onnurineus*, allowing the generation of an ion-gradient via the oxidation of CO to CO_2 coupled to the evolution of H_2 [45].

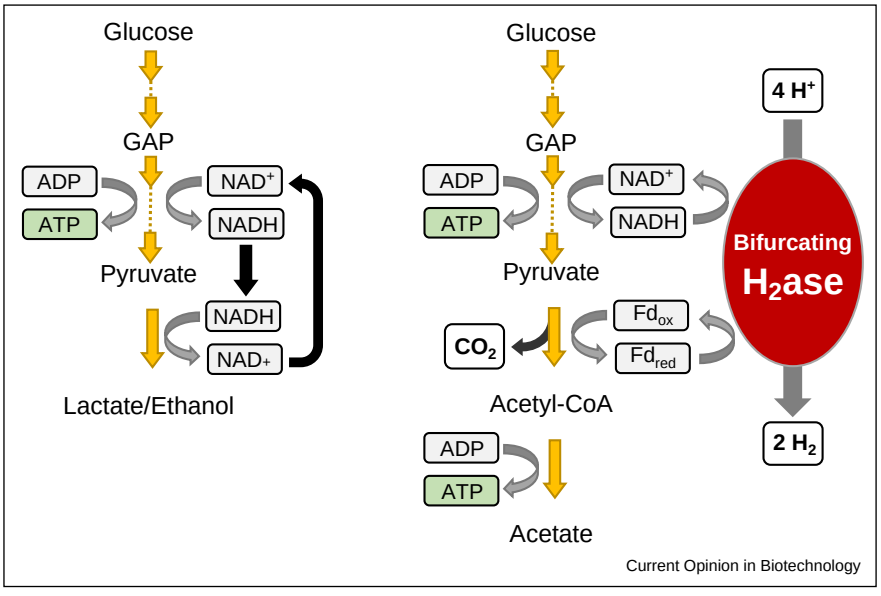
P. furiosus can conserve energy from the ion gradient via an ATP synthase, thus permitting the oxidation of CO to act as the primary energy source. This pathway does not fix carbon and thus a complex carbon source (maltose or peptides) is necessary to provide for biosynthesis. Since *P. furiosus* typically produces acetate as a metabolic by-product from these complex carbon sources, the demonstration of acetate consumption during growth on CO indicates the reverse reaction with regeneration of acetyl-CoA. In another interesting study, a two-step process with *T. onnurineus* and *Thermoanaerobacter kivui* (T_{opt} 65°C) converted a CO-based gas mixture, typical of steel mill waste streams, to acetate. In the first step, *T. onnurineus* converted 70% of the CO to CO_2 and H_2, which was then fed to *Thermoanaerobacter kivui* (T_{opt} 65°C), obtaining acetate (~0.5 g/L) [46]. The two-stage process, utilizing a carboxytrophic thermophilic archaeon and an autoacetogenic thermophilic bacterium, beneficially exploits their native capabilities toward transferring waste syngas-like streams into chemicals. Further genetic engineering efforts may permit more interesting products to be generated in such processes.

Hydrogen/hydrogenases/bifurcation

Many microorganisms, including extreme thermophiles, generate hydrogen gas to recycle reducing equivalents generated during oxidative metabolism. The thermodynamics of this reaction at higher temperatures can be advantageous. A temperature change from mesophilic conditions (37°C) to hyperthermophilic environments (100°C) increases the entropy term of the Gibbs free energy equation ($\Delta G = \Delta H - T \times \Delta S$) by 20%. Given that the majority of biological reactions operate close to equilibrium, this can transform a reaction that may be endergonic at mesophilic conditions to one that is exergonic and favorable at thermophilic conditions. Such is the case with many hydrogenases, since H_2 production from reducing equivalents becomes much more favorable at elevated temperatures [47]. An excellent example is the case of formate oxidation to hydrogen by the archaeon *Thermococcus onnurineus* (T_{opt} 85°C). At ambient temperatures the reduction potentials of the formate/CO_2 and H^+/H_2 couples are almost identical but at 85°C hydrogen production is favored and the organism is able to conserve energy for growth [48].

