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Extreme thermophiles: moving beyond single-enzyme biocatalysis

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Extremely thermophilic microorganisms have been sources of thermostable and thermoactive enzymes for over 30 years. However, information and insights gained from genome sequences, in conjunction with new tools for molecular genetics, have opened up exciting new possibilities for biotechnological opportunities based on extreme thermophiles that go beyond single-step biotransformations. Although the pace for discovering novel microorganisms has slowed over the past two decades, genome sequence data have provided clues to novel biomolecules and metabolic pathways, which can be mined for a range of new applications. Furthermore, recent advances in molecular genetics for extreme thermophiles have made metabolic engineering for high temperature applications a reality.

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Introduction

Microbial life in terrestrial hot springs, such as those present in Yellowstone National Park (USA), has been observed and studied in earnest since at least as early as the 1960s [1–3]. However, the discovery of microbial life in deep sea thermal vents and shallow marine seeps in volcanic regions of the world in the late 1970s and early 1980s [4–6] led to the realization that the so called *extreme thermophiles* ($T_{\text{opt}} \geq 70$ °C) were more phylogenetically, physiologically and geographically diverse than first thought. Many novel genera and species, both archaea and bacteria, were isolated and described, including several that became model microorganisms because of their interesting metabolic features and relative ease of cultivation in laboratory settings (see Table 1). Three archaea, *Pyrococcus furiosus*, a marine fermentative anaerobe (T_{opt} 98–100 °C) [5,7], *Thermococcus kodakaerensis* (T_{opt} 85 °C) also a marine

fermentative anaerobe [8,9], and *Sulfolobus solfataricus*, a terrestrial heterotrophic acidophilic aerobe (T_{opt} 80 °C, pH_{opt} 3.5) [10,11], and one bacterium, *Thermotoga maritima*, a marine fermentative anaerobic bacterium (T_{opt} 80 °C) [6,12,13], became the focus of most fundamental and biotechnological studies addressing life at high temperatures. In fact, much of what is known about extreme thermophile physiology and enzymology to date has been based on the study of these ‘model’ microorganisms (subclassified as *hyperthermophiles* because of their $T_{\text{opt}} \geq 80$ °C). Recently, as discussed below, genetic systems have been established for the three archaea, further enhancing their value as model systems.

Difficulties in isolating extreme thermophiles because of the harsh and often inaccessible environments from which they come, and subsequently cultivating these microorganisms in laboratory settings, initially presented significant challenges to their study and, consequently, to associated biotechnological applications. In the early 1990s, however, successful attempts to clone and express genes from hyperthermophiles in mesophilic recombinant hosts (e.g. *Escherichia coli*) facilitated efforts to produce specific enzymes for characterization and application [14,15]. Furthermore, since 1995, when the genome sequence of the hyperthermophile *Methanocaldococcus jannaschii* was reported [16], related efforts for many high temperature microorganisms have enabled and accelerated projects to not only identify promising biocatalysts for single-step biotransformations but also to discover metabolic pathways, cellular features, and biological phenomena that are relevant to biotechnology. Now, armed with virtually unlimited access to genome sequence data, and aided by molecular genetics and ‘omics’ tools, the prospects for biotechnology at elevated temperatures have never been more promising, and poised to go beyond single-step biocatalysts (i.e. the use of a single enzyme for a single biotransformation).