In fermentative metabolism, the standard EMP pathway generates NADH and the subsequent conversion of pyruvate to acetyl-CoA provides reduced ferredoxin in many thermophilic organisms. The low potential of the ferredoxin (E_m typically near −500 mV) allows molecular hydrogen to be generated ($H_2/2H^+ = -420$ mV) directly by ferredoxin-dependent hydrogenases. However, it is not energetically favorable for NADH ($E_m = -320$ mV) to be used as an electron donor for H_2 production. Thus, NADH oxidation must often be coupled to the production of reduced carbon products, such as lactate or ethanol

Figure 3



(Left) Electrons from reduced electron carriers require high potential electron acceptor in the form of pyruvate or an aldehyde, resulting in energy yield of 2 ATP per glucose. (Right) Bifurcating hydrogenase allows energy yield of 4 ATP per glucose by generation of hydrogen from reduced electron carriers [49].

from pyruvate. The necessity for an electron acceptor mandates that pyruvate, or a downstream product from pyruvate (*e.g.*, acetaldehyde), be dedicated to this purpose. This prevents the ATP formation from pyruvate to acetate, resulting in a less energetically favorable metabolism (Figure 3).

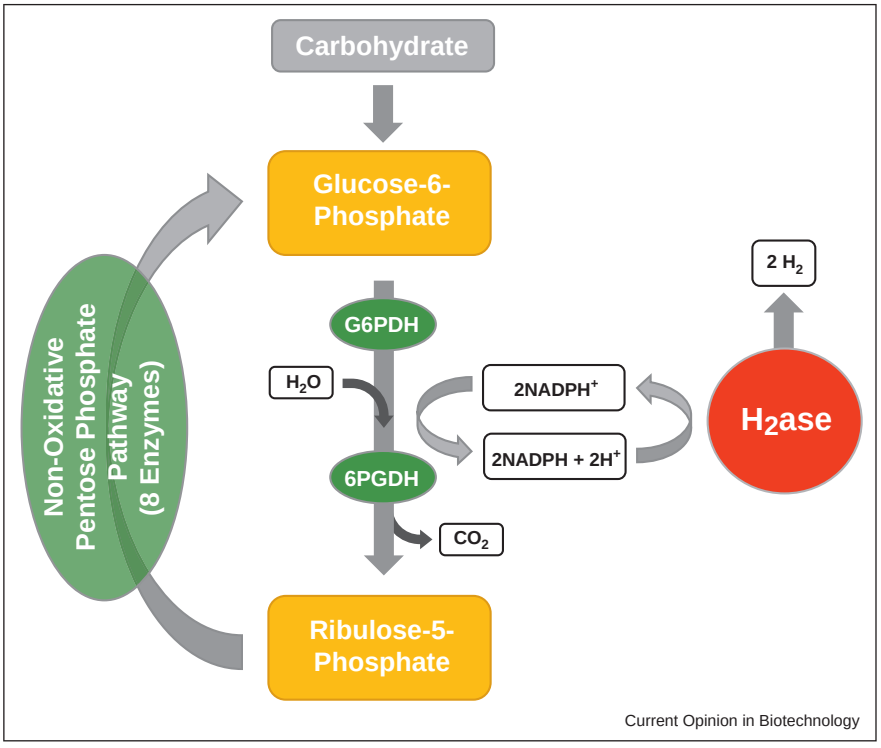
In some microorganisms this problem is overcome by a so-called bifurcating hydrogenase, which was first discovered in the extremely thermophilic bacterium *Thermotoga maritima* [49]. This enzyme uses reduced ferredoxin and NADH simultaneously to produce H_2. In essence, the enzyme uses the excess energy of ferredoxin oxidation to drive the endergonic oxidation of NADH. As shown in Figure 3, this has very important consequences in fermentation, as now all of the reductant generated from glucose oxidation can be used to produce H_2 at the so-called Thauer limit (4 H_2/glucose) where the maximum amount of free energy is conserved (4 ATP/glucose). It is estimated that about one-third of all ferredoxin-dependent hydrogenases are bifurcating enzymes [50].