Isolation of novel extreme thermophiles

For the most part, the isolation of many currently known and studied extremely thermophilic microorganisms happened in the late 1970s through the early ‘90s [17,18], although isolation of several *Sulfolobus* species was reported in the early 1970s [19]. Description of new isolates continues to appear in the literature, but most often reside within genera of previously studied extreme thermophiles. As such, discovery of significantly different extremely thermophilic genera and species is becoming rare. Coupled to this is the fact that the criteria for

2 Biotechnology and bioprocess engineering

Table 1

Model extreme thermophiles						
Organism	Isolation site	T_{opt} (°C)	Domain	Genome size (kb)	Growth physiology	PubMed Citations (May, 2012)
<i>Sulfolobus solfataricus</i> P2	solfatara, Naples, Italy	80 °C	Crenarchaea	2,992,245	Aerobic, extreme thermoacidophile	1252
<i>Thermotoga maritima</i> MSB8	shallow marine sediments, Vulcano Island, Italy	80 °C	Bacteria	1,860,725	Fermentative anaerobe, facultative S ^o reducer	1148
<i>Thermococcus kodakaerensis</i> KOD1	solfatara, Kodakara Island, Japan	85 °C	Euryarchaea	2,088,737	Fermentative anaerobe, facultative S ^o reducer	156
<i>Pyrococcus furiosus</i>	shallow marine sediments, Vulcano Island, Italy	98 °C	Euryarchaea	1,908,255	Fermentative anaerobe, facultative S ^o reducer	1047

designating a new isolate as ‘novel’ have become more stringent, given the availability of genome sequences and related quantitative measures for differentiating among microorganisms. Thus, new reports of truly novel extreme thermophiles based on more than marginal differences in 16S rRNA phylogeny and subtle variations in growth physiology are infrequent.

Nonetheless, interesting isolates continue to be reported. In the last several years, several new fermentative anaerobes have been isolated (Table 2). For example, *Acidilobus saccharovorans* [20,21], a terrestrial thermoacidophilic crenarchaeon (T_{opt} 80–85 °C, pH_{opt} 3.5–4) contributes to closure of the anaerobic carbon cycle in terrestrial hot springs by complete oxidation of organic compounds (acetate, ethanol, and lactate). Also, *Pyrococcus yayanosii* is one of only a handful of microbes (and the only *Pyrococcus* species) shown to be an obligate piezophile (T_{opt} 98 °C, P_{opt} 52 MPa); growth was not observed at atmospheric pressure, but rather between 20 and 120 MPa [22].

One promising approach to the isolation of novel microorganisms from high temperature environments is to consider mixed cultures and consortia. For example, a three-species archaeal consortium, capable of deconstructing crystalline cellulose, was recently described [23^{••}]. This consortium, with members related to archaea from the genera *Ignisphaera*, *Thermofilum*, and *Pyrobaculum*, was able to partially dissolve filter paper after incubating for 30 days. In other cases, inter-species interactions are critical for isolation of new extreme thermophiles. Along these lines, the discovery of nanoarchaea was reported, occurring as parasitic partners with the extremely thermophilic archaeon, *Ignicoccus hospitalis* [24]. Evidence from genome sequence data of lateral gene transfer between *Nanoarchaeum equitans* and *I. hospitalis* was linked to how these two microorganisms adapted to growth by sulfur–H₂ respiration coupled to inorganic carbon and nitrogen fixation. As DNA sequencing rates increase and costs continue to decrease, metagenomes from thermal environments will be examined for hints to unusual physiologies [25], as well

Table 2

Recently described extreme thermophiles						
Organism	T_{opt} (°C)	16S rRNA identity to closest relative	Isolation site	Metabolism	Notes	Ref
<i>Aciduliprofundum boonei</i>	70	83	Deep sea hydrothermal vent	Anaerobic fermentation	First obligate thermoacidophile from deep sea vents	[89]
<i>Nanoarchaeum equitans</i>	70–98	81	Submarine hydrothermal vent	Parasitism	Small (0.5 Mb) genome, parasite of <i>Ignicoccus hospitalis</i>	[90]
<i>Methanocaldococcus villosus</i>	80	95	Submarine hydrothermal vent	Chemolithoautotrophy	Unique striated cell surface pattern observed	[91]
<i>Geoglobus acetivorans</i>	81	97	Deep sea hydrothermal vent	Chemolithoautotrophy	1st hyperthermophile enriched on acetate as e ⁻ donor	[92]
<i>Thermogladius shockii</i>	84	96	Hot spring	Anaerobic fermentation	Unlike close relatives, growth unaffected by sulfur	[93]
<i>Desulfurococcus kamchatkensis</i>	85	98.1	Hot spring	Anaerobic fermentation	Can use keratin as sole carbon and energy source	[94]
<i>Pyrococcus yayanosii</i>	98	99.4	Deep sea hydrothermal vent	Anaerobic fermentation	Obligate piezophile	[22]
<i>Acidilobus saccharovorans</i>	80–85	98.1	Hot spring	Anaerobic fermentation	Unlike <i>A. aceticus</i> , can use monosaccharides and disaccharides	[21]

as clues to new enzymes and metabolic pathways, the components of which can be produced recombinantly (perhaps, in extremely thermophilic hosts) for further analysis.

Genomics of extreme thermophiles

As mentioned above, access to genome sequence information for extreme thermophiles (which has facilitated functional genomics, proteomics and other 'omics-based' approaches) has enabled rapid advances in our understanding of these microbes' physiology over the past 15 years, despite the lack of genetic systems. In some cases, genome sequence information has been used to ask global questions about how thermophilic proteins fold and function at high temperatures [26]. Along these lines, lower levels of structural disorder and functional simplification determined at the level of individual genes and proteins, as well as of whole genomes, was proposed as the basis for prokaryotic thermophily [27]. Genome sequence information led to the provocative proposal that high temperature bacteria and archaea have lower spontaneous mutation rates than mesophiles [28]. The relationship between mesophiles and thermophiles has been given new perspective through comparative genomics. For example, reverse gyrase, an enzyme involved in thermophilic transcriptional processes to deal with uncoiling DNA, was once thought to be a defining feature of extreme thermophiles [29,30]. However, the genome sequence of *Nautilla profundicola* (T_{opt} 40–45 °C) encodes the gene for this enzyme, probably acquired through lateral gene transfer within the submarine hydrothermal environment that it inhabits [31]. The phylogenetic lines between thermophily and mesophily may be more blurred than expected. Mesophilic members of the Order Thermotogales (or 'mesotogas') were recently identified [32], as well as related species that have very broad growth temperature ranges [33], raising questions of about the thermal direction of microbial evolution. It is also clear that extreme thermophiles from the same genus can have differences in their genome sequences that map to subtle but significant differences in their growth physiology [34]. Finally, examination of the *P. furiosus* and *S. solfataricus* proteomes, with respect to metal content, revealed a far more extensive set of metals implicated in protein structure and function, including 'non-biological' metals, such as uranium and vanadium [35]. Since similar results were found for *E. coli*, it seems that the microbial world employs more of the periodic table for biological function than previously thought.

In certain cases, genome sequences of extreme thermophiles have revealed physiological insights not previously known, despite years of microbiological study. The genome sequence of *Metallosphaera sedula*, an extremely thermoacidophilic archaeon (T_{opt} 73 °C, pH_{opt} 2.0) [36], originally isolated and studied for its ability to mobilize metals from sulfidic ores [37], confirmed the presence of a

novel CO₂ fixation pathway (3-Hydroxypropionate/4-Hydroxybutyrate cycle) [38]. Consequently, this archaeon possesses a much more versatile growth physiology than previously thought [39]. In fact, *M. sedula* bioenergetics can be fueled by CO₂/H₂ autotrophy, heterotrophy, and metal/sulfur oxidation, separately or in combination (mixotrophy) [39].

Genome sequencing has also brought renewed interest to extreme thermophiles that had been isolated 20 years ago. For example, the genome sequences of *Caldicellulosiruptor saccharolyticus* [40,41] and *Caldicellulosiruptor bescii* [42] (formerly *Anaerocellum thermophilum*) shed new light on microbial mechanisms [43,44] and enzymology [45–47] of lignocellulose deconstruction. Furthermore, comparative genomics of eight *Caldicellulosiruptor* species revealed key determinants of lignocellulose degradation, based on the core and pan genomes of this genus [48].