The complete oxidation of glucose to CO_2 theoretically yields 12 mol H_2/mol glucose, but fermentative pathways yield a maximum of 4 mol H_2/mol glucose. Because of the necessary energy conservation in the form of ATP, acetate rather than CO_2 is generated. The Thauer limit clearly presents a barrier to biohydrogen production processes [51]. To address this limitation, an *in vitro* pathway was constructed by recruiting thirteen enzymes from six thermophilic organisms, focusing on oxidative and non-oxidative components of the pentose phosphate pathway (Figure 4) [52••]. This achieved nearly the theoretical yield of 12 mol H_2/mol glucose (sucrose), or three times the Thauer limit.

Alcohols

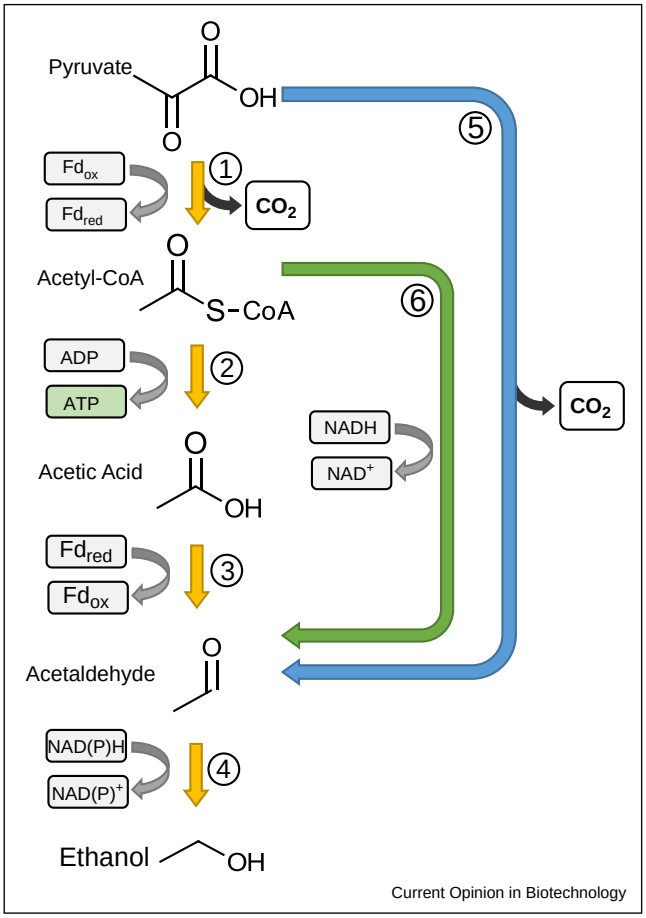
Many ethanologenic organisms, such as *Saccharomyces cerevisiae*, obtain 2 ATP per glucose through traditional EMP glycolysis. The resulting pyruvate is decarboxylated via pyruvate decarboxylase complex (PDC) to acetaldehyde, which is subsequently reduced by NADH to ethanol (Figure 5). However, a thermophilic PDC, while of great potential importance, has not yet been reported. Thus most thermophilic native ethanol-producing organisms follow a three-step pathway from pyruvate to ethanol such as has been demonstrated by moderately thermophilic *Clostridium thermocellum* (T_{opt} 60°C). An ethanol titer of 38 g/L was achieved by removal of three genes that redirected carbon flux away from the organism's other native products, acetate and lactate [53].

Figure 4



In vitro route from carbohydrates to hydrogen at near theoretical yield (24 mol H_2 per dihexose) utilizing eleven thermophilic enzymes recruited from four different species [52••].
*Two mesophilic and two thermophilic enzymes, all from different species, were utilized for conversion of sucrose to glucose-6-phosphate (not shown).

Figure 5



Three routes from pyruvate to ethanol: four enzyme, ATP-generating pathway utilizing single step reduction of acetate to acetaldehyde (1–4—yellow), two enzyme pathway utilizing direct decarboxylation of pyruvate to acetaldehyde (5 and 4—blue), and three enzyme pathway with reduced ferredoxin generated during pyruvate to acetyl-CoA step followed by consumption of NADH during conversion to acetaldehyde. (1, 6, and 4—green).
Acetyl-CoA synthetase (ACS) (1), acetyl-CoA dehydrogenase (ACD) (2), aldehyde oxidoreductase (AOR) (3), alcohol dehydrogenase (AdhA) (4), pyruvate decarboxylase complex (PDC) (5), and acetaldehyde dehydrogenase (ALDH) (6).