Molecular genetics tools for extreme thermophiles

As mentioned above, studies of extreme thermophiles, and hence biotechnological applications, have been hampered to a certain extent by the limited availability of tools for genetic manipulation and metabolic engineering. Selection of thermostable markers, high temperature solid media, need for anaerobic conditions, and lack of defined media are among the challenges faced. Despite these obstacles, in recent years new molecular genetics systems have been developed for several extreme thermophiles, as well as being refined and expanded in cases where these had previously existed [49,50].

Initial success with molecular genetics in extreme thermophiles was achieved with the extremely thermoacidophilic archaeon *S. solfataricus*. The fact that this archaeon grows aerobically and can be cultivated on solid media no doubt contributed to progress in this regard. Most genetic manipulation in this species has utilized *lacS* mutants [51,52]. DNA is introduced to the cell via electroporation and growth on lactose (which requires *lacS* complementation) is used to select for successful transformation. This strategy has been used as the basis to generate deletion mutants for the study of copper response [53,54], toxin-antitoxin pairs [55], and antimicrobial proteins [56]. In addition, virus-based vectors encoding genes under the control of arabinose-inducible and heat-inducible promoters have been used to overexpress proteins in *S. solfataricus* [57,58], and reporter systems for monitoring gene expression have been developed based on β -galactosidase (*lacS*) [58] and β -glucuronidase (*gusB*) [59].

In recent years, success with molecular genetics has gone beyond *Sulfolobus* species. Development of genetic techniques for euryarchaeal Thermococcales has been facilitated by isolation of naturally competent strains of *T. kodakaraensis* and *P. furiosus* [60,61]. Manipulation

4 Biotechnology and bioprocess engineering

of both species initially hinged upon *pyrF*, the gene encoding orotidine-5'-monophosphate (OMP) decarboxylase; 5-fluoroorotic acid (5-FOA) can be used to select for *pyrF* mutants, because cells with the functional gene are sensitive to this compound. On the contrary, media lacking uracil can be used to select for complementation of *pyrF*, as the gene is required for uracil biosynthesis. Once *pyrF* mutants of *P. furiosus* and *T. kodakaraensis* were generated [60,62], methods were developed for performing selections in complex media [63,64], overexpressing proteins, secreting proteins [65], and generating 'markerless' deletions that allow multiple manipulations [62,66].

These tools have facilitated more targeted investigation of fermentative H₂ metabolism, a defining feature of *Pyrococcus* and *Thermococcus* species. One problem, central to understanding hydrogenase function, is producing sufficient amounts of active forms of the enzyme to study and evaluate. This issue was addressed for soluble hydrogenase I (SHI) from *P. furiosus*, which was recombinantly produced in *E. coli* by using an anaerobically driven promoter native to this bacterium [67]. Furthermore, an engineered hydrogenase, consisting of two of the four native subunits, was overexpressed heterologously in the native host, *P. furiosus*, and found to utilize electrons directly from pyruvate ferredoxin oxidoreductase without the involvement of an intermediate electron carrier (NADPH or ferredoxin) [68]. There are biotechnological implications of this work. Hydrogen production is normally growth-associated, but an electron carrier-independent hydrogenase might partially decouple these processes, resulting in higher yields of hydrogen.

Directed gene knockouts have been pivotal in probing metabolic mechanisms related to H₂ generation in *P. furiosus* [69] and *T. kodakaraensis* [70,71]. Deletion of *surR*, which encodes a transcriptional regulator, revealed that this gene is required for expression of the membrane-bound hydrogenase [71], the primary H₂-evolving enzyme [70]. Deletion of cytosolic hydrogenases limited growth of the microbe, but increased specific H₂ production rates, suggesting that re-oxidation of H₂ by these enzymes is an important energy conservation mechanism in this species [70,71]. Deletion of the membrane-bound oxidoreductase complex, which is required for sulfide production [69,70], also resulted in increased specific hydrogen production [69,70,71].

Biocatalysis at elevated temperatures

To date, the largest impact of enzymes from extreme thermophiles have had on science and technology relates to their use in catalyzing the polymerase chain reaction (PCR) [72]. Beyond that, the first thoughts for biotechnological applications for enzymes from these extremophiles turned to thermostable and thermoactive 'drop in'

replacements for industrial enzymes already in use (e.g. proteases, amylases, glucose isomerases). Several insightful reviews have appeared over the past 25 years that examine scientific and biotechnological aspects of biocatalysis at elevated temperatures (for a recent excellent review see [73]). One of the opportunities afforded by biocatalysts that function at temperatures approaching and exceeding 100 °C is the exploitation of the intrinsic features that underlie their unprecedented thermostability and thermoactivity. In this sense, the key strategic questions to consider are whether the biocatalytic process requires high temperatures, and, as a consequence, if high temperatures confer any strategic advantage.

The structural stability of enzymes from extreme thermophiles makes them attractive candidates for protein engineering, based on the premise that these are less susceptible to undesired consequences from genetic manipulations. The extensive protein engineering of β -glycosidases from *S. solfataricus* to catalyze chemo-enzymatic synthesis of oligosaccharides serves as an excellent example in this regard [74]. In another case, cofactor specificity of an alcohol dehydrogenase from *P. furiosus* could be modified by site-directed mutations in the co-factor binding pocket [75]. Building on the theme of enzymes from extreme thermophiles as robust protein engineering targets, the transcription factor Sso7d from *S. solfataricus* was used as a scaffold for creating binding partners to a variety of biomolecules [76]. The thermostability of these enzymes was also a key factor in examining the effect of microwaves on biocatalysis. Under certain conditions, the biocatalytic rates of enzymes from *P. furiosus*, *S. solfataricus* and *T. maritima* could be enhanced under microwave irradiation, a prospect not possible for mesophilic enzymes whose stability was significantly impacted by microwaves [77].

Key to many enzyme applications is the capability to immobilize the biocatalyst for stabilization or for a specific process strategy (e.g. re-use or localization). Enzymes from extreme thermophiles present some opportunities, as well as challenges, when it comes to immobilization [78]. For example, entrapment in a porous gel as a means of immobilization, commonly employed for mesophilic enzymes, is problematic because of the thermolability of the matrix. Alternatively, carbohydrate binding domains from extreme thermophiles can be employed in fusions with extremely thermophilic enzymes for immobilization, as was demonstrated with the chitin-binding domain from a *P. furiosus* chitinase and the xylose isomerase from *Thermotoga neapolitana* [79].

Not surprisingly, enzymes from extreme thermophiles have been examined closely for applications related to the emergence of biofuels. It has been argued that the deconstruction of lignocellulose is best done at elevated

Table 3

Considerations for metabolic engineering bioprocesses at high temperatures

Advantages	Disadvantages
Reduced recalcitrance of plant biomass for biofuels applications	Possible energy burden of heating reactor contents
Reduced risk of contamination	Lower yields of cellular biomass
Use of temperature regulation to optimize product formation	Genetic stability of thermophilic recombinant hosts unknown
Facilitated recovery of volatile products	Lower gas solubilities
Lower risk of release of viable genetically modified organisms	Substrate, product lability at elevated temperatures
Higher temperatures more consistent with chemical processes	Limited to genes encoding thermostable proteins/enzymes
Higher mass transfer rates	Less known about microbial physiology
Improved solubility of carbohydrates, amino acids	Genetics tools are in infancy