While classical EMP provides a net ATP gain from glucose, it sacrifices the ATP-yielding hydrolysis of acetyl-CoA as it requires that pyruvate (via acetaldehyde) be utilized as an electron sink for NAD^+ recycling. By utilizing a unique aldehyde oxidoreductase (AOR) from *P. furiosus*, acetate can be reduced to ethanol with the reducing equivalents available from the pathway. This enzyme is capable of reducing organic acids to aldehydes, utilizing electrons from the low potential electron carrier ferredoxin. Through a NADPH-dependent alcohol dehydrogenase, these aldehydes are subsequently converted to alcohol with high efficiency. This pathway can be extended to other metabolic engineering efforts to convert organic acids to alcohols, since both the AOR and the ADH demonstrated broad substrate activity [54••]. In theory, this pathway allows an ethanol-producing organism with EMP central glycolysis to obtain 4 ATP from glucose to ethanol, thereby doubling the energy yield of most ethanologens.

P. furiosus was also engineered to produce *n*-butanol, utilizing a template pathway from *Clostridium acetobutylicum* [36]. By recruiting seven enzymes from four different thermophilic species, *n*-butanol (~60 mg/L) production was demonstrated. Complementary to this effort, a reaction kinetic model provided insights into optimal enzyme concentrations and promoter engineering for improved *n*-butanol production and selectivity [55].

As mentioned previously, *Caldicellulosiruptor* species utilize the carbohydrate content of lignocellulosic biomass, which is metabolized to acetate, lactate and hydrogen. *C. bescii* was engineered to eliminate lactate formation while producing ethanol, through the insertion of an NADH-dependent bi-functional acetaldehyde/alcohol dehydrogenase from *C. thermocellum* for operation at 65°C. Growth on untreated switchgrass yielded 0.6 g/L ethanol, demonstrating direct conversion of lignocellulose to product without pre-treatment nor exogenous enzyme additions [56]. The NAD(P)H-dependent acetaldehyde/alcohol dehydrogenases from *Thermoanaerobacter pseudethanolicus* were similarly engineered into *C. bescii* to more closely match enzyme thermoactivity with the optimal temperature of the host. While the operating temperature (75°C) may have improved the host metabolism, the comparatively lower ethanol concentrations (0.1 g/L) were likely a result of the limited availability of NAD(P)H [57].

Directing carbon flux away from organic acid production is critical for yield considerations in metabolic engineering efforts. Furthermore, organic acids can be toxic at low concentrations and thus even low concentrations that may be acceptable to yield may prevent the organism from reaching its potential alcohol titer. These undesired by-products, such as acetate, can significantly inhibit growth, yet complete removal of the pathways for these compounds may be infeasible for various reasons. To address these problems, detoxification by metabolite conversion to a less toxic product has been demonstrated in *Thermoanaerobacterium saccharolyticum* (T_{opt} 60°C). By insertion of a three-enzyme heterologous pathway, the low levels of acetate were converted to acetone, thereby removing the majority of acetate inhibition. This resulted in a threefold improvement in ethanol concentrations to 45 g/L, an industrially relevant titer [58].

Conclusions

Not only do extreme thermophiles provide a source of unconventional metabolic pathways, carbon fixation pathways, and ability to metabolize diverse substrates, there are also additional benefits related to thermodynamics at

higher temperatures. As such, extremely thermophilic microorganisms can be valuable resources for addressing the current metabolic engineering barriers. By deliberate and structured design of pathways inspired by these non-model organisms, there now exist new opportunities for commercially relevant processes. Many of these metabolic features have been validated as a 'proof-of-concept', so it remains to be seen whether and how extreme thermophiles will fit into the emerging industrial biotechnology sector.

Acknowledgements

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- • of special interest
- •• of outstanding interest

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