temperatures, either because thermal factors facilitate this process, or because biofuels bioprocessing already involves thermal steps for pretreatment [45]. To this point, computational studies suggest that thermal contributions to enzyme plasticity and molecular motion at high temperatures play a role in enhancing cellulose-binding domain and catalytic domain synergy in cellulose [80^{*}]. The genomes of species within the extremely thermophilic genus *Caldicellulosiruptor* encode a host of multi-domain glycoside hydrolases that contribute to the breakdown of crystalline cellulose and hemicellulose [43^{*},48^{**}]. Recent work has looked at the contributions of the various domains within these enzymes to complex carbohydrate hydrolysis [46,81] and the potential role of certain multi-domain glycoside hydrolases, which also use S-layer homology domains to anchor to the cell envelope [82]. The high temperature, cellulose-degrading consortium of archaea, described above, also gave rise to the discovery of a novel hyperthermophilic cellulase, a multi-domain enzyme exhibiting optimal activity at 109 °C [23^{**}].

Biocatalysis based on whole cells has also been the objective of efforts with extreme thermophiles. For example, the degradation of toxic pollutants has been demonstrated; *S. solfataricus* 98/2 could utilize phenol for growth in a fed-batch bioreactor [83]. The recovery of base, precious and strategic metals through whole cell bio-oxidation processes has been a long-term goal for extreme thermoacidophiles and recent efforts have focused on identifying process bottlenecks and improved processing strategies. It is becoming clear, not surprisingly, that mixed cultures will be the most effective approach to biohydrometallurgy and could be a way to overcome problems with surface passivation by jarosite and elemental sulfur by-products [84].

Metabolic engineering applications and opportunities at high temperatures

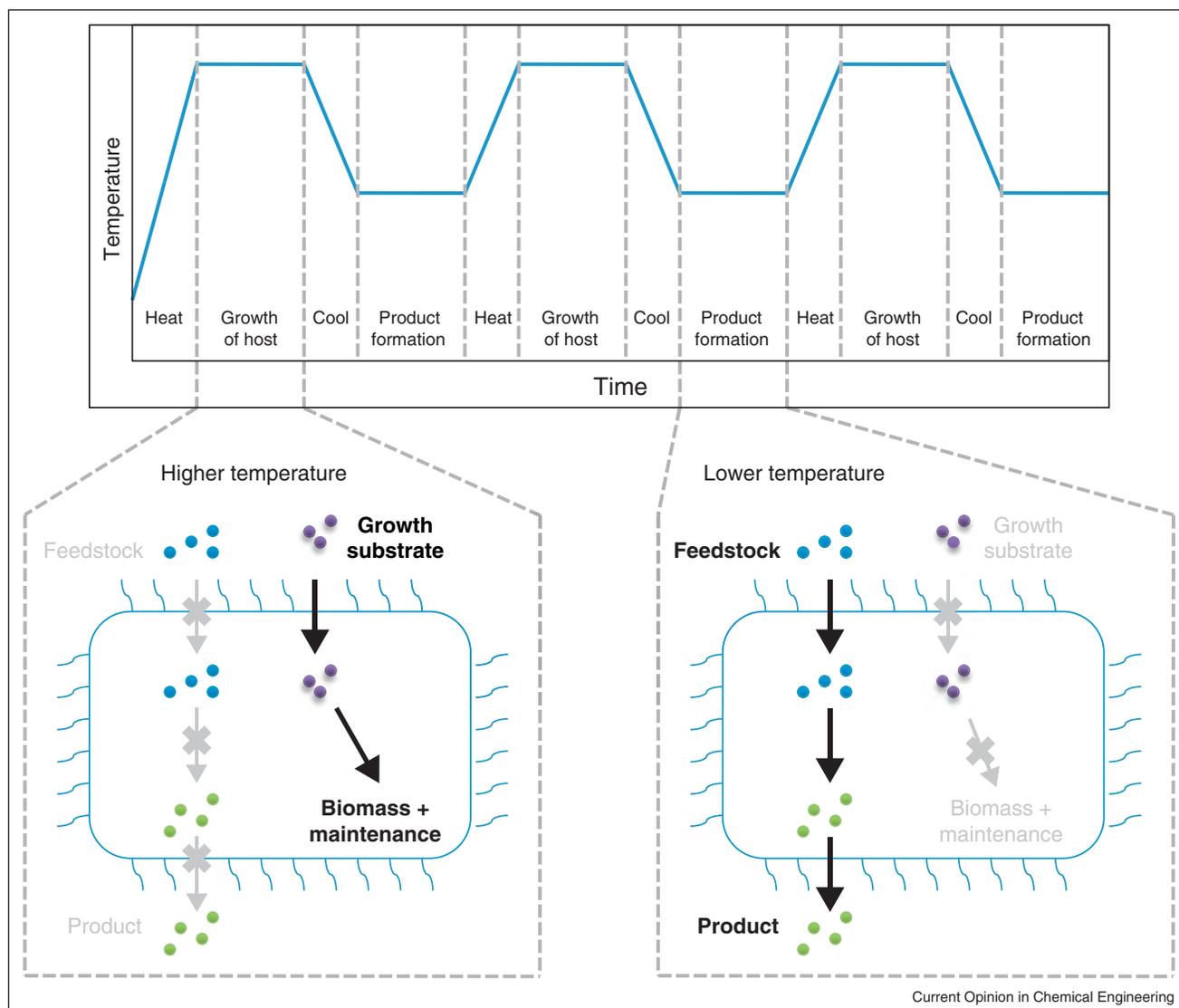
Until very recently, metabolic engineering involving enzyme and pathways from extreme thermophiles were carried out in mesophilic hosts. For example, the archaeal isoprenoid ether lipid biosynthesis pathway was reconstructed in *E. coli* to produce diglycerylglyceryl phosphate (DGGGP) [85]. The strategic use

of temperature for bioprocessing was demonstrated by cloning a hyperthermophilic α -amylase from *T. maritima* into transgenic sweet potato [86^{*}]. Below 40 °C, the normal growth temperature range of the plant, no significant amylase activity could be detected nor was plant growth and development impacted. But upon switching to 80 °C, the hyperthermophilic enzyme was activated such that the plant storage root was rapidly hydrolyzed. This created a mechanism for first producing the sweet potato starch then using a thermal switch to convert the starch into a fermentable sugar for biofuels applications.

Of course, the next frontier is metabolic engineering at high temperatures (Table 3). In fact, the rapid progress with molecular genetics for extreme thermophiles has given rise to the prospect of using these microorganisms as recombinant hosts for metabolic engineering. The three archaea shown in Table 1 offer the most near-term prospects in this regard. For example, *S. solfataricus* P2 differs from *S. solfataricus* 98/2 in that the latter strain is less able grow on surfaces. However, the insertion of two genes from P2 into 98/2, encoding α -mannosidase and β -galactosidase, enabled 98/2 to mimic P2 by attaching to glass and forming static biofilms [87]. The use of thermally driven gene regulation has been elegantly demonstrated using *P. furiosus* as a recombinant host. When microorganisms are employed to generate a desired product, the production pathway often competes with the microbe's natural biosynthesis pathways for key intermediates or cofactors. In this situation, one might envision an ideal two-stage process in which biomass is generated during the first stage with minimal product formation, while cellular activity ceases and the desired product is generated during the second stage (see Figure 1). The recent proof-of-concept work of Basen *et al.* [63^{**}] uses temperature as the switch to halt cell growth and initiate product formation. Lactate dehydrogenase from *Caldicellulosiruptor bescii* ($T_{opt} = 78$ °C) was cloned into *P. furiosus* ($T_{opt} = 98$ °C) under the control of a *P. furiosus* 'cold shock' promoter that is turned on at 70–75 °C. In this situation *P. furiosus* can be cultured at 98 °C until it reaches a high cell density, at which point it can be transferred to 72 °C, resulting in expression of the heterologous lactate dehydrogenase and formation of

6 Biotechnology and bioprocess engineering

Figure 1



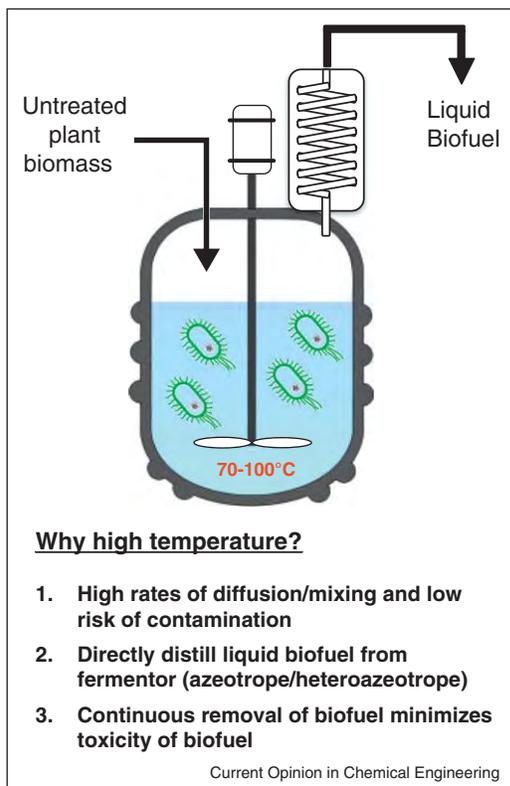
Temperature-dependent regulation of product formation. Two substrates are provided initially: one that can be catabolized by the extremely thermophilic host's endogenous metabolism for growth, another that cannot and is used for product formation. At higher temperature, the host grows but product formation is silenced. After sufficient cell generation has occurred, the temperature is lowered, inhibiting the host's metabolism and inducing a cold shock promoter that controls expression of heterologous enzymes active at the lower temperature. The heterologous enzymes catalyze all reactions necessary to generate the desired product from the substrate that is provided specifically for product formation. When product formation deteriorates, the temperature can be raised to rehabilitate the extremely thermophilic host and regenerate cofactors and intermediates to prepare for another round of product formation. Additional growth substrate can be provided if necessary, or the extreme thermophile may be able to subsist by catabolizing the heterologous enzymes.

lactate (a product that *P. furiosus* is unable to produce naturally).

In the near future, we will probably see the first demonstrations of high temperature strains used as hosts for biotechnological applications. For example, in biofuels production, the ultimate goal is to create metabolically engineered extreme thermophiles that breakdown

lignocellulose and convert fermentable sugars to liquid biofuels (so-called 'consolidated bioprocessing'). At high temperatures, there is the possibility that biofuels can be recovered directly through direct evaporation and distillation (see Figure 2). Other strategic uses of high temperatures will probably emerge as tools for molecular genetics in extreme thermophiles become more firmly established.

Figure 2



Consolidated bioprocessing at high temperatures for biofuels production. Metabolic engineering tools for extreme thermophiles could be used to create trains capable of deconstruction of plant biomass and conversion to volatile liquid biofuels that can be recovered directly by distillation from fermentation broths.

Conclusions

Although discovered more than 40 years ago, in many ways extremely thermophilic microorganisms are just at the beginning when it comes to biotechnological applications. Virtually any enzyme that is identified in a mesophile has a homologous version in an extreme thermophile, typically with significantly higher levels of thermostability, if not thermoactivity. As genetic systems for extreme thermophiles become more widely used and more tractable, the challenge will be to exploit elevated temperatures to improve upon existing bioprocessing strategies, or even better, make possible novel multi-step, biotransformations. Ideas along these lines have already been proposed [88], and it is only a matter of time before these process concepts are demonstrated and put into practice.

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10 Biotechnology and bioprocess engineering

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